THE ROLE OF PODOCALYXIN IN ADHESION AND CELL MORPHOGENESIS

by

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B.Sc., The University of Victoria, 2000

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2006

ABSTRACT

Podocalyxin is a sialomucin expressed on kidney podocytes, vascular endothelia, and hematopoietic progenitors. Although podocalyxin and its close relative, CD34 have been studied for many years, their precise functions have remained elusive, and roles in blocking differentiation, preventing cell adhesion, and establishing cell polarity have all been proposed. Despite this ambiguity, the perinatal lethality of podocalyxin knockout mice (as a result of kidney defects) and podocalyxin's close association with cancer progression highlight its biological importance. I therefore used several strategies to clarify podocalyxin's functions and mechanisms of action.

Podocalyxin was overexpressed in epithelial cells, and I observed a striking decrease in cell adhesion and an induction of microvillus formation. Microvillus formation was then used as the endpoint to assess the activity of podocalyxin mutants: the extracellular domain was essential while most of the cytoplasmic tail could be deleted without loss of this function. These *in vitro* studies also demonstrated that podocalyxin recruits the scaffolding protein, NHERF1, which may have important implications in the regulation of NHERF-related processes, such as interaction with ion transporters and signalling molecules.

In order to study podocalyxin *in vivo*, generation of conditional podocalyxin overexpressing mice was attempted. The intention was to generate a single floxed *podxl* transgenic mouse line that could be crossed with numerous Cre mice in order to induce

podocalyxin expression in selected tissues. For example, podocalyxin overexpression in mammary tissue was intended to facilitate evaluation of podocalyxin's role in breast cancer progression. Similarly, these mice could be used to selectively rescue defects in podocalyxin-deficient mice. Unfortunately, chromosomal abnormalities in the parental embryonic stem cells temporarily prevented completion of this study.

As an alternative strategy, we attempted to selectively rescue the kidney defects observed in podocalyxin-null mice by creation of mice with a kidney-specific *podxl* transgene. Surprisingly, although transgenic podocalyxin was appropriately expressed, and podocyte morphology appeared relatively normal in contrast to podocalyxin-null mice, transgenic mice still died perinatally. This suggests the presence of other serious, as yet undetermined, abnormalities in podocalyxin-deficient animals. Continued assessment of these defects and podocalyxin's role in cancer progression is underway. In summary, this thesis reveals a new mechanistic role for podocalyxin in the process of cell morphogenesis and suggests that in addition to its vital role in kidney development, podocalyxin may play an essential role in other aspects of mammalian development.

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LIST OF ABBREVIATIONS

aa	amino acid
AGM	aorta-gonad-mesonephros
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
bp	base pair
BM	bone marrow
BSA	bovine serum albumin
B6	C57BL/6 wildtype mouse strain
β-gal	β-galactosidase
β_2-AR	β_2-adrenergic receptor
CFTR	cystic fibrosis transmembrane regulator
CFU	colony forming unit
ch	chicken
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovary
CKII	casein kinase II
DAB	diaminobenzidine
DAPI	4', 6'-diamidino-2-phenylindole
DDS	Denys-Drash syndrome
DKO	podocalyxin/CD34 double knockout
ΔDTHL	podocalyxin mutant lacking C-terminal DTHL

ΔEC	podocalyxin mutant lacking most of the extracellular domain
Δtail	podocalyxin mutant lacking cytoplasmic tail
E	embryonic day
EBP50	ezrin binding phosphoprotein of 50 kDa
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
EGFP	enhanced green fluorescent protein
ELAM	endothelial leukocyte adhesion molecule
ER	estrogen receptor
ERM	ezrin-radixin-moesin
ESC	embryonic stem cell
E3KARP	NHE3 kinase A regulatory protein
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FC	flow cytometry
FTL/FL	fetal liver
GBM	glomerular basement membrane
GFP	green fluorescent protein
GLEPP	glomerular epithelial protein
GlyCAM	glycosylation-dependent cell adhesion molecule
GRK	G protein-coupled receptor kinase
HCC	hepatocellular carcinoma

HEV	high endothelial venule
HGEC	human glomerular epithelial cells
HRP	horse radish peroxidase
HSC	hematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
ICAM	intracellular adhesion molecule
IF	immunofluorescence
Ig	immunoglobulin
IHC	immunohistochemistry
IL-6	interleukin 6
ip	immunoprecipitation
IRES	internal ribosome entry site
kDa	kiloDalton
KO	knockout
LIF	leukemia inhibitory factor
LSK	Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$
LTR	long term repopulating
MAdCAM	mucosal addressin cell adhesion molecule
MDCK	Madin-Darby canine kidney
MEP	Myb-Ets transformed progenitor
ms	mouse
nd	not determined
NHE	Na^+/H^+ exchanger

NHERF	Na^+/H^+ exchanger regulatory factor
NSGCT	nonseminomatous germ cell tumour
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAN	puromycin aminonucleoside
PB	peripheral blood
PBS	phosphate buffered saline
PC	post coitum
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PDZ	PSD-95/Dlg/ZO-1
PE	phycoerythrin
PFA	paraformaldehyde
PKC	protein kinase C
PMSF	phenylmethanesulfonyl fluoride
Podo	podocalyxin
PolyA/pA	polyadenylation
PP	post partum
PS	protamine sulfate
P2Y1R	purinergic receptor
rb	rabbit
RNAi	RNA interference
RT-PCR	reverse transcriptase-polymerase chain reaction

SA	streptavidin
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
siRNA	small interfering RNA
SPL	spleen
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus 0.05 % Tween 20
TEM	transmission electron microscopy
TER	transepithelial resistance
UTR	untranslated region
WT	wildtype
WT1	Wilms' tumour 1
YS	yolk sac

ACKNOWLEDGEMENTS

It has been quite a journey, and I have had the opportunity to work with many great people over the past few years. I am very grateful to Dr. Kelly McNagny for giving me the chance to carry out my studies in his lab and for always believing in me; his support has really made all this possible. I want to thank everyone in the McNagny lab, past and present, for the advice and especially for the laughs. I particularly thank Shierley Chelliah for countless hours of help, always with a smile. I also thank Helen Merkens, Dr.'s Regis Doyonnas and Sebastian Furness, Jamie Haddon, Steve Maltby, and Poh Tan, who have all contributed in many ways and also made the McNagny lab an enjoyable place to be.

The Biomedical Research Centre is a fabulous environment for research and collaboration, and I am lucky to have been a part of this group. I would particularly like to thank Dr. Wilf Jefferies and Matt Finlay for generating my kidney-specific transgenic mice. In addition, I would like to thank Dr.'s Hermann Ziltener, Fabio Rossi, John Schrader, Muriel David, Doug Carlow, Pete Schubert, Maya Kotturi, Jason Grant, Stéphane Corbel, Brock Grill, and Gary McLean, as well as Phil Owen, Wooseok Seo, Bernhard Lehnertz, Jeff Duenas, and Mike Williams for advice and reagents. I am also grateful to Andy Johnson for hours of sorting cells, and to the amazing core staff at the BRC, particularly Nicole Voglmaier, Taka Murakami, Lea Wong, and Samantha Hoium.

I am also grateful to many people outside of the BRC. Dr.'s Carolyn Brown, Pauline Johnson, and Rob Kay of my supervisory committee have always provided me with good

advice and kept me on track, and I really appreciate their critical reviews of my thesis. One of the most productive and enjoyable aspects of my thesis research has been my collaboration with Dr. Cal Roskelley, Dr. Wayne Vogl, Marcia McCoy, and Jane Cippolone, and I am truly grateful for all their contributions to the microvilli project. I also appreciate the expert electron microscopy assistance from Derrick Horne, as well as advice from Garnet Martens, at the UBC BioImaging Facility, the use of Dr. Robert Nabi's confocal microscope, and contributions from the Department of Medical Genetics, especially those of Cheryl Bishop. I am also grateful to Dr. Corrinne Lobe and Raymond Wong at Sunnybrook Health Sciences Centre and Dr. Jill Lahti at St. Jude Children's Research Hospital for assistance with the inducible podocalyxin expression project, and to Dr.'s David Kershaw and Atsushi Miyajima for invaluable reagents. I am also thankful for funding from NSERC and UBC and travel funding from the Stem Cell Network.

None of this would have been possible without the tremendous support from my family and friends. At UBC, I especially want to thank Marcia McCoy, Jasmeen Merzaban, Nicole Voglmaier, Julie Wong, and Jane Cippolone for their constant encouragement; the many frustrations of research are so much easier to deal with when you have good friends who are in similar situations. In addition, my parents have always been an incredible source of encouragement for me, and I cannot thank them enough. My grandparents, who continue to live life to the fullest every day, have also been a constant source of inspiration for me. Lastly, there are no words to express how much I appreciate the unwavering support of my husband, Christian Nielsen. I am exceptionally grateful for his encouragement, every single day throughout my doctoral studies.

CHAPTER 1 : INTRODUCTION

1.1 Podocalyxin is a Member of the CD34 Family of Sialomucins

Podocalyxin is a cell surface protein essential for kidney development (Doyonnas et al., 2001) and implicated in a wide range of cancers (Casey et al., 2006; Chen et al., 2004; Kelley et al., 2005; Schopperle et al., 2003; Somasiri et al., 2004; Stanhope-Baker et al., 2004). It was first identified by Dr. Marilyn Farquhar's group and termed "podocalyxin" as it is the major component of the glycocalyx of kidney glomerular podocytes (Kerjaschki et al., 1984). It has also been called podocalyxin-like protein 1 (PCLP-1), thrombomucin, Myb-Ets transformed progenitor (MEP)-21, and gp135, and it is encoded by the *podxl* gene (Kershaw et al., 1995; McNagny et al., 1992; McNagny et al., 1997; Meder et al., 2005)*.

- Some figures in this chapter have been published in the following articles:

1) Doyonnas, R., Nielsen, J.S., Chelliah, S., Drew, E., Hara, T., Miyajima, A. and McNagny, K.M. (2005) Podocalyxin is a CD34-related marker of murine hematopoietic stem cells and embryonic erythroid cells. *Blood*, **105**, 4170-4178.

2) Nielsen, J.S., Doyonnas, R. and McNagny, K.M. (2002) Avian models to study the transcriptional control of hematopoietic lineage commitment and to identify lineage-specific genes. *Cells Tissues Organs*, **171**, 44-63.

3) Somasiri, A., Nielsen, J.S., Makretsov, N., McCoy, M.L., Prentice, L., Gilks, C.B., Chia, S.K., Gelmon, K.A., Kershaw, D.B., Huntsman, D.G., McNagny, K.M. and Roskelley, C.D. (2004) Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression. *Cancer Res*, **64**, 5068-5073.

1.1.1 Protein Structure

Podocalyxin is a type I transmembrane protein with a predicted mass, based on its protein backbone, of approximately 55 kDa (Kershaw et al., 1997a; McNagny et al., 1997). Its serine-threonine-proline rich extracellular domain is extensively O-glycosylated and sialylated, resulting in an actual mass of 140-170 kDa and characterizing it as a sialomucin (Doyonnas et al., 2001; Hilkens et al., 1992; Kerjaschki et al., 1984; Kershaw et al., 1997a; McNagny et al., 1997; Miettinen et al., 1999; Orlando et al., 2001; Sassetti et al., 1998; Takeda et al., 2000). The extracellular domain also contains several potential sites of N-linked glycosylation, a globular domain consisting of four cysteine residues, and a juxtamembrane stalk region (Doyonnas et al., 2001; Kershaw et al., 1997a; Orlando et al., 2001). A 26 amino acid (aa) hydrophobic region encodes a single pass transmembrane domain, which is followed by a well-conserved cytoplasmic tail (Kershaw et al., 1997a; McNagny et al., 1997; Sassetti et al., 1998; Takeda et al., 2000). This intracellular domain contains putative phosphorylation sites for protein kinase C (PKC) and casein kinase II (CKII) as well as the C-terminal PDZ-binding motif, DTHL (Doyonnas et al., 2001; Kershaw et al., 1997a; McNagny et al., 1997). Like other sialomucins, the extracellular domain of podocalyxin demonstrates a very low degree of sequence conservation (<33%) (Kershaw et al., 1997a; Orlando et al., 2001; Takeda et al., 2000). In contrast, the 75 aa cytoplasmic tail exhibits a high level of aa sequence identity (~95 % between rat, rabbit, and human, and slightly less in comparison to chicken), indicative of an important, conserved function (Kershaw et al., 1997a; Li et al., 2002; McNagny et al., 1997; Miettinen et al., 1999).

Amino acid sequence, protein structure, genomic organization, and patterns of alternative splicing (described below) suggest that podocalyxin is most closely related to CD34 and endoglycan (McNagny et al., 1997; Nielsen et al., 2002; Sassetti et al., 2000). Each of these proteins contains a highly glycosylated and sialylated extracellular domain, a globular domain, and a stalk followed by a single pass membrane spanning domain (Figure 1-1) (Brown et al., 1991; He et al., 1992; Kershaw et al., 1997a; Krause et al., 1996; Krause et al., 1994; McNagny et al., 1997; Nakamura et al., 1993; Nielsen et al., 2002; Sassetti et al., 2000; Simmons et al., 1992; Suda et al., 1992). The cytoplasmic tails contain the highest degree of similarity across species and between family members, with sequences suggestive of roles in cell signaling or cellular localization: podocalyxin, endoglycan, and CD34 all contain consensus phosphorylation sites, as well as a C-terminal PDZ-binding motif (Brown et al., 1991; Krause et al., 1996; McNagny et al., 1997; Nielsen et al., 2002; Sassetti et al., 1998; Sassetti et al., 2000; Simmons et al., 1992).

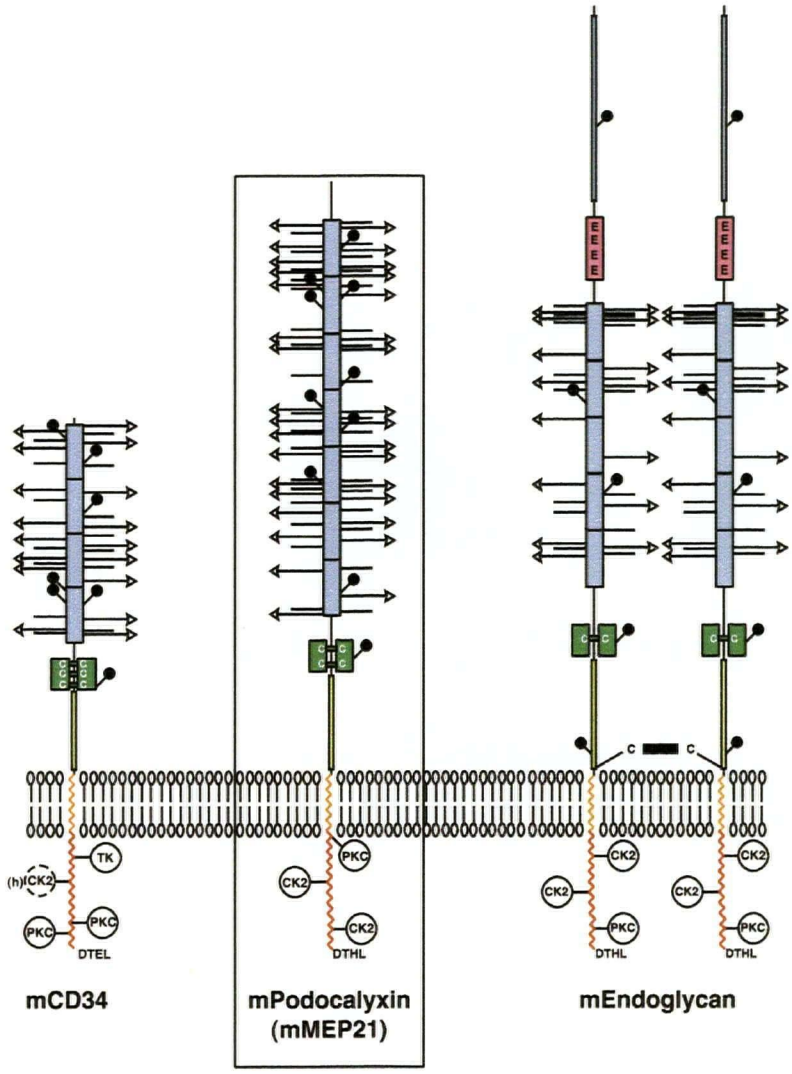

Figure 1-1: Schematic of CD34 Family Members.

Blue: mucin domains, black circles: potential N-linked carbohydrates, horizontal lines: potential O-linked carbohydrates, triangles: potential sialic acid residues, green: globular motifs, yellow: stalk, orange: transmembrane domains, red: cytoplasmic tails, large circles: potential phosphorylation sites. Reproduced with kind permission of S. Karger AG, Basel (Nielsen et al., 2002).

There are, however, several notable differences between these three sialomucins. Firstly, the mucin domains vary in length, with CD34 being the shortest protein overall (Krause et al., 1994; Sassetti et al., 1998; Sassetti et al., 2000). Secondly, in the globular region podocalyxin and CD34 have four and six cysteine residues, respectively, while endoglycan has two, with an additional unpaired juxtamembrane cysteine, likely involved in dimerization (Brown et al., 1991; Fieger et al., 2003; Kershaw et al., 1997a; Sassetti et al., 2000). Thirdly, while podocalyxin and endoglycan share the C-terminal DTHL motif, CD34's C-terminal sequence is slightly altered: it is DTEL (He et al., 1992; Kershaw et al., 1997a; McNagny et al., 1997; Sassetti et al., 2000; Simmons et al., 1992). Finally, endoglycan has a unique N-terminal region rich in glutamic acid residues; while this motif is found in many intracellular regulators of transcription, it is rarely a characteristic of extracellular domains (Sassetti et al., 2000). Thus, although this family is closely related, the three proteins do have some unique, and potentially important, features.

1.1.2 Genomic Organization and Alternative Splicing

The genomic organizations of *podxl*, *cd34*, and *endoglycan* strongly suggest an evolutionary relationship (Figure 1-2) (Li et al., 2001; Nielsen et al., 2002). Each protein is encoded by eight exons, and, across the family, individual exons encode equivalent protein motifs and are very similar in size (Li et al., 2001; Nielsen et al., 2002; Satterthwaite et al., 1992). Throughout the family, intronic distances are also strikingly similar, and splicing to an additional exon between exons seven and eight generates a longer transcript encoding a protein lacking much of the cytoplasmic tail (Figure 1-3) (Kershaw et al., 1997a; Li et al., 2001; McNagny et al., 1997; Nakamura et al., 1993;

Nielsen et al., 2002; Sassetti et al., 2000; Suda et al., 1992). Thus, these three sialomucins have been grouped into a single family based on protein structure, genomic organization, and patterns of alternative splicing.

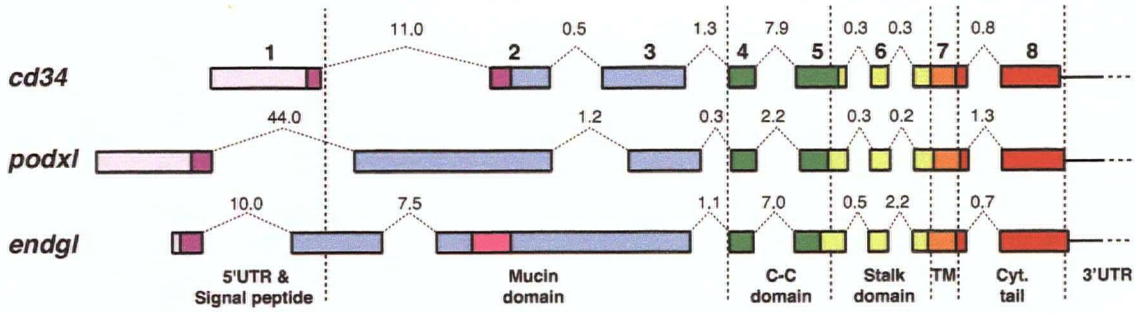

Figure 1-2: Genomic Organization of CD34 Family Members.

Dark purple: signal peptide, blue: mucin domain, green: globular motif, yellow: stalk, orange: transmembrane domain, red: cytoplasmic tail. Numbers refer to intron sizes in kilobase pairs. Reproduced with kind permission of S. Karger AG, Basel (Nielsen et al., 2002).

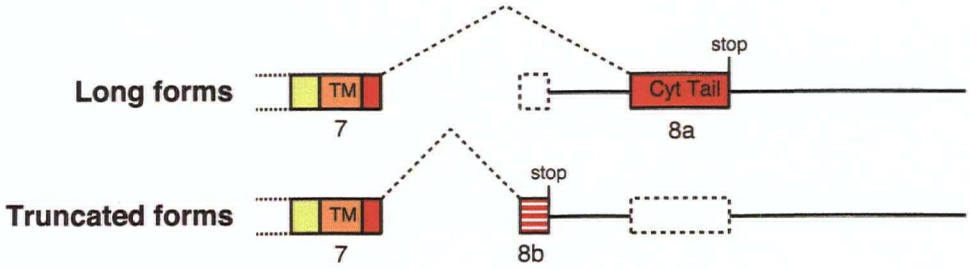

Figure 1-3: Alternative Splicing Patterns for CD34 Family Members.

Splicing produces a longer transcript encoding a shorter cytoplasmic tail. Reproduced with kind permission of S. Karger AG, Basel (Nielsen et al., 2002).

1.1.3 Expression Pattern

The high level of podocalyxin expression on the surface of renal glomerular epithelial cells (podocytes) enabled its initial isolation and characterization from these cells in 1984 (Kerjaschki et al., 1984). Podocalyxin is also expressed on the luminal face of vascular endothelia in a wide variety of vessels, by hematopoietic stem cells and progenitors, and even on hemangioblasts, which are the precursors of hematopoietic and endothelial cells (Delia et al., 1993; Doyonnas et al., 2005; Hara et al., 1999; Horvat et al., 1986; Kershaw et al., 1997a; McNagny et al., 1997; Miettinen et al., 1990; Sassetti et al., 1998). In more mature hematopoietic cells, it is only expressed on platelets, as well as their precursors, megakaryocytes (McNagny et al., 1997; Miettinen et al., 1999). Outside of the hematopoietic system, podocalyxin is expressed by mesothelial cells lining many organs (Doyonnas et al., 2001; McNagny et al., 1997), and during development, it is expressed in all three germ layers, as well as embryonic stem cells and a subset of neurons (Doyonnas et al., 2005; Vitureira et al., 2005).

Like podocalyxin, CD34 is expressed on vascular endothelial cells as well as hematopoietic progenitors and stem cells (Andrews et al., 1989; Baumhueter et al., 1994; Berenson et al., 1988; Ema et al., 1990; Fina et al., 1990; Sato et al., 1999; Young et al., 1995). There has, however, been considerable controversy about expression of CD34 on long term repopulating hematopoietic stem cells (LTR-HSCs). It is now believed that CD34 expression fluctuates depending on developmental stage and cell activation: in older adult mice CD34 is expressed only on activated HSCs, whereas expression is more widespread during development (Dao et al., 2003; Ito et al., 2000; Nakamura et al., 1999;

Ogawa, 2002; Osawa et al., 1996; Sato et al., 1999; Tajima et al., 2000; Zanjani et al., 2003). Thus, it may be that the most primitive and quiescent HSCs are CD34$^-$. Then, in order for cells to contribute to engraftment, CD34 may be upregulated. This could be followed by a decrease in expression as the bone marrow achieves steady state levels of hematopoietic cells. Additionally, CD34 is expressed on mature murine mast cells, but it is not found on platelets (Drew et al., 2002). There is therefore considerable overlap between podocalyxin and CD34 expression, but there are some notable differences.

Although much less is known about endoglycan, its expression pattern displays some similarity to those of CD34 and podocalyxin. Some hematopoietic cells, including bone marrow macrophages and Toll-like receptor-activated B cells, as well as a subset of neuronal cells and vascular smooth muscle cells all express endoglycan ((Nielsen et al., 2002; Sassetti et al., 2000), and Helen Merkens and Kelly McNagny unpublished). There is also some evidence for expression on endothelial cells lining vessels (Sassetti et al., 2000).

1.2 Cloning of Podocalyxin

Although podocalyxin was initially identified and characterized in rat in the 1980's (Kerjaschki et al., 1984), it was not until 1995 that it was first cloned using a rabbit glomerular cDNA library (Kershaw et al., 1995). A bacterial cDNA library expression system was screened with monoclonal antibodies generated against a podocalyxin-like protein expressed in glomeruli. Six positive clones were identified, and these corresponded to a 5.5 kb transcript highly expressed in glomeruli. Analysis of the

predicted amino acid sequence demonstrated that it contained many characteristics of podocalyxin, so the molecule was named "podocalyxin-like protein 1 (PCLP1)". Subsequently, rabbit cDNA encoding podocalyxin was used to screen a human renal cortex cDNA library, and human PCLP1 was cloned (Kershaw et al., 1997a). cDNA corresponding to the cytoplasmic tail of human PCLP1 was then used to probe the genomic DNA of a variety of species. Homologous sequences were found in monkey, rat, mouse, dog, cow, rabbit, and chicken, but not yeast, demonstrating the remarkable conservation of this region of the gene in vertebrates (Kershaw et al., 1997a).

Concurrently, the chicken homolog of podocalyxin was cloned and named "thrombomucin" (McNagny et al., 1997). To begin with, primitive hematopoietic progenitors derived from chicken embryo yolk sac were transformed with the Myb-Ets oncoprotein-encoding acute leukemia virus, E26; these cells were termed **M**yb-**E**ts-transformed **p**rogenitors, or MEPs (Graf et al., 1992). A panel of monoclonal antibodies was generated against surface antigens on these cells by using them to immunize mice (McNagny et al., 1992). One of the antibodies, MEP21, specifically recognized an antigen present on MEPs and platelets, but not on other mature hematopoietic cells. The MEP21 antigen could only be purified in very low quantities, so nanoelectrospray mass spectrometry was used to sequence the protein, and degenerate oligonucleotides were used to clone the MEP21-encoding cDNAs (McNagny et al., 1997). Three distinct types of clones were obtained. The first encoded a 524 base pair (bp) 5' untranslated region (UTR), a 1347 bp 3' UTR, and an open reading frame coding for 571 aa, which included a putative N-terminal signal peptide, a mucin domain, a globular domain, a stalk, a

putative transmembrane domain, and a cytoplasmic tail containing consensus PKC and CKII phosphorylation sites. The second type of clone was very similar, but it encoded an insertion of 18 additional amino acids in the juxtamembrane region of the cytoplasmic tail. The third type of clone contained a novel sequence beginning at the same site as the insertion in type two clones, and it encoded a shorter cytoplasmic tail with an earlier stop codon. This was the first evidence of alternative splicing to generate a truncated cytoplasmic tail in podocalyxin. Expression of this mucin on platelets, or thrombocytes, led to it being called thrombomucin.

Subsequently, rat *podxl* was cloned using cDNA libraries from kidney. It was partially cloned using rabbit cDNA as a probe (Miettinen et al., 1999); it was later completely cloned using primers designed from homologous sequences in the cytoplasmic tails of rabbit and human podocalyxin (Takeda et al., 2000). Interestingly, although dog podocalyxin (gp135) has been used as an apical marker of Madin-Darby canine kidney (MDCK) epithelial cells for many years (Ojakian and Schwimmer, 1988), it was only recently recognized as podocalyxin (Meder et al., 2005). It was previously known simply as "gp135," but after purification and identification of tryptic peptides by nanoelectrospray tandem mass spectrometry, it became clear that it was, in fact, podocalyxin. EST's corresponding to podocalyxin have now been identified in human, dog, rat, mouse, squirrel, chicken, frog, and zebrafish.

1.3 Transcriptional Regulation of Podocalyxin

The process of transcriptional regulation of CD34 has proven difficult to decipher, and *podxl*'s promoter and untranslated regions are likely complicated as well. In fact, transfection of 54 kb of human genomic DNA is not sufficient to drive hematopoietic progenitor-specific expression of CD34, while 160 kb is, suggesting that considerable upstream, downstream, and intronic sequences are required for tissue-specific expression of CD34 (Radomska et al., 1998; Yamaguchia et al., 1997). Preliminary experiments with the *podxl* locus suggest that it may be regulated in an equally complex manner (unpublished observations, Jon Frampton and Regis Doyonnas (Nielsen et al., 2002)). However, there are a few clues about podocalyxin's regulation. It is positively regulated by the Wilms' tumour 1 (WT1) zinc finger transcription factor, negatively regulated by p53, and also affected by Ets-1 expression (Guo et al., 2002; Palmer et al., 2001; Stanhope-Baker et al., 2004; Teruyama et al., 2001).

1.3.1 Positive Regulation by WT1

WT1 itself is complex: alternative splicing, RNA editing, and multiple translation initiation sites allow the production of 24 different proteins from the *wt1* gene, and biochemical experiments suggest that it acts as a transcription factor as well as playing a role in RNA processing, depending on its cellular context and splice variants ((Bruening and Pelletier, 1996; Guo et al., 2002; Haber et al., 1991; Scharnhorst et al., 1999; Sharma et al., 1994), and reviewed in (Discenza and Pelletier, 2004)). Furthermore, there is confusion as to whether it activates or represses transcription (Maheswaran et al., 1993;

Wang et al., 2001), and although it was initially identified as a tumour suppressor it may also function as an oncogene (reviewed in (Loeb and Sukumar, 2002)). WT1 is normally expressed in the developing glomerulus, as well as in the urogenital ridge, mesothelium, spleen, brain, and spinal cord during embryogenesis and in the uterus, oviduct, granulosa cells of the ovary, Sertoli cells of the testes, hematopoietic progenitor or stem cells, and kidney podocytes in adult (reviewed in (Discenza and Pelletier, 2004)).

Several studies have implicated WT1 in regulation of podocalyxin expression. Splicing generates two major variants of WT1 whereby a three-amino acid (KTS) sequence between the third and fourth zinc fingers is either omitted or included (Palmer et al., 2001; Wang et al., 2001). The (–KTS) variant binds DNA and acts as a transcriptional regulator, while the (+KTS) variant is a poor transcriptional regulator but can instead interact with splicing machinery (Roberts, 2005). Within hours of inducible expression of the (-KTS) isoform in rat embryonic kidney cell lines, a dramatic increase in podocalyxin is detected at both the mRNA and protein levels (Palmer et al., 2001). This effect is reversible upon removal of WT1. Additional experiments identified a WT1 (-KTS) responsive domain in the *podxl* promoter (Palmer et al., 2001), and an independent group used chromatin immunoprecipitation (ChIP) to demonstrate that WT1 does, in fact, bind the promoter (Stanhope-Baker et al., 2004). Furthermore, in a transgenic mouse expressing a mutant form of WT1, there is a statistically significant decrease in podocalyxin expression in newborn kidneys, although normal levels are achieved within several months of birth (Gao et al., 2004). Moreover, podocalyxin expression in the developing glomerulus, and in other cell types, corresponds to that of WT1. In the

glomerulus, WT1 is expressed during the renal vesicle and S-shaped body stages of development, and it is restricted to visceral epithelial cells (podocytes) of developing and mature glomeruli (Armstrong et al., 1993; Palmer et al., 2001). Podocalyxin is first evident at a similar stage of glomerular development, and expression is greatest in mature podocytes (Palmer et al., 2001; Pritchard-Jones et al., 1990). In addition, WT1 and podocalyxin are co-expressed in mesothelium and hematopoietic precursors (Doyonnas et al., 2001; Doyonnas et al., 2005; Ellisen et al., 2001; Pritchard-Jones et al., 1990). Thus, WT1 is the most conclusive regulator of podocalyxin expression to date.

1.3.2 Negative Regulation by p53

In contrast, there is some evidence to suggest that p53 represses podocalyxin expression. Using cDNA microarray analysis, podocalyxin was identified as a p53 target in the Wilms' tumour model cell line, WiT49 (Stanhope-Baker et al., 2004). A luciferase reporter construct was then used to confirm the considerable decrease in podocalyxin levels upon p53 expression.

Interestingly, p53 and WT1 have been shown to physically interact and modulate the functions of one another in some situations. While p53 can alter the transcriptional regulatory activity of WT1, WT1 can stabilize p53, alter its activity, and prevent p53-induced apoptosis (Maheswaran et al., 1995; Maheswaran et al., 1993). However, in the WiT49 cell line, p53-induced podocalyxin repression is not related to p53's effects on WT1: a minimal *podxl* promoter containing the WT1-reponsive portion is not sufficient

to decrease podocalyxin expression upon activation of p53 (Stanhope-Baker et al., 2004). Thus, p53 can modulate podocalyxin's expression in a WT1-independent manner.

1.3.3 Positive Regulation by Ets-1

The only additional information regarding control of podocalyxin expression comes from a study based on cDNA microarray analysis of human umbilical vein endothelial cells (HUVEC) infected with an adenovirus encoding the Ets-1 transcription factor (Teruyama et al., 2001). Ets-1 expression in endothelial cells is known to promote angiogenesis. Its expression in HUVECs leads to a 3.3-fold upregulation of podocalyxin in comparison to null-virus infected cells, although there is no evidence to suggest that podocalyxin expression is directly regulated by this factor. There is therefore considerable work still to be done in unraveling the complex transcriptional regulation of podocalyxin.

1.4 Intracellular Binding Partners for Podocalyxin

Clues regarding the function of novel proteins can often be obtained by identifying binding partners with known functions. The high degree of sequence conservation in podocalyxin's cytoplasmic tail implies that intracellular binding partners might exist; these potential interactors are thus the focus of much research.

1.4.1 Ezrin

The first indication of an intracellular binding partner for podocalyxin came from Dr. Marilyn Farquhar's group in 2001 (Orlando et al., 2001). They had previously noticed that apical podocalyxin localization in podocytes coincides with that of ezrin, a member of the ezrin-radixin-moesin (ERM) family of cytoskeletal linker proteins (Kurihara et al., 1995). ERM proteins contain a C-terminal actin binding motif and an N-terminal FERM protein module, thought to anchor them to membrane proteins (Chishti et al., 1998; Turunen et al., 1994). They regulate cell adhesion and morphogenesis, including formation of microvilli and membrane ruffles (reviewed in (Mangeat et al., 1999)). The importance of podocalyxin in maintaining podocyte foot process integrity (discussed below), and its colocalization with ezrin, suggested a possible link to the actin cytoskeleton. For these reasons, this group assessed the potential association of podocalyxin with ezrin and actin (Orlando et al., 2001).

Overlapping localization of podocalyxin and ezrin in podocytes was confirmed by immunofluorescence and dual immunogold labelling of ultra-thin cryosections of rat kidney (Orlando et al., 2001). Both proteins are concentrated along the apical plasma membrane of podocyte cell bodies and foot processes above the level of slit diaphragms, and they form a stable, co-immunoprecipitable complex (Orlando et al., 2001). Importantly, selective detergent extraction and co-sedimentation assays in podocalyxin-transfected MDCK cells demonstrate that a significant portion of podocalyxin is associated with actin filaments and that this interaction is dependent on the interaction of podocalyxin with ezrin.

Subsequent experiments identified an ezrin binding site in the juxtamembrane region of podocalyxin's cytoplasmic tail (Schmieder et al., 2004). The HQRIS sequence of podocalyxin is similar to the HQRS found in intracellular adhesion molecule (ICAM)-3 (Serrador et al., 2002). The histidine, arginine, and several serine residues are required for the recognition of ICAM-3 by ezrin (Serrador et al., 2002), so these residues were mutated in a His-tagged podocalyxin tail construct in order to assess binding to GST-tagged N-terminal ezrin (Schmieder et al., 2004). Mutation of serine to alanine, histidine and arginine to alanine, or all three residues to alanine, or deletion of the 12 N-terminal residues of the cytoplasmic tail altogether decreases binding by 22, 46, 95, or 100 %, respectively. These results strongly suggest that ezrin and podocalyxin are capable of direct interaction.

1.4.2 NHERF Proteins

There is also increasing evidence that podocalyxin interacts with members of the Na^+/H^+ exchanger (NHE) regulatory factor (NHERF) family of scaffolding proteins (reviewed in (Donowitz et al., 2005; Shenolikar et al., 2004; Thelin et al., 2005; Voltz et al., 2001; Weinman, 2001)). NHERF1/EBP50 (ezrin binding phosphoprotein of 50 kDa) and NHERF2/E3KARP (NHE3 kinase A regulatory protein) both interact with podocalyxin (discussed below), while the more distantly related and recently discovered family members NHERF3/PDZK1 and NHERF4/IKEPP have not yet been shown to bind podocalyxin. NHERF molecules all have multiple protein-protein interaction modules. NHERF1 and NHERF2 each have two tandem PSD-95/Dlg/ZO-1 (PDZ) domains and a

C-terminal ERM domain. In contrast, NHERF3 and NHERF4 both lack ERM domains, but each have four PDZ domains. ERM domains facilitate interaction with ERM family members and indirect linkage to the actin cytoskeleton, while PDZ domains, which represent one of the most common modular domains in the human genome, recognize specific sequences, generally at the C-terminus of proteins. There are several classes of PDZ domains, but the second PDZ domains of both NHERF1 and NHERF2 recognize the consensus sequence X-(S/T)-X-(I/V/L/M) where X represents any amino acid.

NHERF proteins have the capacity to homodimerize or heterodimerize with each other, and their multiple protein-interaction domains could enable the formation of large protein complexes connected to the actin cytoskeleton. Early studies of this family also suggested that NHERF proteins were involved in apical membrane localization of other proteins, but recent data has raised doubts about this proposed function. NHERF family members are thought to be involved in a wide variety of biological processes, including trafficking, transport, and signalling, based on their interaction with over 30 target proteins. They can interact with ion transporters, such as NHE3, the cystic fibrosis transmembrane regulator (CFTR), and the Na^+-phosphate transporter (Npt2), and G protein-coupled receptors, including β_2-adrenergic receptors (β_2-AR), the purinergic receptor P2Y1R, and the κ-opioid receptor. They can also associate with signalling proteins, scaffolds, and nuclear proteins, such as platelet-derived growth factor receptor (PDGFR), phospholipase Cβ1, 2, and 3, the G protein-coupled receptor kinase (GRK), and β-catenin. It must be noted, however, that many of these interactions have not yet been confirmed *in vivo*.

Shortly after identifying ezrin as a podocalyxin binding protein, Dr. Marilyn Farquhar's group also identified NHERF2 as a binding partner for podocalyxin (Takeda et al., 2001)). Using the cytoplasmic tail of podocalyxin as bait, NHERF2 was detected in a yeast two-hybrid screen of a rat glomerular cDNA library. Furthermore, in GST pull-down assays, *in vitro* translated NHERF2 binds strongly to GST-tagged podocalyxin tail. This assay also demonstrates that the related molecule, NHERF1 can interact with podocalyxin. When podocalyxin and NHERF family members are co-transfected *in vitro*, podocalyxin is co-immunoprecipitated with both NHERF1 and NHERF2. The interaction of podocalyxin with these two PDZ-containing proteins is not surprising in that it has a conserved PDZ binding motif, DTHL, at its C-terminus; deletion of this motif prevents binding in transfected cells (Takeda et al., 2001). Additional yeast two-hybrid and GST pull-down experiments using *in vitro* translated fragments of NHERF1 and NHERF2 showed that podocalyxin interacts with the second PDZ domain of both molecules, but not with the first domain, and that the interaction between podocalyxin and NHERF2 is much stronger than the interaction with NHERF1.

In kidney, NHERF1 is expressed in proximal tubules, but not glomeruli, while NHERF2 is found along the apical plasma membrane of podocyte foot processes, where it colocalizes with both podocalyxin and ezrin (Takeda et al., 2001). In addition, co-immunoprecipitation (co-ip) experiments from glomerular extracts demonstrate that podocalyxin can interact with both NHERF2 and ezrin, but not with NHERF1, in glomeruli (Orlando et al., 2001; Takeda et al., 2001). Since NHERF proteins contain an ERM binding domain in addition to their PDZ domains, it is likely that podocalyxin,

NHERF2, and ezrin form a multimeric complex in podocytes. Association of podocalyxin with the cytoskeleton may anchor it to specific membrane microdomains or determine its residence time at the cell surface (Takeda et al., 2001).

At around the same time, Dr. David Kershaw's group also identified NHERF2 as a podocalyxin binding protein by screening a rabbit glomerular cDNA library (Li et al., 2002). In contrast to the previous work, they demonstrated that podocalyxin can interact with both PDZ domains of NHERF2, although the interaction with the second PDZ sequence is much more convincing. Again, disruption of podocalyxin's PDZ binding domain, this time by deletion of only the C-terminal leucine residue, completely abolishes binding. Co-immunoprecipitation and immunofluorescence of glomeruli provided additional support for an association between podocalyxin and NHERF2.

Confocal microscopy and surface biotinylation experiments with MDCK cells transfected with podocalyxin, or podocalyxin lacking the PDZ binding domain, have been performed in an attempt to address the functional significance of podocalyxin/NHERF family interactions (Li et al., 2002). Confocal microscopy demonstrates that full-length podocalyxin is detected on the apical surface, while some mutated podocalyxin is found in the cytoplasm. Biotinylation of the apical surface of cells confirms that the majority of podocalyxin is located on this surface, while a portion of the mutant podocalyxin is intracellular; time course experiments indicate that full-length podocalyxin also persists at the cell surface longer than the mutant form does. There is a precedent for a NHERF-dependent role in membrane retention: the C-terminal PDZ binding motif of the γ-

aminobutyric acid transporter is required for its retention at the basolateral surface of cells (Perego et al., 1999). Linkage of podocalyxin to the actin cytoskeleton through NHERF and ezrin may also serve to maintain podocalyxin's localization in specialized membrane subdomains, and perhaps vascular endothelial podocalyxin would be redistributed according to the direction of blood flow by interaction with cationic molecules if it were not anchored to the actin cytoskeleton (Li et al., 2002). Thus, current data suggest that interaction with NHERF proteins assists in efficient apical localization and stability of podocalyxin.

Podocalyxin is able to interact with ezrin both directly, and indirectly through NHERF proteins (Schmieder et al., 2004). Pull-down assays with GST-tagged podocalyxin tail or a mutant lacking the DTHL sequence confirm that the N-terminal portion of ezrin can interact with both forms, although the absence of any potential NHERF binding leads to a weaker association between podocalyxin and ezrin (Schmieder et al., 2004). Moreover, differential detergent extraction demonstrates that podocalyxin lacking the NHERF binding domain does not strongly associate with actin. Although the rationale for both direct and indirect mechanisms of podocalyxin/ezrin interactions is not known, it may be that direct interaction transiently disrupts binding of podocalyxin to NHERF proteins, thereby enabling regulatory events such as phosphorylation and consequent conformational changes (He et al., 2001).

Until very recently, the search for podocalyxin binding partners had focused on the glomerulus, but podocalyxin is also expressed in the hematopoietic system. In order to

find hematopoietic specific interactors, an early hematopoietic cDNA expression library (McNagny et al., 1996) was screened with a biotinylated peptide corresponding to the cytoplasmic tail of podocalyxin (Tan et al., 2006). This screen identified NHERF1 as a podocalyxin interacting protein in the hematopoietic system (Tan et al., 2006). The podocalyxin peptide was also used to purify interacting proteins from hematopoietic progenitor cell extracts; mass spectrometry confirms the association of podocalyxin and NHERF1. As expected, this interaction is dependent on the C-terminal PDZ recognition sequence of podocalyxin. These two proteins also colocalize and form a co-immunoprecipitable complex in hematopoietic progenitors. Interestingly, strong colocalization corresponds to cells in which podocalyxin is capped to one pole; cells expressing uniform levels of podocalyxin over the entire surface do not display colocalization of the two proteins. Importantly, essentially all hematopoietic cells, including those displaying cell surface markers indicative of stem cells, express NHERF1, which suggests that it may be an important ligand for podocalyxin in these cells (Tan et al., 2006).

1.4.3 Other CD34-Family Binding Proteins

To date, there has been only one study investigating potential intracellular binding partners for endoglycan. Using the hematopoietic cDNA library screen described above, the cytoplasmic tail of endoglycan, like that of podocalyxin, was also shown to bind NHERF1 (Tan et al., 2006). Similarly, affinity purification from hematopoietic progenitor lysates indicates that NHERF1 can associate with a peptide corresponding to the C-terminus of endoglycan. Strikingly, the same experiments reveal a lack of

interaction of NHERF1 with CD34. Although CD34 does contain a C-terminal PDZ recognition sequence, it differs from both podocalyxin and endoglycan, which are identical: podocalyxin contains a DTHL motif, while the corresponding sequence in CD34 is DTEL (Kershaw et al., 1995; Suda et al., 1992). This difference likely conveys the specificity for interaction of podocalyxin and endoglycan with NHERF proteins, and suggests that while the three proteins may have some overlapping functions, there are probably some key differences.

Although CD34 does not appear to interact with NHERF family members, in hematopoietic progenitor cells it has been shown to interact with CrkL, a member of the Crk family of adapter proteins (Felschow et al., 2001). These proteins link proteins that do not possess kinase activity to intracellular signaling cascades, thereby enabling them to indirectly transmit signals. Although the exact binding site for CrkL on CD34 has not yet been determined, it is dependent on a highly conserved intracellular 10 aa juxtamembrane sequence present in both isoforms of CD34. The intracellular sequences of podocalyxin and endoglycan are more similar to each other than to CD34, so the existence of diverse binding partners is not surprising (He et al., 1992; Kershaw et al., 1997a; McNagny et al., 1997; Sassetti et al., 2000; Simmons et al., 1992).

1.5 Proposed Functions of CD34 Family Members

Remarkably, although there are over 12 000 CD34-related publications to date, the functions of CD34 and its family members remain to be ascertained. A variety of potential functions have been proposed, however, including roles in enhancing

proliferation, blocking differentiation, acting as adhesive ligands, preventing cell adhesion, establishing polarity, and regulating asymmetric cell division.

1.5.1 Enhancing Proliferation and Blocking Differentiation

There are two reasons for CD34's hypothesized role in enhancing proliferation or blocking differentiation. First, its expression on multipotent hematopoietic progenitors and progressive downregulation on more mature cells suggests a role in maintenance of the stem cell phenotype (reviewed in (Krause et al., 1996)). Second, in one strain of CD34 knockout (KO) animals, there are less progenitor cells in embryonic and adult tissues, and adult-derived progenitors appear to have a proliferation defect (Figure 1-4A) (Cheng et al., 1996). In comparison to wildtype animals, CD34 knockouts have a significant decrease in total colony forming unit (CFU) progenitor cells in adult as well as a reduction in fetal liver-derived erythroid and myeloid progenitors, despite the fact that there is no clear defect in absolute numbers of mature cells in the hematopoietic system of adult animals. Although this phenotype could also be explained by decreased survival of progenitors or a decline in their retention in the bone marrow, the fact that there is no increase in these cells in the periphery, and the observation that although progenitors survive *in vitro*, there is little expansion in comparison to wildtype-derived cells, both favour the initial idea.

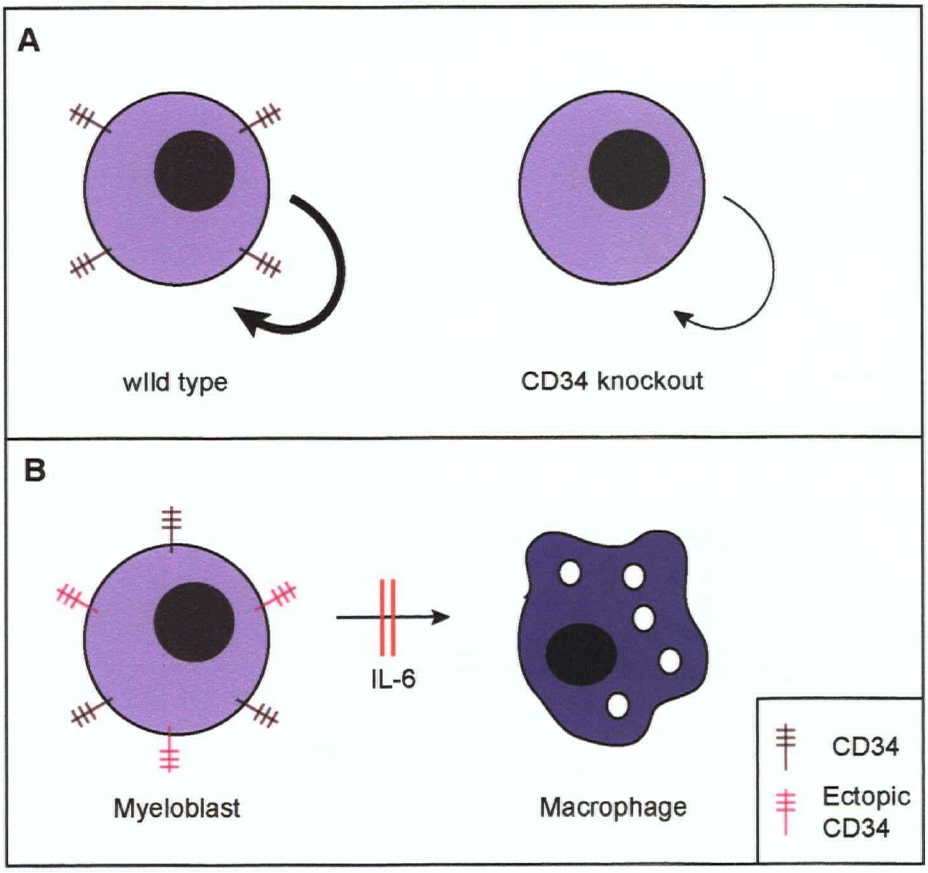

Figure 1-4: Proposed Functions for CD34 Family: Enhancing Proliferation and Blocking Differentiation.

(A) Hematopoietic progenitor cells from CD34-null mice proliferate less in comparison to cells from wildtype mice. (B) The $CD34^+$ progenitor cell line, M1, (light blue) can be induced to differentiate into macrophages (dark blue) upon addition of IL-6 or LIF. Ectopic expression of CD34 in this cell line appears to block differentiation.

A CD34-dependent block in differentiation is supported by one set of *in vitro* overexpression experiments (Fackler et al., 1995). The $CD34^+$ murine myeloblastic leukemia cell line, M1 can be induced to terminally differentiate into macrophages upon treatment with interleukin-6 (IL-6) or leukemia inhibitory factor (LIF), at which point CD34 is downregulated (Suda et al., 1992). Within 24 hours of LIF or IL-6 treatment, there is a maximal reduction in CD34 mRNA levels, followed by differentiation into morphologically mature, functionally active macrophages within three days. When CD34 is ectopically expressed in M1 cells, it appears to block IL-6 and LIF induced differentiation: cells are morphologically immature as they display minimal vacuolation, a high nucleus to cytoplasm ratio, open chromatin, and prominent nucleoli, and they are virtually non-phagocytic (Figure 1-4B) (Fackler et al., 1995). Notably, the naturally occurring splice variant of CD34 is incapable of blocking differentiation. Thus, there is evidence to suggest that CD34 may be involved in blocking differentiation or enhancing progenitor proliferation.

However, there is also mounting evidence to indicate that CD34 family proteins are <u>not</u> involved in enhancing proliferation or blocking differentiation. Regarding the CD34-null phenotype, there is a second strain of mice lacking CD34, and these mice do not display detectable defects in progenitor cell populations (Suzuki et al., 1996). Total bone marrow cell number and relative ratios of Ter119, B220, Mac-1, Gr-1, CD3, c-Kit, and Sca-1 expressing cells are all normal, as are numbers of platelets, red blood cells, white blood cells, and white blood cell subpopulations in peripheral blood. Importantly, culture of bone marrow cells with stem cell factor (SCF), IL-3, and erythropoietin *in vitro* produces

normal numbers of progenitors, as measured by colony assays. Furthermore, when mast cells (the only mature CD34 expressing hematopoietic cells) from these mice are cultured *in vitro*, there is no difference in kinetics of proliferation, differentiation, or degranulation (Drew et al., 2002; Drew et al., 2005). Moreover, in contrast to the overexpression of CD34 in M1 cells, ectopic expression in two other cell lines fails to block differentiation (Fackler et al., 1995). Also with respect to the M1 experiments, it should be kept in mind that two characteristics used to measure differentiation were adhesion and phagocytosis, which could both potentially be affected by an alternative CD34 function: blocking adhesion (Nielsen et al., 2002) (discussed below). Thus, it is unlikely that CD34 family members play an important role in enhancing proliferation or blocking differentiation. Instead, the observed phenotypes may be the result of other CD34-related functions.

1.5.2 Pro-Adhesion

There is convincing evidence showing that CD34 family members can act in an adhesive manner for recruitment of lymphocytes. Leukocytes are constantly recruited from the blood into secondary lymphoid organs and sites of chronic inflammation in a multi-step process involving low affinity binding (known as rolling) followed by integrin-mediated firm arrest and transendothelial migration (reviewed in (Butcher and Picker, 1996; Lasky, 1992)). The initial step, which requires adhesion of leukocytes to endothelial cells under conditions of vascular blood flow, depends upon association of selectin molecules with their ligands. Recruitment of lymphocytes into peripheral lymph nodes requires binding of L-selectin on lymphocytes to particular carbohydrate modifications on proteins expressed on the specialized postcapillary venules known as high endothelial venules

(HEV). These sulfated and sialylated O-linked carbohydrates are presented to L-selectin by mucin-like glycoproteins, including glycosylation-dependent cell adhesion molecule (GlyCAM)-1, mucosal addressin cell adhesion molecule (MAdCAM)-1, and potentially endomucin ((Samulowitz et al., 2002), and reviewed in (Rosen et al., 1997)).

The sialomucins CD34, podocalyxin, and endoglycan can all act in a pro-adhesive manner as ligands for L-selectin on HEV (Figure 1-5). The role of CD34 as an L-selectin ligand was first demonstrated in 1993, when it was shown that a sulfated, HEV restricted form of CD34 is recognized by L-selectin (Baumhueter et al., 1993). Similarly, podocalyxin is also modified with the appropriate glycosylations for L-selectin binding in HEV, and *in vitro* it supports L-selectin dependent tethering and rolling of lymphocytes under conditions of flow (Sassetti et al., 1998). Endoglycan can also act as a ligand for L-selectin, if expressed in conjunction with the necessary enzymes for appropriate post-translational modification (Fieger et al., 2003). Endoglycan binding does, however, involve alternative glycosylations from those present in CD34 and podocalyxin. Thus, all three molecules can function as adhesive ligands for L-selectin.

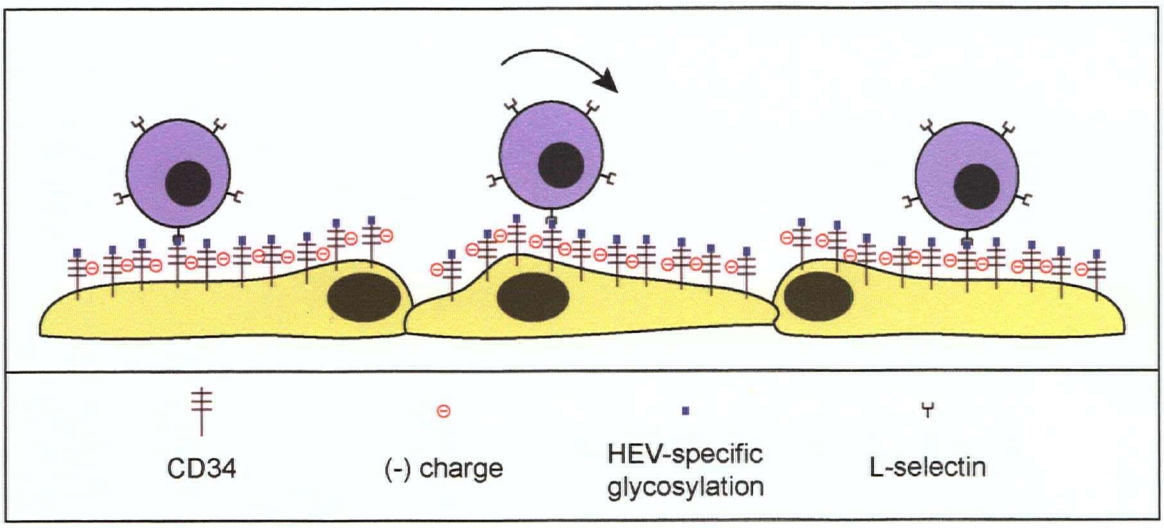

Figure 1-5: Proposed Function for CD34 Family: L-selectin Mediated Adhesion.

CD34 family members expressed on HEV enable L-selectin-mediated adhesion. Adapted from (Nielsen et al., 2002).

Although the evidence to support an adhesive function for CD34 family proteins is convincing, it is unlikely that this is their universal role. Optimal recognition of glycoproteins by L-selectin requires exquisitely specific modifications, including sialylation, fucosylation, and sulfation (Rosen et al., 1997). In the case of CD34 and podocalyxin, the epitope recognized by L-selectin is termed MECA-79; this epitope is not found on CD34 or podocalyxin expressed on other vascular endothelial cells or on podocalyxin on glomerular podocytes (Baumhueter et al., 1993; Michie et al., 1993; Sassetti et al., 1998; Segawa et al., 1997). Moreover, I have demonstrated that podocalyxin and CD34 expressed on fetal liver cells do not interact with L-selectin, or any other common selectin molecules (Doyonnas et al., 2005). Of note, it has been shown that two sulfotransferases that can modify CD34 have distinct expression patterns (Bistrup et al., 1999). *In vitro* expression of these two enzymes, along with a fucosyltransferase, facilitates modification of CD34 for L-selectin recognition (Bistrup et al., 1999). Absence of individual sulfotransferases does not prevent production of L-selectin ligands, but when both enzymes are present, they synergize to produce a ligand with much greater affinity for L-selectin. Importantly, while the Gal-6-sulfotransferase displays a wide tissue distribution, the GlcNAc-6-sulfotransferase is highly restricted to high endothelial cells. Thus, the expression pattern of this, or other modifying enzymes, may explain the interaction of L-selectin with CD34 family members only in HEV. The question, then, is what the alternative function is for these sialomucins in other tissues.

1.5.3 Anti-Adhesion

A proposed podocalyxin function that is gaining considerable support is a contrasting role in blocking adhesion. Cells regulate adhesion by adjusting levels of adhesion molecules, such as integrins, as well as anti-adhesins. Anti-adhesins are often cell surface associated mucins (Wesseling et al., 1996). Mucins are heavily sialylated, O-linked glycoproteins that can block adhesion either by steric hindrance or by charge repulsion, both properties conveyed by their bulky, negatively charged extracellular domains. Since podocalyxin and the related family members have this type of extracellular domain, they have been proposed to act in this capacity (Figure 1-6). Sialomucins are often found on the luminal surface of vessels where they prevent blockage of the lumen caused by weak, non-specific interaction of molecules on opposing luminal membranes (Hilkens et al., 1992). Similarly, expression by malignant tumours can decrease adhesion and prevent immune recognition. Podocalyxin expression on circulating platelets may also prevent their inappropriate adhesion to vessel walls (McNagny et al., 1997). Thus, it seems plausible that the CD34 family may have global anti-adhesive functions.

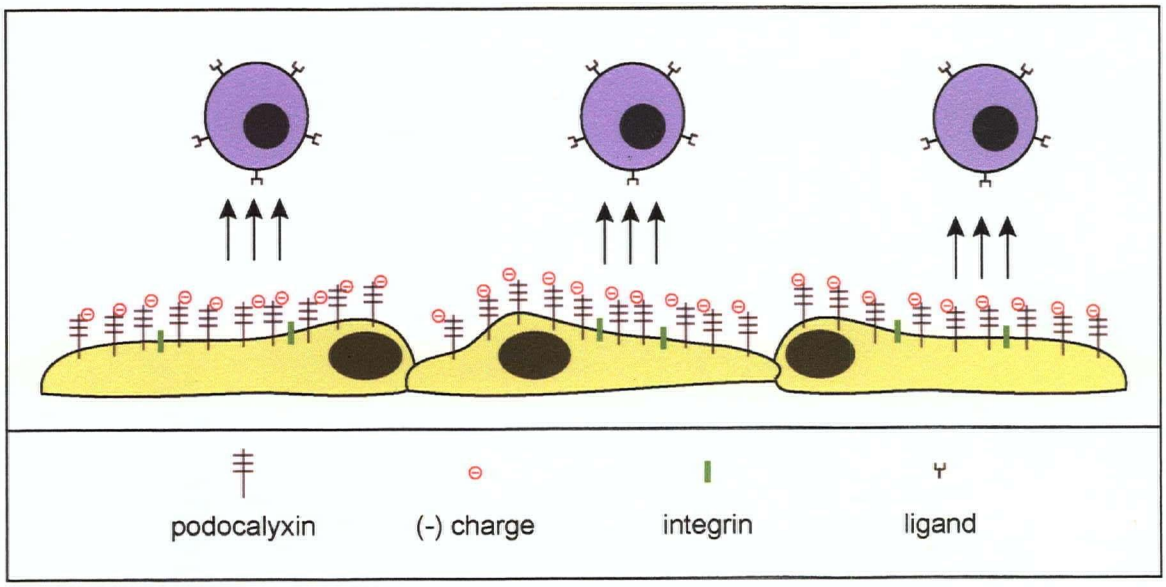

Figure 1-6: Proposed Function for CD34 Family: Blocking Adhesion.

CD34 family members can block cell-cell adhesion by charge repulsion or steric hindrance. Adapted from (Nielsen et al., 2002).

There are several lines of evidence supporting the role of podocalyxin and CD34 in blocking cell adhesion. Ectopic expression of podocalyxin in Chinese hamster ovary (CHO) cells completely blocks cell-cell adhesion in aggregation assays (Takeda et al., 2000). This effect is due to charge repulsion, as treatment with sialidase to remove podocalyxin's negatively charged sialic acid residues abrogates the effect. A similar effect is seen in aggregation assays using podocalyxin-transfected MDCK cells. In addition, podocalyxin expression decreases the strength of tight junctions in MDCK cell monolayers. Localization of junctional proteins is more variable, and transepithelial resistance (TER) decreases slightly. Podocalyxin thus blocks cell-cell adhesion and interferes with cell-cell junctions.

The potential role of signalling in the podocalyxin-related reorganization of cell junctions was recently assessed (Schmieder et al., 2004). Since podocalyxin interacts with ezrin, which is maintained in an open and active conformation in a Rho-dependent manner, this pathway was examined (Chen et al., 1995; Matsui et al., 1999; Schmieder et al., 2004; Shaw et al., 1998). In this pathway, it is thought that RhoA activates ezrin, which in turn binds RhoGDI, a negative regulator of Rho GTPases (Chen et al., 1995; Matsui et al., 1999; Shaw et al., 1998; Takahashi et al., 1997). Sequestration of RhoGDI thereby disrupts the RhoA/RhoGDI complex and permits RhoA activation; this, in turn, maintains ezrin activation. Active RhoA is then able to translocate to the plasma membrane where it interacts with effector proteins to mediate downstream signalling and induce actin reorganization (Schmieder et al., 2004). Of interest, RhoGDI$\alpha^{-/-}$ mice exhibit massive

proteinuria and disruption of foot process architecture, implying a role for proper regulation of Rho GTPases in podocyte structure (Togawa et al., 1999).

In MDCK cells transfected with full-length podocalyxin or podocalyxin lacking the NHERF binding site, RhoA activation status and distribution of RhoA, RhoGDI, and actin have all been assessed (Schmieder et al., 2004). In this system, ectopic podocalyxin expression increases RhoA activation in a manner dependent on the interaction of podocalyxin with NHERF. Likewise, stimulation of β_2-adrenergic receptors and purinergic receptors, both of which are connected to actin through ezrin and NHERF1, also leads to RhoA activation, suggesting that perhaps NHERF proteins recruit a molecule responsible for activation of RhoA (Sauzeau et al., 2000; Schmieder et al., 2004; Yamauchi et al., 2001). However, NHERF is not responsible for redistribution of either RhoA or RhoGDI. In control cells, RhoA is found throughout the cytoplasm and is concentrated in the juxtanuclear region. In contrast, RhoA is partially redistributed toward the plasma membrane in cells ectopically expressing full-length or truncated podocalyxin. Similarly, RhoGDI is relocalized toward the apical membrane. Since activated ezrin binds RhoGDI, this relocalization is likely the result of a direct interaction with ezrin and apically expressed podocalyxin (Schmieder et al., 2004). Importantly, the podocalyxin-dependent activation and relocalization of RhoA, along with the association of RhoA with regulation of the structure and function of tight junctions provide a possible explanation for podocalyxin's effects on junctional proteins (Jou et al., 1998; Schmieder et al., 2004).

An independent group confirmed the effect of podocalyxin overexpression on cell-cell junctions in MDCK cells (Li et al., 2002). Furthermore, they demonstrated that ectopic expression of podocalyxin lacking the C-terminal PDZ binding domain is also sufficient to decrease TER, albeit to a lesser extent than full-length podocalyxin. It can therefore be concluded that while interaction with NHERF proteins greatly enhances the weakening of cell-cell junctions, it is not absolutely required for this effect. A similar reorganization of cell-cell junctions is thought to exist in podocalyxin-expressing glomerular podocytes (discussed in section 1.6.3below).

Additionally, immortalized human glomerular epithelial cells (HGEC), which mimic the phenotype of podocytes, have been used to assess podocalyxin function (Economou et al., 2004). When these cells are cultured on laminin or a complex mixture of glomerular basement membrane components, podocalyxin expression is upregulated. Unlike the normal protein expression pattern, however, some podocalyxin is expressed on the basolateral surface of these cells, but this is likely an artifact of the system. Regardless, podocalyxin expression leads to decreases in adhesion to laminin in cell adhesion assays. Thus, in this independent model system, podocalyxin is also able to block cell adhesion.

Moreover, there is some evidence to suggest that CD34 family members interfere with integrin-mediated adhesion. Cell-substrate adhesion in HGEC is mediated by integrins, as addition of saturating concentrations of anti-β_1 integrin antibodies blocks adhesion. Strikingly, however, when non-saturating concentrations of anti-β_1 integrin antibodies are added in combination with increasing concentrations of anti-podocalyxin antibodies, the

podocalyxin antibodies block podocalyxin's anti-adhesive effects, and adhesion increases. Similarly, anti-CD34 antibodies trigger integrin-mediated adhesion of progenitor cells (Majdic et al., 1994). This adhesion has been proposed to be a result of CD34 signalling (Cheng et al., 1996), but capping of CD34 may expose integrins and provide an alternative explanation for the increase in adhesion. Most experiments involving overexpression of CD34 family members therefore suggest an anti-adhesive function for these proteins.

Expression of CD34 by a subset of mature hematopoietic cells (mast cells) and the existence of viable CD34-null animals provides another excellent tool for assessing its function (Cheng et al., 1996; Drew et al., 2002; Drew et al., 2005; Suzuki et al., 1996). Comparison of bone marrow derived mast cells from wildtype and CD34 knockout animals demonstrates that CD34 is necessary and sufficient to prevent cell-cell adhesion in this system (Drew et al., 2005). Wildtype cells form single cell suspensions, whereas cells lacking CD34 form small aggregates. The defect can be reversed by ectopic expression of CD34, and the naturally occurring splice variant is an even more potent anti-adhesin. It is possible that the full-length isoform can be relocalized in the plasma membrane in order to expose adhesion molecules, whereas the isoform lacking much of the cytoplasmic tail may not interact with proteins involved in its membrane localization. An important observation, however, is that the distantly related sialomucin, CD43, is a much more effective anti-adhesin: loss of CD34 leads to aggregation of 16 ± 7 % of cells, while 70 ± 20 % of CD43-null cells form aggregates.

Further evidence for the anti-adhesive effects of podocalyxin and CD34 have been provided by *in vivo* repopulation studies. In the first set of experiments, short-term homing assays were performed with wildtype, podocalyxin-null, CD34-null, and double-deficient fetal liver cells (Doyonnas et al., 2005). Single knockout cells are less efficient at homing to bone marrow, and cells lacking both molecules display an additive defect. Although this could be explained by a loss of specific homing molecules, there is no decrease in binding to the only known receptors for these molecules, the selectins. Thus, it is more likely that the loss of anti-adhesion molecules results in non-specific adhesion to endothelial cells of vessels *en route* to the bone marrow.

The second experiment involved repopulation of the peritoneal cavity by mast cells after their ablation by injection of water into wildtype, CD34-null, CD43-null, and double-deficient mice (Drew et al., 2005). Double-deficient mice display delayed mast cell repopulation kinetics. While individual loss of CD34 also appears to delay repopulation, the results are not statistically significant. In another system, wildtype and CD34- or CD43-deficient bone marrow cells were injected into sub-lethally irradiated mast cell deficient W/W^v mice in competitive repopulation experiments, and mast cell reconstitution was assessed after 11-12 weeks. Notably, almost all mast cells in mice injected with a combination of wildtype and double-deficient cells are derived from wildtype bone marrow cells. Again, although the trend is the same with single knockout cells, the results are not statistically significant. Strikingly, in the same mice, there is a significant decrease in CD34-deficient hematopoietic progenitor cell engraftment, whereas $CD43^{-/-}$ cells engraft normally. Again, loss of CD34 may lead to increased non-

specific adhesion to vessels *en route* to the bone marrow. Thus, *in vivo* data supports the anti-adhesion hypothesis.

One other interesting observation is that CD34 expression is downregulated upon IL-1 induced upregulation of the adhesion molecules ICAM-1 and endothelial leukocyte adhesion molecule (ELAM)-1 (Delia et al., 1993). This is further support for the idea that podocalyxin and CD34 may inhibit adhesive functions of vascular endothelial cells, and downregulation (Delia et al., 1993) or relocalization may enable adhesion when necessary. As discussed, there is therefore mounting evidence supporting an anti-adhesive function for CD34 and podocalyxin.

1.5.4 Establishing Polarity

An alternative and particularly interesting podocalyxin function was recently proposed involving establishing polarity in epithelial cells (Meder et al., 2005). Although many cells polarize transiently, epithelial cells of kidney, intestine, and other organs become terminally polarized upon creation of monolayers. This requires separation of apical and basolateral membrane domains by intracellular sorting or selective retention of membrane components as well as the formation of junctional complexes (Mellman and Warren, 2000; Mostov et al., 2003). In this study, it was hypothesized that epithelial polarization begins with establishment of an apical pole in single cells (Meder et al., 2005).

In order to assess polarization of MDCK cells, individual cells were plated and stained for cell surface markers shortly thereafter, or after four hours upon formation of small

clusters of cells (Meder et al., 2005). Although localization of numerous apical and basolateral markers was assessed, all proteins display uniform expression patterns with the exception of E-cadherin, which is enriched at cell-cell contact sites, and gp135, which is apically localized on single cells within one hour. At the time, the identity of gp135 was unknown, but its unique expression exclusively on the free surface of cells prompted further study; as discussed in section 1.2, it is now known to be canine podocalyxin (Meder et al., 2005). The early polarized distribution of podocalyxin, in contrast to other typical markers, suggests that it may play a role in this process. This was investigated further by depleting endogenous podocalyxin expression by RNA interference (RNAi). Polarization, as assessed by marker distribution on MDCK monolayers, was delayed in cells lacking podocalyxin (Meder et al., 2005).

A model often used to assess polarization in MDCK cells is that of cyst formation in a collagen matrix. In this assay, single cells grow and form multicellular, polarized cysts each with a central lumen. Strikingly, many podocalyxin-depleted MDCK cells form cysts with multiple lumens, or cysts completely devoid of lumens (Meder et al., 2005). Although the 2005 publication by Meder *et al* (Meder et al., 2005) was the first to suggest a role for podocalyxin in establishing polarity, apical expression of gp135 (later identified as podocalyxin) was noted in MDCK cells lacking cell junctions in a paper published over a decade earlier (Ojakian and Schwimmer, 1988). In addition, removal of surface expressed gp135 by trypsinization is followed by rapid reinsertion of podocalyxin into the plasma membrane, but only at the apical surface (Ojakian et al., 1990). Thus, recent

results suggest the exciting possibility that podocalyxin in involved in regulating cell polarity.

The same group addressed the mechanism involved in podocalyxin-induced cell polarization (Meder et al., 2005). Since mutant podocalyxin lacking the C-terminal DTHL sequence displays slightly less restricted localization, it was hypothesized that NHERF proteins could also play a role in early cell polarization. MDCK cells were transfected with a green fluorescent protein (GFP)-tagged NHERF2 fusion protein in order to follow NHERF2 localization during MDCK cell polarization. Localization of this protein is similar to that of endogenous podocalyxin throughout all stages of early polarization, and the two proteins form a co-immunoprecipitable complex that appears to strengthen as cells become more polarized. Since NHERF proteins contain tandem PDZ domains with differing binding specificities, they are able to dimerize and cross-link multiple ligands (Shenolikar et al., 2004). In the case of epithelial polarization, perhaps NHERF proteins can link multiple ligands with the ERM family of cytoskeletal adapters in order to establish a pre-apical membrane scaffold for directing junction formation and membrane trafficking (Meder et al., 2005).

1.5.5 Podocalyxin and NHERF: A Role in Asymmetric Cell Division?

Admittedly the potential role of podocalyxin in asymmetric cell division is based on very little evidence, but it is an exciting possibility worth considering. Asymmetric cell division has recently gained much attention in the hematopoietic system, as stem cells must divide in this manner in order to produce daughter cells with differing properties.

Maintenance of the stem cell pool requires that one daughter cell remain a stem cell, while the other begins to populate the hematopoietic system by virtue of its increased progress along the differentiation pathway (reviewed in (Ho, 2005; Wilson and Trumpp, 2006)).

There are two models that address the mechanisms underlying asymmetric division. The first suggests that external stimuli following cell division provide cues that determine cell fate. This is based on the idea of a stem cell niche and division along a plane in which one daughter cell remains in contact with the niche, and therefore remains an HSC while the other does not. The second model suggests that molecules within a cell are redistributed unequally before division, such that those required for maintaining an immature cell are on one side while determinants of a more mature cell are on the opposite side. The expression of podocalyxin in early hematopoietic progenitor cells, but perhaps not in the most immature hematopoietic stem cells, and its association with the actin cytoskeleton and the free surface of cells suggests that it could play a role in either situation (Figure 1-7). In the first case, podocalyxin's association with the free surface of cells may enable an immature HSC to adhere to the niche via adhesion molecules segregated from the podocalyxin-containing domain. Then, after division, the podocalyxin-containing cell will be destined to become more mature, while the adherent cell will remain in contact with the niche, thereby receiving the signals required for maintaining the immature state. On the other hand, perhaps podocalyxin's interaction with NHERF1 facilitates segregation of a multitude of fate determining proteins on one side of the cell before cell division. The fact that podocalyxin and NHERF1 colocalize to

one side of cells upon IL-3 stimulation of a hematopoietic progenitor cell line provides some evidence for relocalization prior to cell division (Tan et al., 2006).

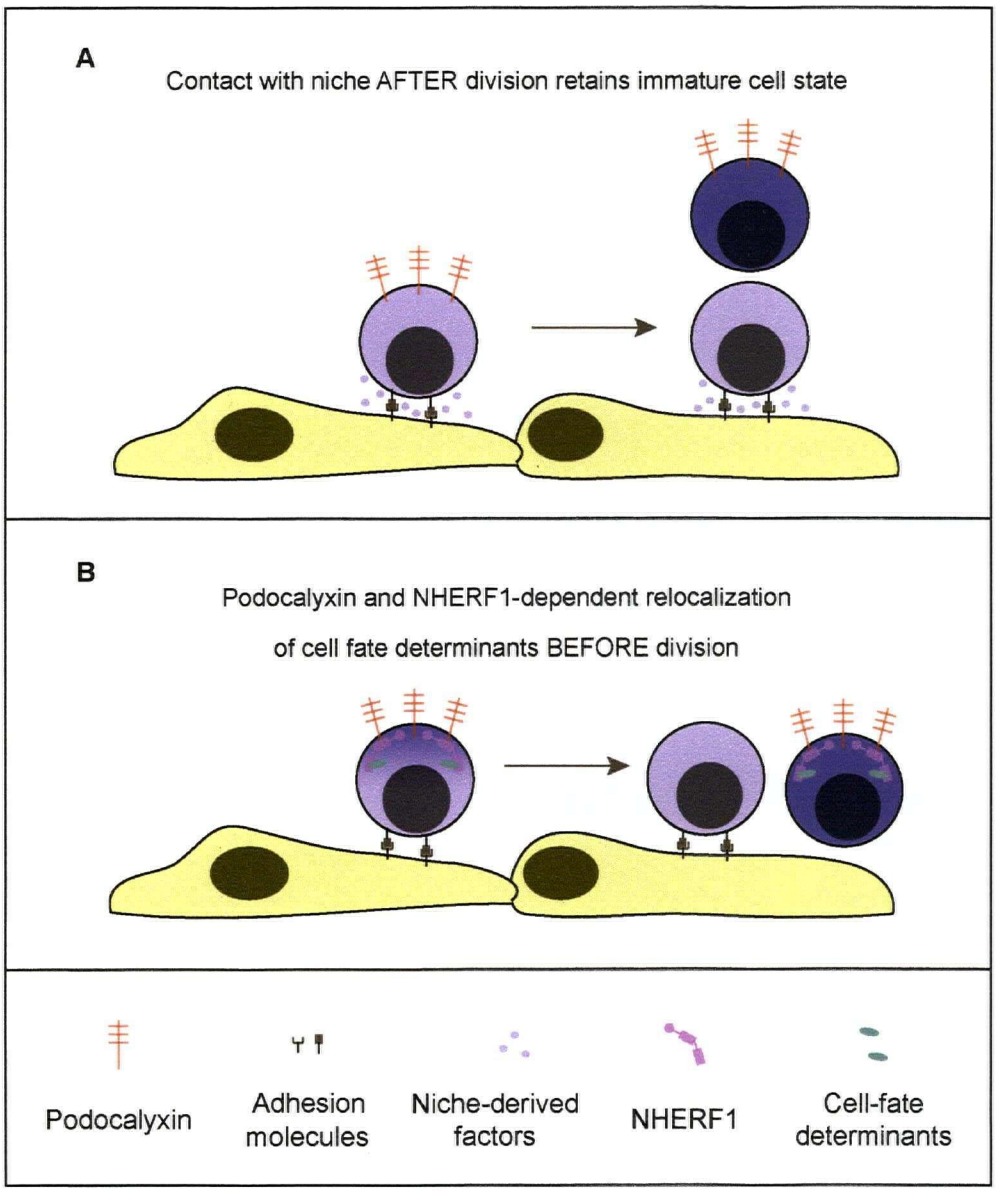

Figure 1-7: Two Models of Podocalyxin-Dependent Asymmetric Cell Division.

(A) Podocalyxin is segregated from adhesion molecules, which allows the HSC to adhere to the niche. After division, the cell remaining in contact with the niche receives signals to remain immature, while the podocalyxin-containing cell becomes more mature. (B) Prior to division, cell-fate determinants are relocalized to one side of the cell through interactions with NHERF1 and podocalyxin. Thus, after division, the cell containing podocalyxin is more mature. Lighter blue cells are less mature.

Podocalyxin, CD34, and endoglycan are related sialomucins with overlapping expression patterns and likely overlapping functions. Although a role in enhancing proliferation or blocking differentiation was initially proposed, there is more evidence in support of alternative functions. They can all act as adhesive tethers for L-selectin in HEV but are not appropriately modified for this function in other tissues. Globally, it is more likely that podocalyxin and CD34, at least, act as blockers of adhesion. In addition, there is recent evidence to suggest that podocalyxin may regulate cell polarity, and it is possible that the CD34 family could also be involved in regulation of asymmetric cell division. These functions all rely on a link to the actin cytoskeleton, which, in the case of podocalyxin, has been established to occur via interactions with ezrin and NHERF proteins.

1.6 Podocalyxin in Kidney Development

While much information can be gained through overexpression experiments and *in vitro* analysis, it is also necessary to investigate functional implications of a protein's expression in tissues where it is normally found. In the case of podocalyxin, it was first identified in the glomerulus of rat kidney, and its expression in this tissue is not only abundant, but also essential for podocyte development (Doyonnas et al., 2001; Kerjaschki et al., 1984).

1.6.1 Overview of Glomerular Development

Glomeruli are responsible for blood filtration and urine production in the kidney (Gao et al., 2004). They are composed of capillary loops lined with fenestrated endothelial cells, supporting mesangial cells, a glomerular basement membrane (GBM), and glomerular epithelial cells called podocytes (Gao et al., 2004). While vascular endothelial cells provide the first barrier in production of the glomerular filtrate, endothelial fenestrae provide an exit route for small molecules (Gelberg et al., 1996). The GBM consists of a network of collagen IV, laminin, heparan sulfate proteoglycans, and fibronectin (Economou et al., 2004); its size- and charge-selectivity provide the main selective barrier enabling retention of macromolecules in the circulation (reviewed in (Farquhar, 1975; Kanwar, 1984)).

Podocytes have a unique architecture with three main parts. The cell body contains the majority of the cell's cytoplasmic organelles, including the nucleus, mitochondria, rough and smooth endoplasmic reticulum, and well-developed Golgi, which are likely to be involved in formation and degradation of the GBM components (Takeda, 2003). Projections, termed major processes, extend from the cell body and surround the capillary loops (Andrews, 1979). Smaller projections, called foot processes, extend from the major processes, interdigitate with foot processes from neighbouring podocytes, and connect the basal surface of podocytes to the GBM, mainly through $\alpha_3\beta_1$ integrin-mediated focal contacts (Adler, 1992; Andrews, 1979). Foot processes lack cytoplasmic organelles, and instead contain a dense network of actin filaments, which stabilize the foot process structure through adhesion to the GBM and by forming connections with proteins of the

slit diaphragm complex (Figure 1-8) (Kobayashi et al., 2004; Takeda, 2003). Slit diaphragms are tenuous bridges between adjacent foot processes through which the glomerular filtrate must pass (Kurihara et al., 1992). Recent evidence suggests that, along with the GBM, slit diaphragms also play a role in filtration (reviewed in (Salant and Topham, 2003; Tryggvason, 1999)). The apical surface of podocytes, which faces the urinary space, is coated by a sialic acid-rich glycocalyx designated the epithelial polyanion, and now known to be mainly composed of podocalyxin (Kerjaschki et al., 1984; Michael et al., 1970).

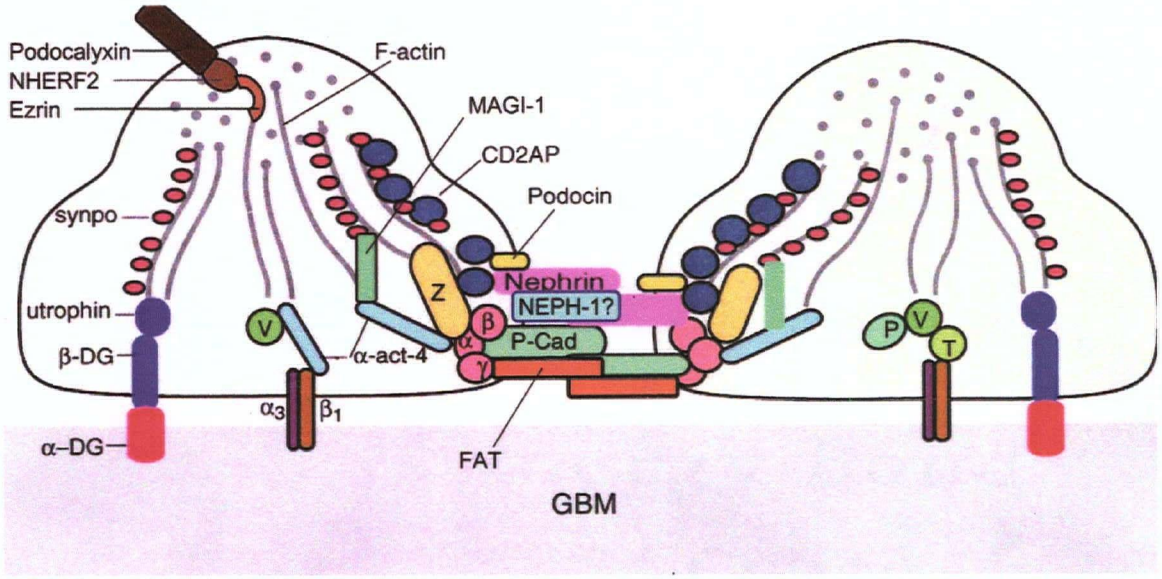

Figure 1-8: Schematic of Molecules Related to Podocyte Architecture.

This diagram shows two adjacent podocyte foot processes bridged by a complex of proteins that form the slit diaphragm. Proteins found in the basal domain, such as $\alpha_3\beta_1$ integrin ($\alpha_3\beta_1$), interact with the actin cytoskeleton and link podocytes with the glomerular basement membrane (GBM). Podocalyxin interacts with the actin cytoskeleton through NHERF2 and ezrin, and molecules of the slit diaphragm complex also interact with the cytoskeleton. Other abbreviations include α-act4: α-actinin-4, α-DG: α-dystroglycan, β-DG: β-dystroglycan, P: paxillin, P-cad: P-cadherin, Synpo: synaptopodin, T: talin, and V: vinculin. Reproduced with kind permission of Lippincott Williams & Wilkins (Mundel and Shankland, 2002).

During glomerular development, which proceeds via a series of loosely defined stages, mesenchymal cells are induced to condense and undergo mesenchymal to epithelial transition (Guo et al., 2002). They initially form a cluster of cells known as the renal vesicle (Guo et al., 2002; Reeves et al., 1978). The renal vesicle is invaginated by mesenchymal cells, and the cluster of cells present at this stage rearranges and matures via the comma- and S-shaped body stages to form a central lumen, which later becomes Bowman's space (Guo et al., 2002; Reeves et al., 1978). During the S-shaped body stage, mesangial cells develop from the mesenchymal cleft, while the cells on one side of the cleft begin to form the glomerular epithelium and those on the other side differentiate to form the proximal tubule and capillary endothelium (Reeves et al., 1978). The glomerular epithelial cells initially have tight junctions at their apices, but upon expression of podocalyxin the junctions begin to migrate laterally toward the GBM.

Throughout the next stage, known as the developing capillary loop stage, glomerular epithelial cells (podocytes) proliferate, and movement of the cell-cell junctions enlarges the intracellular spaces, which are continuous with Bowman's space (Reeves et al., 1978). Extensive morphological rearrangements occur whereby foot processes extend and junctions redistribute between foot processes. Slit diaphragms later replace these junctions (Reeves et al., 1978; Schnabel et al., 1989). Podocalyxin redistribution follows that of the junctions: it is always found along the apical surface of podocyte cell bodies and foot processes above the level of the slit diaphragms (Kerjaschki et al., 1984; Kurihara et al., 1992; Schnabel et al., 1989). The maturing glomerulus stage involves further maturation of podocytes, maturation of the GBM into a complex structure with

several layers, and fenestration of endothelial cells to form the final glomerular structure (Reeves et al., 1978).

1.6.2 Glomerular Diseases and Model Systems

Maintenance of the intricate architecture of glomerular podocytes is essential for optimal kidney function; numerous human diseases and animal models of glomerular malfunction involve disruption of these unique structures (Economou et al., 2004). Loss of foot process architecture is the main morphologic abnormality detected in patients with nephrotic syndrome (Farquhar et al., 1957). Diabetic nephropathy, a leading cause of chronic kidney failure and end-stage renal disease, also involves broadening of foot processes and is accompanied by a decrease in glomerular sialic acid content (Cardenas et al., 1991; Pagtalunan et al., 1997).

Puromycin aminonucleoside (PAN) nephrosis and protamine sulfate (PS) perfusion are two rodent models of glomerular disease (Farquhar and Palade, 1961; Kurihara et al., 1992). Sialylation of podocalyxin is reduced in PAN nephrosis, and PS neutralizes podocalyxin's negative charge (Kerjaschki et al., 1985; Seiler et al., 1977). Thus, in both models, the negative charge on podocalyxin is decreased, foot process architecture is disrupted, filtration slits are reduced in number, and slit diaphragms are displaced or completely replaced by leaky, discontinuous tight junctions; overall, the epithelium resembles that of immature glomeruli (Gao et al., 2004; Kurihara et al., 1992). Injection of sialidase intraperitoneally causes similar morphological alterations as a result of loss of sialic acid residues from the surface of podocytes (Gelberg et al., 1996). Features of these

models include decreased glomerular filtration and a perhaps counterintuitive increase in protein in the urine (proteinuria) (Bohrer et al., 1977; Kurihara et al., 1992). Reduction in glomerular charge selectivity results in an increase in GBM permeability and subsequent proteinuria. However, there is an overall decline in glomerular filtration as the collective slit pore area, and thereby the exit path for filtrate, decreases (Bohrer et al., 1977; Kurihara et al., 1992). Blockage of the exit path may also lead to a reactive elevation in glomerular blood pressure and a subsequent dramatic increase in permeability of the glomerular filter (Gelberg et al., 1996). Podocytes, their extensive expression of podocalyxin, and alterations in disease states have therefore been the focus of much research for several decades.

1.6.3 Early Studies of Podocalyxin

Although podocalyxin was not identified until 1984, the importance of the "epithelial polyanion" in maintenance of podocyte morphology was noted more than ten years earlier (Kerjaschki et al., 1984; Michael et al., 1970). A reduction in foot process number in association with loss of the epithelial polyanion was first proposed in 1970 (Michael et al., 1970). This was confirmed by experiments in which rat kidneys were perfused with polycations to neutralize the epithelial polyanion. This leads to distortion of podocyte morphology: slit pores are narrower or absent and foot processes are fused together (Seiler et al., 1977). In contrast, perfusion with polyanions and neutral molecules has no effect, although perfusion of anionic molecules <u>after</u> perfusion of cationic molecules partially reverses their disruptive effects. The phenotypes observed upon cation perfusion are analogous to those observed in proteinuric conditions, where podocyte morphology is

similar to that observed in immature glomeruli (Seiler et al., 1977). Thus, it is not surprising that appearance of the epithelial polyanion is associated with extension of foot processes during development: it was demonstrated in 1978 by Reeves *et al* that the epithelial polyanion, as detected by colloidal iron staining, first appears on the surface of glomerular epithelial cells just before formation of foot processes and slit pores (Reeves et al., 1978).

A major contributor to the negative charge on the epithelial polyanion is its high sialic acid content (Michael et al., 1970; Mohos and Skoza, 1969). This was demonstrated by the observed reduction in colloidal iron staining of podocytes after treatment with neuraminidase, which removes sialic acid residues. Similar to the effects seen with polycation perfusion, neuraminidase treatment also disrupts foot process architecture (Andrews, 1979). Foot processes fuse together, slit diaphragms are displaced, and junctional complexes, lost during development, reappear. Furthermore, it has been suggested that the defects observed in PAN models of nephrosis may be caused by interference with sialic acid metabolism in podocytes and a corresponding loss of the sialic acid content in the glycocalyx.

It was at this stage that the molecular identity of the epithelial polyanion was finally determined (Kerjaschki et al., 1984). Several characteristics of the epithelial polyanion had previously been demonstrated, and these features, along with it being the most abundant membrane glycoprotein in the glomerulus, enabled its isolation. Firstly, it is highly negatively charged, as demonstrated by its recognition by cationic histochemical

stains (Michael et al., 1970; Mohos and Skoza, 1969). Secondly, neuraminidase treatment alters its staining properties, thereby demonstrating its extensive sialic acid content. Thirdly, it is recognized by wheat germ agglutinin before neuraminidase treatment and peanut agglutinin afterwards (Holthofer et al., 1981). Using these characteristics, a single 140 kDa protein, composed of approximately 20 % hexose and 4.5 % sialic acid by weight, was isolated, characterized, and named podocalyxin (Kerjaschki et al., 1984). The sialic acid rich nature of podocalyxin can be fully appreciated when it is realized that it contains the majority of the protein-bound sialic acid of the glomerulus. The presence of O-linked glycosylations was implied based on the binding of peanut lectin after neuraminidase treatment; the simultaneous presence of some N-linked oligosaccharide chains was also suggested by the binding of concanavalin A. Considerable analysis has been undertaken to begin to characterize the oligosaccharide chains of podocalyxin's mucin-like domain, including their sialylations and sulfations (Dekan et al., 1991). Mucin domains are predicted to be rigid, extended structures, so presumably podocalyxin's extracellular domain functions as an attachment site for the many negatively charged moieties that contribute to the anionic glycocalyx of podocytes ((Kershaw et al., 1997a), and reviewed in (Jentoft, 1990)). Thus, 15 years after detection of the epithelial polyanion, its molecular nature was determined.

The identification of podocalyxin and generation of specific monoclonal antibodies facilitated a detailed assessment of its expression pattern (Kerjaschki et al., 1984). In adult, podocalyxin is highly expressed on the apical surface of podocytes and at 5-10 fold lower levels on endothelial cells (Dekan et al., 1990; Kerjaschki et al., 1984). It covers all

surfaces of podocytes facing the urinary space, including the apical surface of cell bodies and foot processes above the level of slit diaphragms; it is not expressed on the basal surface of podocytes or on the abluminal face of endothelial cells (Kerjaschki et al., 1984; Sawada et al., 1986). These detailed studies also provided some insights into podocalyxin's function. It had previously been proposed that the purpose of its prominent negative charge was to provide a charge-selective barrier for glomerular filtration. However, the main barrier performing this function is located at the level of the GBM, where podocalyxin expression is lacking, so it is instead postulated to be involved in maintenance of structural integrity of foot processes (Farquhar, 1975; Kerjaschki et al., 1984; Sawada et al., 1986).

Podocalyxin expression and localization during development has also been followed carefully in rat (Schnabel et al., 1989). Rodent kidneys provide an excellent model for studying kidney development for two reasons. First, throughout development new renal vesicles are continually formed at the periphery of the kidney cortex, with more mature nephrons near the cortical-medullary junction, so all stages of maturation are visible in a single kidney. And second, this process is not completed until one week after birth in rodents, in contrast to the situation in humans, so glomerular development can easily be investigated in newborn rodents (Reeves et al., 1978). The earliest detectable podocalyxin is expressed on capillary endothelial cells in the mesenchymal tissue surrounding the renal vesicle (Schnabel et al., 1989). Subsequently, the first podocalyxin expression on cells that eventually become podocytes is detected as the pre-Bowman's space lumen forms. Podocalyxin is initially localized to the apical surface of these glomerular

epithelial cells, but it then moves along the lateral surface toward the basal surface as cell-cell junctions migrate in a similar fashion. Podocalyxin is always expressed on the entire apical surface and along the lateral membrane to the position immediately above tight junctions. Its expression extends along the tops of foot processes, but it is never expressed on the basal surface facing the GBM. Upon replacement of tight junctions with slit diaphragms, podocalyxin expression is maintained on the entire podocyte surface above this level. Moreover, in immature podocytes podocalyxin is detected in the endoplasmic reticulum, Golgi apparatus, and carrier vesicles of the biosynthesis pathway demonstrating that it is synthesized at a high rate in these cells.

Since neutralization of the epithelial polyanion (podocalyxin) leads to alterations in podocyte morphology, disease models were used to gain further insights into the mechanisms involved. For example, in PAN nephrosis, actin filament reorganization occurs (Whiteside et al., 1993); since podocalyxin is linked to actin through NHERF2 and ezrin in podocytes (Li et al., 2002; Orlando et al., 2001; Takeda et al., 2001), co-immunoprecipitation and sequential detergent extraction experiments were performed in order to investigate potential disruptions of this complex in disease states (Takeda et al., 2001). In PAN nephrosis, there is little, if any, disruption of the podocalyxin/NHERF2/ezrin complex, but the overall amount of phosphorylated (active) ezrin decreases and the entire complex dissociates from the actin cytoskeleton. Upon protamine sulfate perfusion, NHERF2 and ezrin remain bound to actin, but podocalyxin dissociates from the complex. Sialidase treatment leads to dissociation of podocalyxin from the NHERF2/ezrin complex, which also dissociates from actin. Thus, in all three

models, the association of podocalyxin with the actin cytoskeleton is disrupted, providing a possible explanation for the morphological changes noted in podocyte foot processes. How exactly an alteration in the surface charge of podocalyxin affects the complex is not known, but it may be that neutralization of the charge induces a conformational change in the cytoplasmic tail of podocalyxin which prevents binding. All of the early studies of the epithelial polyanion and podocalyxin in the kidney suggest a very important role for this molecule in kidney development. Generation of podocalyxin-null mice demonstrated exactly how important this molecule is for glomerular development.

1.6.4 Lessons from the Podocalyxin Knockout

Podocalyxin knockout mice were generated by homologous recombination in which the majority of exons five, six, seven, and eight were deleted; loss of expression was confirmed by Northern blot (Doyonnas et al., 2001). Since CD34-null mice do not have any major defects in tissues that normally co-express podocalyxin and CD34 (Cheng et al., 1996; Suzuki et al., 1996), abnormalities were not expected in these tissues in podocalyxin-null mice either. Instead, cells that express only podocalyxin, particularly podocytes, were expected to have more obvious defects (Doyonnas et al., 2001).

1.6.4.1 Podocalyxin is Essential for Podocyte Morphogenesis

The expected defects in podocytes lacking podocalyxin made the glomeruli an excellent initial target for analysis in podocalyxin-null animals (Doyonnas et al., 2001). For the most part, however, podocyte maturation is unaffected in knockout mice. Nephrin is a

component of the slit diaphragm (discussed in section 1.6.5.1, below), and glomerular epithelial protein (GLEPP)-1 is a transmembrane tyrosine phosphatase of podocytes; both are considered markers of podocyte differentiation (Doyonnas et al., 2001; Kawachi et al., 2002; Wang et al., 2000). Immunohistochemical staining for these two proteins is normal in mice lacking podocalyxin (Doyonnas et al., 2001). However, ultrastructural analysis of podocytes by transmission electron microscopy (TEM) reveals dramatic morphological abnormalities. There is a significant reduction in the number of major processes and a complete absence of foot processes and slit diaphragms in podocalyxin-null mice. Moreover, podocyte cell bodies completely envelop the capillary loops, and there is a striking presence of junctional complexes between adjacent podocytes. These defects block normal filtrate production, as the bladders of knockout mice are completely empty just before birth, in contrast to wildtype littermates. Other observations include vacuoles within podocytes, thickening of the capillary endothelial cell layer, fewer endothelial fenestrae, and an apparent reduction in the volume of urinary spaces (Doyonnas et al., 2001). The electron micrograph in Figure 1-9, included for orientation purposes, depicts a normal kidney podocyte extending major processes and foot processes around a glomerular capillary. Similarly, cross-sections taken through podocytes and capillaries of wildtype and podocalyxin-null mice are depicted in Figure 1-10, where many of the differences described above are clearly visible.

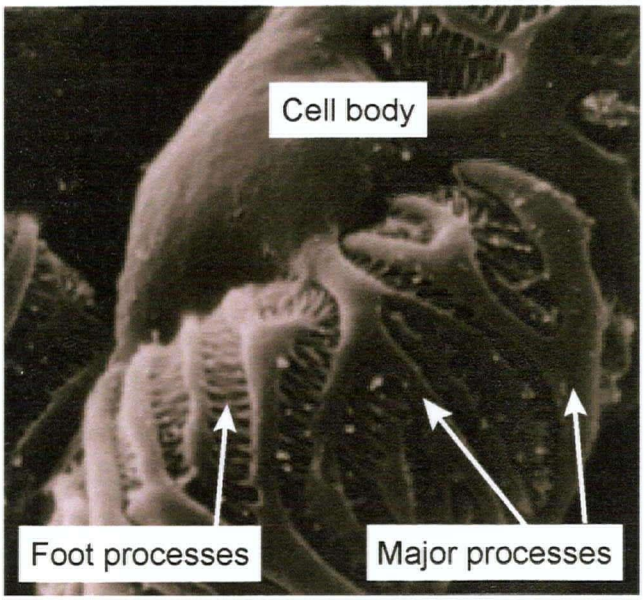

Figure 1-9: Electron Micrograph of a Normal Glomerular Podocyte with Extended Major Processes and Foot Processes Surrounding a Blood Vessel.

Original magnification: 7300X. Reproduced and adapted with kind permission of Springer Science and Business Media (Mundel and Kriz, 1995).

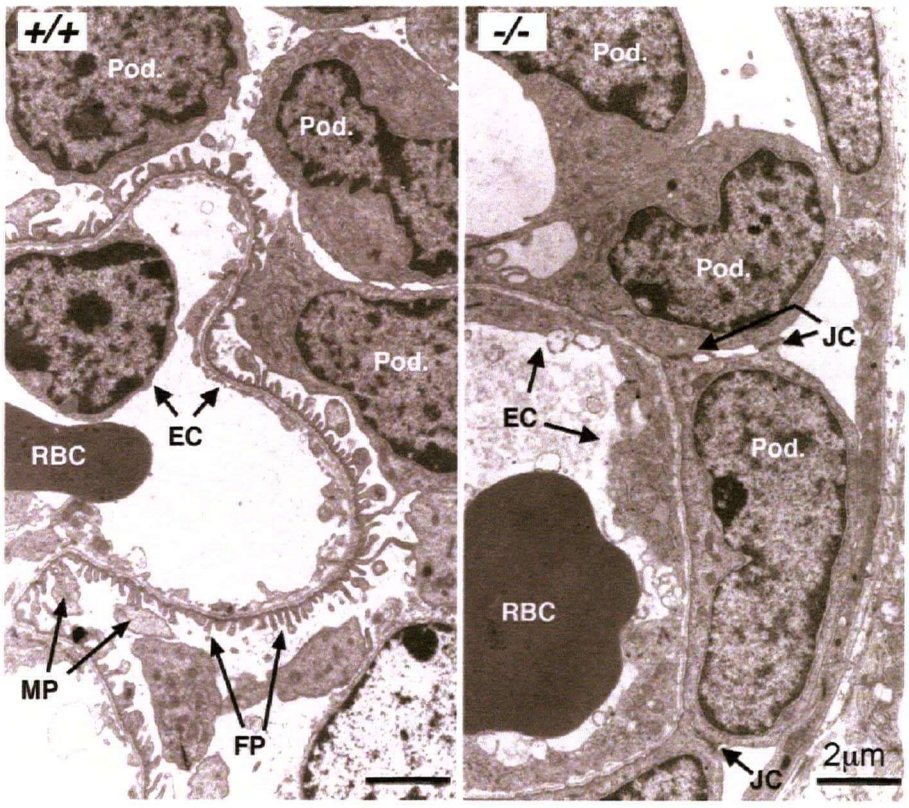

Figure 1-10: TEM's of Kidneys from Wildtype and Podocalyxin Knockout Mice.

Junctional complexes (JC) are visible between podocytes (Pod) in podocalyxin-null mice. These mice also lack major processes (MP) and foot processes (FP) and have thickened endothelial cells (EC). RBC: red blood cell. Reproduced with kind permission of The Rockefeller University Press (Doyonnas et al., 2001).

The podocyte phenotypes observed in podocalyxin knockout mice are consistent with a role for podocalyxin in decreasing cell adhesion and affecting cell morphology. Although podocyte maturation markers appear normal, the cells have an immature morphology overall. As in developing glomeruli, the podocytes in podocalyxin-null animals retain tight junctions instead of forming slit diaphragms (Doyonnas et al., 2001). Thus, it seems likely that under normal conditions, either podocalyxin alters distribution of tight junction proteins by means of its cytoplasmic interaction partners, or simply that expression of high levels of this bulky, negatively charged molecule on the apical surface may physically displace junctions to a more basal location. The lack of foot processes may imply a more specific role for podocalyxin in affecting cell morphology through its extensive negatively charged domain in addition to its conserved cytoplasmic tail and interactions with the actin cytoskeleton. Regarding the increased presence of cytoplasmic vacuoles, this is also a characteristic of human and rodent models of renal disease, and it may be an alternative pathway for filtrate production, since the presence of tight junctions and the lack of slit pores block the normal pathway (Toth and Takebayashi, 1992). The thickening of capillary endothelial cells in podocalyxin knockout mice may be related to the loss of endothelial podocalyxin or it may be due to an increase in capillary pressure as a result of the blockage in filtrate production (Doyonnas et al., 2001). Thus, podocalyxin-null mice certainly demonstrate that podocalyxin is a vital component of the developing kidney.

1.6.4.2 The Kidney Defect in Podocalyxin-Null Mice is the Apparent Cause of Perinatal Lethality in these Animals

The strikingly abnormal podocytes in podocalyxin-deficient animals clearly represent a serious defect in these animals and, in fact, it is the apparent cause of their lethality within the first day of birth (Doyonnas et al., 2001). All genotypes are present in expected frequencies throughout development, but this changes shortly after birth, such that there are no live podocalyxin-null mice one day later (Table 1-1). Unfortunately, death at this early time point precludes a more thorough assessment of potentially widespread implications of the kidney defect.

Age of embryos (post coitum)	Number of animals/genotype +/+	+/-	-/-	**Frequency of -/- mice**
15 d	19	28	17	**26**
16 d	5	21	10	**27**
17 d	8	15	10	**30**
18 d	35	71	30	**22**
19 d	6	10	8	**33**
Age of newborns (post partum)				
1 d	52	100	0	**0**

Table 1-1: Frequency of *podxl*$^{+/+}$, *podxl*$^{+/-}$, and *podxl*$^{-/-}$ Animals during Development.

Although normal ratios of podocalyxin-null animals are present throughout development, they all die within 24 hours of birth. Adapted from (Doyonnas et al., 2001).

Other newborn mice with anuric renal failure have also been described to die within the first day of birth, making it likely that this is the reason for the death of podocalyxin knockout animals (Bullock et al., 1998; Davis et al., 1995). It is important to remember, however, that other mice with anuric renal failure generally have other significant defects or lack kidneys entirely. The podocalyxin knockout was thus the most selective, lethal anuric condition described at the time, but confirmation that the kidney defect is the definitive cause of death is not easy to obtain (Doyonnas et al., 2001).

1.6.5 Other Molecules Involved in Glomerular Development

Some patients with glomerular diseases have mutations in genes encoding structural proteins involved in integrity of the podocyte cytoskeleton or slit diaphragms, such as α-actinin-4, nephrin, podocin, and CD2-associated protein (Boute et al., 2000; Kaplan et al., 2000; Kestila et al., 1998; Kim et al., 2003). However, most patients with glomerulosclerosis do not have mutations in these genes. Instead, expression of these genes may be dysregulated by mutations in other genes, such as WT1 (Gao et al., 2004). In fact, it has been shown that mutations in WT1 can be a factor in early-onset glomerulosclerosis. It is also possible that abnormal podocyte function caused by mutation of a single gene may initiate a series of responses that includes dysregulation of other podocyte genes. Although there are obviously numerous proteins required for glomerular development, I have selected two molecules to discuss that are particularly relevant for my studies. Nephrin is initially expressed on the basolateral surface of developing podocytes at the S-shaped body stage of glomerular development, not unlike podocalyxin (Kawachi et al., 2002; Schnabel et al., 1989)). I have taken advantage of this

fact when generating transgenic mice (described in chapter five). In addition, WT1 is essential for glomerular development, and it is known to be a regulator of podocalyxin expression (Gao et al., 2004; Guo et al., 2002; Kreidberg et al., 1993; Palmer et al., 2001; Stanhope-Baker et al., 2004).

1.6.5.1 Nephrin

Nephrin is a 180 kDa transmembrane protein belonging to the immunoglobulin (Ig) superfamily (reviewed in (Salant and Topham, 2003; Tryggvason, 1999)). It has eight extracellular Ig-like motifs, and in kidneys it is localized exclusively to the slit diaphragm region. It was first identified in a genome-wide screen to locate the gene responsible for the autosomal recessive congenital nephrotic syndrome of the Finnish type (CNF). The filtration barrier in CNF patients is disrupted, resulting in symptoms of nephrotic syndrome. The effects are seen early, with severe proteinuria *in utero*, loss of foot processes, and death within two years in the absence of a kidney transplant. Close to 50 different mutations in the gene encoding nephrin (*NPHS1*) have been detected in these patients.

In the developing glomerulus, nephrin is expressed on the basolateral surface of podocytes below junctional complexes (Kawachi et al., 2002). It migrates in conjunction with junctional proteins and is eventually restricted to the site of the slit diaphragm. Nephrin knockout mice rapidly develop severe proteinuria, they exhibit partial foot process effacement, loss of slit diaphragms, and narrowing of filtration slits, and they die within 24 hours of birth (Putaala et al., 2001). Although nephrin expression is decreased

in PAN, other studies addressing the role of nephrin in glomerular diseases are inconclusive (reviewed in (Salant and Topham, 2003)).

The precise role of nephrin in maintaining slit diaphragm integrity is unknown, but some evidence suggests that the slit diaphragm is a zipper-like structure (reviewed in (Tryggvason, 1999)). A model for the involvement of nephrin in this type of structure relies on it interacting homophilically with other nephrin molecules on neighbouring foot processes, as expected for Ig-like adhesion molecules. There are two Ig-like domains proximal to the plasma membrane and six more distal, separated by a spacer section. Based on the predicted 40 nm slit pore width, it is possible that the six distal Ig-like domains would interact with those on opposing nephrin molecules and that unpaired cysteine residues would form intermolecular disulphide bridges. Slit pores may then form in between the non-interacting, proximal Ig-like domains of neighbouring nephrin molecules. This model has yet to be proven, but the necessity for nephrin in glomerular development and slit diaphragm structure is clear.

1.6.5.2 WT1

As described in section 1.3.1, WT1 is a zinc finger transcription factor expressed in glomerular podocytes (Palmer et al., 2001; Roberts, 2005), and there is considerable evidence implicating WT1 in normal podocyte function (Guo et al., 2002). It is first detectable at day nine of mouse development in the lining of the coelomic cavity and in the urogenital ridge (Armstrong et al., 1993). Expression is maintained in mesothelial cells lining major organs throughout development, but it is also strongly expressed in the

renal vesicle and S-shaped body stages by cells that later become podocytes. It is then restricted to podocytes in the adult kidney (Roberts, 2005). Denys-Drash syndrome (DDS) generally involves development of glomerular nephropathy with glomerulosclerosis; 94 % of DDS patients have mutations in *wt1* (Guo et al., 2002). Moreover, one of the common *wt1* mutations seen in these patients can also cause glomerulosclerosis in mice (Patek et al., 1999). In addition, some patients with nephrotic syndrome and isolated cases of glomerulosclerosis have *wt1* mutations (Ito et al., 1999; Yang et al., 1999).

Loss of WT1 has profound effects on glomerular development in mice. Targeted inactivation of WT1 by deletion of the first exon prevents mesenchymal induction and results in renal agenesis; null mice die between embryonic day 13 and 14 (Kreidberg et al., 1993). WT1 ablation by small interfering RNA (siRNA) in developing kidney explants confirms this result (Davies et al., 2004). When siRNA is added to explants corresponding to embryonic day 9 (E9), ureteric buds fail to grow. However, if siRNA is instead added at E11, ureteric buds remain intact, but nephrogenesis is severely impaired. WT1 is therefore required at several stages of development, where it plays numerous roles.

When WT1-null mice are rescued using a human *wt1* transgene, nephrogenesis is recovered, but mice can still develop crescentic glomerulonephritis or mesangial sclerosis after birth, depending on the level of WT1 expression (Guo et al., 2002). WT1-deficient mice with a single copy of the human transgene survive at least until birth. However,

only 26 % have two kidneys; 14 % have one kidney, and 60 % lack kidneys completely and die within 48 hours of birth. The surviving mice all suffer from delayed nephrogenesis and congenital nephrotic syndrome with severe albuminuria and die within 20 days. Transgenic mice with two copies of human *wt1* all have at least one kidney, and 76 % have two. They also have a less striking delay in nephrogenesis, but they still develop adult-onset nephrotic syndrome with albuminuria, and 26 % die within 150 days. Similarly, mice with one copy of murine *wt1* develop both kidneys, but also exhibit adult-onset nephrotic syndrome, and 11 % die within 150 days. WT1 is therefore clearly important for nephrogenesis and normal kidney function throughout life.

1.7 Podocalyxin in the Hematopoietic System

Although podocalyxin is most highly expressed in kidney, other significant areas of expression include the hematopoietic and vascular systems.

1.7.1 Overview of Hematopoiesis

During development of the hematopoietic system, there are thought to be two main waves of hematopoiesis, although there is still some controversy regarding exactly when and where each cell type is first generated. Primitive hematopoiesis begins at E7.5 in mice and involves production, in the blood islands of the yolk sac, of the first circulating blood cells (reviewed in (Dzierzak et al., 1998; Keller et al., 1999)). These cells are large, nucleated erythroid cells and are required for survival and rapid growth of the embryo. This is followed by production of yolk sac-derived hematopoietic progenitors, which

enter the vasculature and circulate until replaced by definitive hematopoietic cells. Definitive hematopoiesis begins in the aorta-gonad-mesonephros (AGM) region at E10.5-11.5 and is characterized by production of enucleated erythrocytes, lymphoid cells, and LTR-HSCs. Within three days, LTR-HSCs migrate to the fetal liver (FTL), the main site of fetal hematopoiesis. Hematopoiesis also occurs transiently in the fetal spleen (SPL) before becoming firmly established in the bone marrow (BM), where an elaborate set of environmental signals regulate proliferation and differentiation (reviewed in (Wilson and Trumpp, 2006)).

1.7.2 Podocalyxin's Role in the Hematopoietic System

Podocalyxin is expressed in all hematopoietically active tissues throughout development (Doyonnas et al., 2005). For example, most hematopoietic cells in E10-12 murine yolk sac and peripheral blood express podocalyxin (Figure 1-11). Virtually all podocalyxin-positive cells at this stage express markers of the erythroid lineage, but a very small percentage instead express the stem cell factor receptor c-Kit and the pan-hematopoietic marker CD45. In colony forming assays, these cells give rise to erythroid and myeloid colonies, suggesting that podocalyxin is expressed on primitive erythrocytes as well as primitive myeloid or multilineage progenitors. Podocalyxin expression in yolk sac and peripheral blood gradually decreases over time, both in terms of expression levels and frequency of podocalyxin-positive cells.

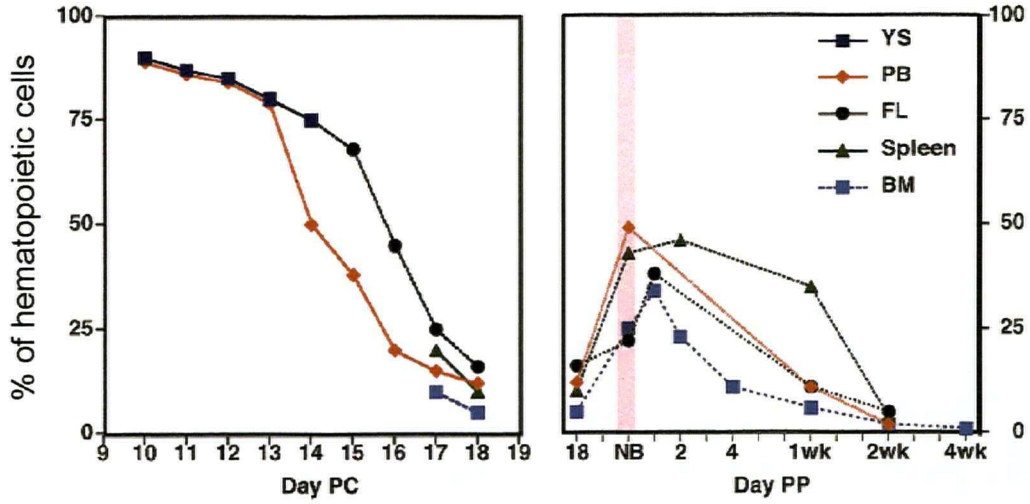

Figure 1-11: Podocalyxin is Expressed in Hematopoietic Tissues throughout Development.

PC: post coitum, PP: post partum. Adapted from (Doyonnas et al., 2005).

Then, as hematopoiesis shifts to FTL at E15, 75 % of cells in this tissue express podocalyxin (Figure 1-11) (Doyonnas et al., 2005). At this stage, there are two distinct populations of podocalyxin expressing cells. The cells expressing lower levels of podocalyxin are either primitive or definitive erythroid cells, while the population expressing the highest levels contains definitive hematopoietic progenitors. Again, podocalyxin expression in this tissue declines over the next few days. Similarly, SPL and BM both contain podocalyxin-positive populations upon acquisition of hematopoietic activity. In perinatal mice, most cells expressing podocalyxin are erythroblasts or early hematopoietic progenitors. Podocalyxin expression then decreases to virtually undetectable levels by birth. However, there is a distinct burst of podocalyxin expression in hematopoietic tissues immediately after birth, but this is again followed by a gradual decline. Thus, the establishment of each hematopoietically active tissue coincides with increased expression of podocalyxin.

Podocalyxin expression in the developing hematopoietic system has also been assessed in chicken (McNagny et al., 1997; Suonpaa et al., 2005). As in mice, there are two populations of podocalyxin expressing cells in early avian embryos (McNagny et al., 1997). Erythroid cells express low levels of podocalyxin, while podocalyxin is more highly expressed on multipotent hematopoietic progenitors. Significantly, podocalyxin-positive cells are found adhering to the ventral wall of the dorsal aorta before E4; this is the precise location of the first definitive HSCs (McNagny et al., 1997; Suonpaa et al., 2005). Podocalyxin is also found on hematopoietic progenitor cells in bone marrow of 1-

week old chicks. Thus, podocalyxin's expression pattern is comparable in developing mammals and avians.

In adult, podocalyxin expression is restricted to cells of the platelet/megakaryocytic lineage and a rare population of cells with a stem cell phenotype (Doyonnas et al., 2005; McNagny et al., 1997; Miettinen et al., 1999). These Lin$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) podocalyxin$^+$ cells give rise to myeloid and lymphoid lineages in serial transplantation experiments, suggesting that podocalyxin may be a marker of LTR-HSCs (Doyonnas et al., 2005). Although erythroid cells in adult do not normally express podocalyxin, it is rapidly upregulated by erythroid progenitors in response to hemolytic anemia (Doyonnas et al., 2005). This suggests that podocalyxin is expressed by early erythroid progenitors only when high rates of erythropoiesis are required, such as throughout development and under conditions of erythropoietic stress. One study also reported expression of podocalyxin mRNA in numerous mature hematopoietic lineages, including B lymphocytes, T lymphocytes, and myeloid cells, but the corresponding protein is not expressed in these cells (Kerosuo et al., 2004). Notably, however, podocalyxin mRNA levels are much higher in kidney and in endothelial cells than in any hematopoietic cells. Thus, podocalyxin is expressed by hematopoietic stem cells and platelets, but not in other mature hematopoietic lineages under steady state conditions in adult.

1.7.3 Hematopoiesis in the Podocalyxin Knockout

Since podocalyxin is expressed by a subset of hematopoietic cells, including early hematopoietic progenitors, the hematopoietic system of podocalyxin knockout animals

was assessed for defects (Doyonnas et al., 2001; Doyonnas et al., 2005). E15 FTL (Figure 1-12), E18 SPL, and E18 BM were stained and examined by flow cytometry with antibodies against hematopoietic progenitors (Sca-1 and c-Kit), erythroid cells (Ter119), megakaryocytes and platelets (CD41), myeloid cells (Mac1), B cells (B220), T cells (CD3), and granulocytes (Gr-1) (Figure 1-12 and (Doyonnas et al., 2001)). There are no detectable differences in the frequencies of any hematopoietic lineages in podocalyxin-deficient mice throughout development. The related protein, CD34, is also expressed by hematopoietic progenitors, thereby providing the possibility of functional compensation by this family member (Andrews et al., 1989; Berenson et al., 1988; Doyonnas et al., 2001). For this reason, compound knockout mice lacking both molecules were also generated and analyzed (Doyonnas et al., 2005). Surprisingly, however, hematopoiesis is also normal in mice lacking both molecules (Figure 1-12).

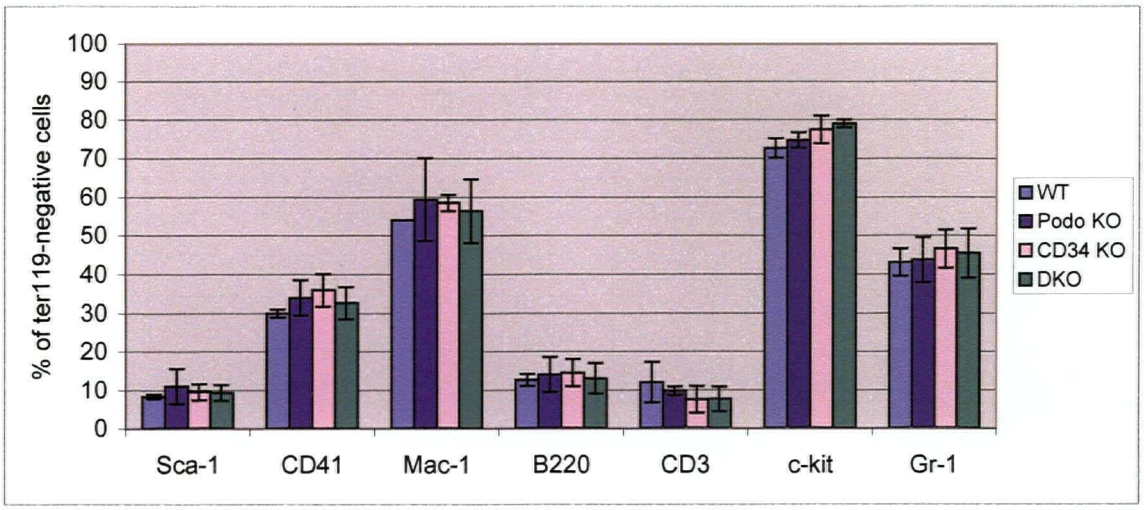

Figure 1-12: Steady State Levels of All Hematopoietic Lineages in E15 Fetal Liver in Podocalyxin (Podo)-Knockout (KO), CD34-KO, and Double-KO (DKO) Mice are Similar to those in Wildtype (WT) Mice.

Although the ratios of all hematopoietic lineages are normal in podocalyxin-, CD34-, and podocalyxin/CD34-null mice, there is a functional defect in these hematopoietic cells. Short-term homing assays demonstrate that injected cells lacking either podocalyxin or CD34 migrate with 20-30 % lower efficiency to the bone marrow than their wildtype counterparts, and that loss of both molecules has an additive effect (Doyonnas et al., 2005). However, despite this defect, when cells from any of these mice are injected in sufficient numbers, they can fully reconstitute lethally irradiated recipients. This result, along with the expression pattern of podocalyxin, in particular, implies that while these molecules are not essential, they may facilitate the crossing of endothelial barriers for entry into or exit from, hematopoietic microenvironments (Doyonnas et al., 2005). Hematopoietic cells of the yolk sac become less adherent in order to leave blood islands, precursors in FTL must cross into the vasculature to migrate to SPL and BM, and severe anemia leads to an efflux of erythroid progenitors from the BM in order to establish additional sites of erythropoiesis. Each of these situations corresponds to an increase in podocalyxin expression.

1.7.4 Podocalyxin in the Vasculature

In addition to its expression in the hematopoietic system, podocalyxin is also a universal marker of vascular endothelial cells (Doyonnas et al., 2005; Horvat et al., 1986; Miettinen et al., 1990). It is expressed in blood vessels of kidney, lung, heart, brain, small intestine, and other tissues (Horvat et al., 1986). Podocalyxin is found on endothelial cells lining a wide range of vessels, from the coronary artery to the sinusoids of liver and spleen and the specialized postcapillary venules in lymph nodes termed HEV (Horvat et

al., 1986; Miettinen et al., 1990; Sassetti et al., 1998). By immunoTEM, it has been shown that podocalyxin is localized in a patchy pattern on the luminal face of vascular endothelial cells (Horvat et al., 1986).

Despite the widespread expression of podocalyxin on blood vessels, the vasculature appears normal in developing podocalyxin knockout animals (Doyonnas et al., 2001). There are no detectable differences in the staining pattern of the endothelial marker, PECAM-1 in E16 brain, kidney, lung, or gut. Furthermore, electron microscopy of newborn lungs shows the formation of well-developed pulmonary capillaries. The related protein, CD34, is also ubiquitously expressed in vasculature, however, so it is possible that it could be functionally compensating for loss of podocalyxin (Doyonnas et al., 2001; Fina et al., 1990). In fact, upregulation of CD34 in lung of E18 podocalyxin-deficient animals has been detected by real time RT-PCR and immunohistochemistry (Doyonnas et al., 2001). Although there are no obvious defects in the vasculature, approximately 25 % of podocalyxin-deficient embryos exhibit mild to severe edema (Doyonnas et al., 2001). This may be a result of leaky vessels or, alternatively, the kidney defect would likely lead to an increase in blood pressure, which may be relieved by loss of fluid from the vessels. Functionally, of course, it would be easier to assess defects in the vascular system in adult animals, but perinatal lethality precludes this analysis.

1.7.5 Podocalyxin Expression in Hemangioblasts

Expression of podocalyxin by hematopoietic and vascular endothelial cells implies that it could also be expressed by the common precursor of both cell types, the hemangioblast.

Podocalyxin is also expressed by $CD45^+$ and $CD45^-$ cells in the AGM region at E11.5 (Hara et al., 1999). Podocalyxin$^+$CD45$^-$ cells from the AGM differentiate into both angioblasts and hematopoietic cells *in vitro*, depending on the growth conditions, and they are also capable of long-term lymphoid and myeloid reconstitution in transplantation experiments, suggesting the presence on LTR-HSCs in this population, although this cannot be conclusively demonstrated without performing single cell transplants (Hara et al., 1999). Thus, although not confirmed, this data is suggestive of podocalyxin expression on hemangioblasts.

1.8 Podocalyxin in the Brain

Another interesting site of podocalyxin expression is the brain, where it is detected in the marginal zone of the cerebral cortex, the cerebellum, and the superior colliculus at E15-16 (Garcia-Frigola et al., 2004). Expression in migrating cells in the developing cerebellum hints that it may be involved in enabling neurons to migrate and detach from radial glia. *In situ* hybridization demonstrates that podocalyxin mRNA is, in fact, expressed in many regions throughout development and in adult brain, with the highest expression levels in cerebral cortex and cerebellum postnatally (Vitureira et al., 2005). In the forebrain, podocalyxin is expressed in the olfactory bulb, neocortex, and hippocampus. It is expressed in developing neurons and along specific axonal pathways. The functional significance of podocalyxin expression in the brain and any potential defects in podocalyxin knockout animals are still under investigation, but its pattern of expression hints at a role in proliferation, migration, or neuronal differentiation in the central nervous system (Vitureira et al., 2005). Anti-adhesive forces may aid in the

detachment and migration of neuronal cells and leading processes, or podocalyxin may be actively involved in axonal path finding through its interactions with PDZ domain-containing proteins (Orlando et al., 2001; Vitureira et al., 2005).

1.9 Additional Phenotypes in Podocalyxin-Null Animals

Podocalyxin is expressed by podocytes, hematopoietic progenitors, platelets, vascular endothelia, a subset of neurons, and mesothelial cells lining many organs; defects are therefore expected in these tissues in mice lacking podocalyxin (Doyonnas et al., 2001; Horvat et al., 1986; Kerjaschki et al., 1984; Kershaw et al., 1997a; McNagny et al., 1997; Miettinen et al., 1999; Sassetti et al., 1998). CD34 may functionally compensate for podocalyxin loss in hematopoietic progenitors and blood vessels, potential neuronal defects are being actively investigated, and podocytes are clearly abnormal (Doyonnas et al., 2001). The final area of interest is therefore the lining of body cavities. Strikingly, approximately 30 % of podocalyxin-null mice are born with a herniation of the gut, known as an omphalocele (Doyonnas et al., 2001). This is actually a normal physiological process that occurs at E12 as the rapidly growing organs exceed the limiting space of the peritoneal cavity; however, in normal mice it is resolved as the peritoneal cavity expands, generally by E16 (Kaufman, 1998). Using timed matings, it was determined that resolution of the omphalocele is delayed in all podocalyxin-null mice, but 70 % do retract the gut before birth (Doyonnas et al., 2001). The delay may be a result of increased adhesion upon loss of podocalyxin from exposed surfaces. Thus, in another tissue where CD34 cannot functionally compensate for loss of podocalyxin, there is an apparent increase in cell adhesion.

1.10 Podocalyxin in Cancer

Up to this point I have concentrated on the normal function and expression pattern of podocalyxin, but I will now focus on cases where podocalyxin expression is dysregulated. Podocalyxin has been implicated in numerous malignant situations, including breast cancer, testicular cancer, prostate cancer, and leukemia (Casey et al., 2006; Kelley et al., 2005; Schopperle et al., 2003; Somasiri et al., 2004).

1.10.1 Podocalyxin in Breast Cancer

According to analysis of a tissue microarray of 272 invasive breast carcinomas and corresponding long-term outcome data, we have shown that podocalyxin upregulation is correlated with poor outcome in a distinct subset of tumours (Somasiri et al., 2004). Immunohistochemistry was used to compare podocalyxin expression in tumour samples. A score of "0" corresponds to a lack of podocalyxin staining in tumour cells, and was found in 60 % of cases. Less than 10 % of tumour cells were stained (group 1) in 23 % of samples, 12 % of cases exhibited intense staining in less than half of the cells or diffuse staining in more than 10 % (group 2). The final group (group 3) made up 6 % of the array and had intense podocalyxin staining in the majority of tumour cells. Survival rates were compared between patients from each group, and a statistically significant difference was noted between group 3 and the rest of the patients, combined (Figure 1-13). The mean survival time was 9.0±1.8 years for patients in group 3, while those patients with tumours expressing low or no podocalyxin lived on average 15±0.5 years. Although there are no

significant differences in histological subtype, tumour size, or lymph node metastasis between groups, there are proportionally more high grade, estrogen receptor (ER)-negative tumours in the high podocalyxin group. Furthermore, podocalyxin overexpression is a statistically significant independent predictor of poor outcome with more than an eight-fold relative risk compared to podocalyxin low or negative samples.

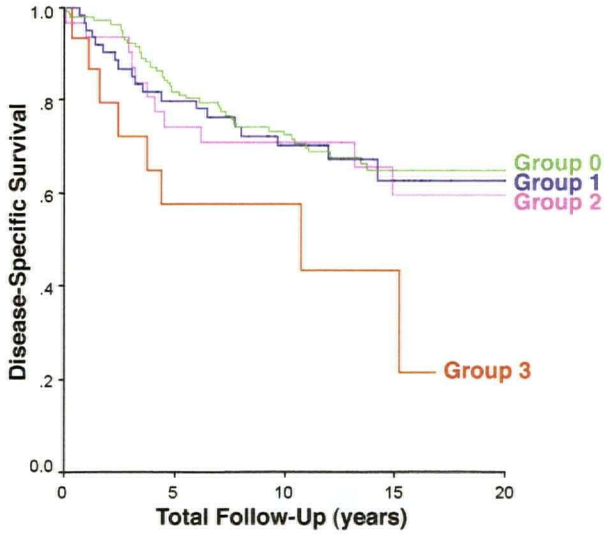

Figure 1-13: Survival Rates for Patients Diagnosed with Breast Tumours Shown to be Expressing Varying Levels of Podocalyxin.

Groups 0-2: low/no podocalyxin expression and group 3: high podocalyxin expression (Somasiri et al., 2004).

The mechanism leading to increased podocalyxin expression has not yet been determined (Somasiri et al., 2004). However, the human *podxl* gene is located on chromosome 7q32-q33, which is in between two regions previously identified as chromosomal gain sites in ductal breast carcinoma and breast tumour cell lines (Aubele et al., 2000; Forozan et al., 2000; Kershaw et al., 1997b). Moreover, the podocalyxin binding protein, NHERF1 is often expressed at lower levels in ER-negative breast tumours in comparison to ER-positive tumours, and many of the tumours expressing high levels of podocalyxin were ER-negative, which may be a contributing factor (Somasiri et al., 2004; Stemmer-Rachamimov et al., 2001). Thus, although the events leading up to it can only be hypothesized at this stage, podocalyxin is upregulated in a subset of breast tumours in patients with poor outcomes. There is currently a larger screen of breast tumours underway to confirm these results.

1.10.2 Podocalyxin in Prostate Cancer

Genetic approaches were used to identify podocalyxin dysregulation as a factor in prostate cancer (Casey et al., 2006). Since prostate cancer aggressiveness, which varies widely among patients, is influenced by family history, a genetic component is suspected (Klein et al., 1998). A genome-wide screen of over 500 affected siblings strongly implicated chromosome 7q32-q33 in tumour aggressiveness (Witte et al., 2000), and this result has been confirmed in subsequent studies (Paiss et al., 2003; Witte et al., 2003). In prostate tumours, this region also exhibits a high frequency of allelic imbalance, with the *podxl* gene contained within the smallest region of imbalance (Neville et al., 2002).

Mutational analysis was performed on genomic *podxl* from the probands of 17 families identified previously (Casey et al., 2006). The relationship between prostate cancer, tumour aggressiveness, and *podxl* variants was then assessed in a family-based association study. Several common mutations were identified, including a variable in-frame deletion in the first exon, four missense mutations, and two silent variants. The presence of one or two copies of the in-frame deletion variant, which results in loss of serine and proline residues in the extracellular domain, increases the relative risk, with two copies doubling the risk of developing more aggressive prostate cancer. The presence of missense mutations in exon two increases the risk of developing prostate cancer by approximately 50 %, but has no effect on aggressiveness. The other mutations were not risk factors, and additional variants outside of the *podxl* locus did not show any association with prostate cancer either, implying that the mutations described above are genuine risk factors. While the functional implications of these mutations are not yet known, it may be that they result in decreased negative charge on podocalyxin, which may disrupt the association of podocalyxin with the actin cytoskeleton, or cell motility and invasiveness may be increased as a result of perturbation of downstream targets.

1.10.3 Podocalyxin in Testicular Cancer

Podocalyxin expression in testicular cancer, the most common type of solid tumour in young adult males, was the first report of podocalyxin in malignant cells (Schopperle et al., 2003). It is important to find markers to distinguish between the two types of testicular germ cell tumours, seminomatous and nonseminomatous (NSGCT), because they require different treatments. Podocalyxin is preferentially expressed by NSGCT, and

not in protein lysates from normal tissue. Podocalyxin is also found in the supernatants of cultured embryonal carcinoma cell lines, and it may therefore be a useful serum marker for detection of NSGCT (Schopperle et al., 1992). Interestingly, the NSGCT form of podocalyxin is considerably larger than the form found in kidney, suggesting that it may undergo additional post-translational modifications in these tumours (Schopperle et al., 2003).

1.10.4 Podocalyxin in Leukemia

Potential expression of podocalyxin in leukemia was assessed for several reasons: the related protein, CD34 is expressed by many, but not all, leukemic blasts, podocalyxin is expressed by normal hematopoietic progenitors, and the podocalyxin transcriptional regulator, WT1 is expressed by the majority of blasts in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (Doyonnas et al., 2005; Kelley et al., 2005; Kerosuo et al., 2004; Menssen et al., 1995; Palmer et al., 2001). AML and ALL tissue microarrays and biopsy specimens were assayed for podocalyxin expression (Kelley et al., 2005). Podocalyxin was detected in blasts in 77 % of 39 AML cases, with strong expression in 41 % of samples. It was also detected in 81 % of 27 ALL cases, with strong expression in 22 %. Although CD34 is also expressed in many leukemic blasts, there is no significant correlation between CD34 and podocalyxin expression. Podocalyxin expression in myeloid sarcomas was also assessed in order to address a proposed role in facilitating tissue infiltration; it was expressed in 87 % of 15 cases with strong expression in 53 %. Since podocalyxin expression is regulated by WT1 in podocytes, expression of WT1 was assessed in leukemic blasts: 44 % of AML cases and 78 % of ALL cases

exhibited nuclear WT1, but there was no correlation with podocalyxin expression. This suggests that podocalyxin expression is somehow dysregulated in leukemia or, since podocalyxin is normally expressed by hematopoietic progenitors, perhaps expression by leukemic blasts just follows the normal expression pattern, which may be independent of WT1 in hematopoiesis. Again, the functional relevance of podocalyxin expression in this type of cancer is unknown, but its presence could be used as a marker to increase the sensitivity of assays designed to detect leukemia.

1.10.5 Podocalyxin in Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the five leading causes of cancer death worldwide (Pisani et al., 1999). Unfortunately, this type of cancer is often detected too late, thereby limiting treatment options, and making the five-year survival rate only five percent (El-Serag et al., 2001). Finding additional markers of HCC may facilitate earlier detection and better outcome (Chen et al., 2004). One major difference between HCC and normal liver tissue can be in the type of blood vessels they contain. Normal vessels lining the hepatic sinusoid allow free diffusion of macromolecules, but not larger particles, via small fenestrations (Chen et al., 2004). In contrast, vasculature within tumours is often abnormally permeable, allowing passage of larger molecules, and even metastasis of cancerous cells, through widened cell-cell junctions, larger fenestrations, transcellular holes, and an irregular basement membrane (Hashizume et al., 2000). Strikingly, CD34 is highly upregulated in endothelial cells of HCC in comparison to normal liver tissue (Ruck et al., 1995). Notably, CD34 is generally not detectable in most solid tumours, such as breast cancer, lymphoma, myeloma, or neuroblastoma (reviewed in (Silvestri et

al., 1992)), while podocalyxin has been found in several, such as those already discussed, Wilms' tumours (described below), and high grade ovarian tumours (unpublished observations, ML McCoy, CB Gilks, CD Roskelley, as cited in (Somasiri et al., 2004)). In a search for other markers of HCC using cDNA microarray analysis of normal tissue and tumours from 58 patients, podocalyxin and CD34 were both found to be upregulated in HCC (Chen et al., 2004). This result was confirmed by immunohistochemistry: 11 of 12 HCC tissue sections were positive for podocalyxin, while all 5 normal tissue sections lacked podocalyxin. There was also a highly statistically significant increase in podocalyxin and CD34 expression in HCC on a tissue microarray containing 350 samples (Chen et al., 2004). It is thought that perhaps this dramatic upregulation contributes to the leakiness of vasculature in HCC. Regardless of the functional implications of CD34 and podocalyxin expression, they may be useful markers for earlier detection of this type of cancer.

1.10.6 Podocalyxin in Wilms' Tumours

Wilms' tumour is the most frequently occurring pediatric kidney cancer and the fourth most common childhood malignancy (Miller et al., 1995). The podocalyxin transcriptional regulator, WT1 is mutated in 10-15 % of Wilms' tumours, and there is also evidence to implicate p53 in some cases; however, the majority of Wilms' tumours have no known cause. In contrast to the other cancers described above, podocalyxin expression is significantly reduced in Wilm's tumours relative to normal fetal kidney, according to cDNA microarray analysis of 64 tumour samples (Stanhope-Baker et al., 2004). Surprisingly, however, podocalyxin and WT1 expression levels were not

correlated, at least at the level of mRNA. It must be kept in mind, though, that protein levels, and the potential presence of WT1 mutations in these samples, have not yet been assessed. More in keeping with the other published literature, however, there was a significant increase in podocalyxin expression in the more aggressive, anaplastic tumours. Since p53 is generally mutated in anaplastic Wilms' tumours, and in that it has been shown to negatively regulate podocalyxin expression, this may explain why podocalyxin is expressed more highly in these cases. Functionally, podocalyxin may contribute to the increased metastasis of this subset of tumours.

Podocalyxin has now been associated with a wide variety of cancers, and, strikingly, it is often associated with more aggressive cases. The most likely functional implication of podocalyxin overexpression is increased metastasis, although this has yet to be proven.

1.11 Thesis Objectives

1) The dramatic upregulation of podocalyxin in numerous cancers, and particularly in subsets with poor outcome, is an important area for further investigation. Understanding the functional role of podocalyxin in these situations may facilitate the design of new therapies. I have approached this goal by overexpressing podocalyxin in cell lines in order to determine its function. I have also generated a panel of podocalyxin mutants and overexpressed these *in vitro* as well. This has helped to unravel podocalyxin's mechanism of action.

2) Although considerable information has been gained by studying normal podocalyxin expression patterns and podocalyxin-null mice, an *in vivo* gain-of-function model is lacking. I have therefore attempted to generate transgenic mice overexpressing podocalyxin. I am using the versatile Cre-loxP system in order to create mice expressing podocalyxin in select tissues. For example, overexpression in mammary tissue will provide a model for deciphering podocalyxin's role in breast cancer. Alternatively, overexpression in vascular tissue may create leaky vessels. Thus, a single transgenic strain could be crossed to multiple Cre mice in order to produce mice overexpressing podocalyxin in numerous tissues.

3) The absolute requirement for podocalyxin expression in podocytes is important and interesting in itself, but it prevents assessment of podocalyxin-deficient tissues, such as the hematopoietic system and vasculature as well as the brain, in adult mice. I have therefore repaired the kidney defect in podocalyxin-null mice by specifically expressing podocalyxin in podocytes using a tissue-specific promoter, with the intention of investigating the effect of podocalyxin loss in other tissues.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Cloning and Mutagenesis of Podocalyxin

Murine *podxl* cDNA was a generous gift from Dr. David Kershaw; chicken podocalyxin cDNA was cloned from HD100 hematopoietic progenitor cells (McNagny et al., 1997).

2.1.1 Conditional Podocalyxin Overexpression Transgenic Construct

Murine *podxl* cDNA was excised from pBluescript using BamHI and XhoI restriction enzymes, and the transgenic pCCALL-2 vector (generously provided by Dr. Corrinne Lobe) was digested with BglII and XhoI. After ligation of murine *podxl* into this vector and *in vitro* assessment of podocalyxin expression in NS0 cells, the strategy was modified slightly to include expression of the marker gene, GFP. Thus, the IRES-GFP sequence between XhoI and SacII was excised from the pCCALL-2 derivative Z/EG and ligated into the pCCALL-2-*podxl* construct, such that podocalyxin and GFP were expressed concurrently.

2.1.2 Podocyte-Specific Podocalyxin Transgenic Construct

The podocyte-specific transgenic construct was generated using the murine *NPHSI* promoter provided by Dr. Sue Quaggin. Two oligonucleotides (5'-TCGAGCGGCCTTAATTAAG-3' and 5'-AATTCTTAATTAAGGCCGC-3') were first annealed to generate a linker sequence containing a PacI restriction site. This was ligated

into the pIRES2-EGFP vector (BD Biosciences, Mississauga ON) using the XhoI and EcoRI sites of the multiple cloning site. Murine *podxl* was then excised from the pBluescript SK cloning vector via the SacII and BglII sites and ligated into pIRES2-EGFP between SacII and BamHI. Finally, the murine *NPHS1* promoter was cloned into this plasmid between XhoI and the new PacI site. After testing CMV promoter-driven podocalyxin expression from this construct *in vitro*, XhoI and SfiI restriction enzymes were used to isolate the *NPHS1* promoter and murine *podxl* for generation of transgenic mice.

2.1.3 Murine Podocalyxin Expression Vector

The murine *podxl* expression vector used for *in vitro* studies was created as described in section 2.1.2, but the ubiquitous CMV promoter was retained and the podocyte-specific *NPHSI* promoter was not included.

2.1.4 Chicken Podocalyxin Expression Vectors

A construct had previously been generated for expression of a fusion protein that included the extracellular (N-terminal) portion of chicken *podxl* in the pcDNA3.1 expression vector (pcDNA3.1-chMEP21Fc). PCR, using primers 5'-TCTCTCACTTTCCAGTCATCGTCC-3' and 5'-GCTCTAGAGTTTCAGGGGGTTGTTTTTTGC-3', was used to amplify the C-terminal portion of chicken *podxl* from the original pBluescript cloning construct (clone 4D1 (McNagny et al., 1997)) and to add an XbaI cloning site at its C-terminus. This PCR

product was ligated into the pCR2.1-TOPO cloning vector (Invitrogen) from which it was then excised with BamHI and XbaI. This fragment was ligated into the same sites of the pcDNA3.1-N-terminal *podxl* construct, in order to generate a full-length chicken *podxl* expression construct.

Podocalyxin expression levels were low in cells transfected with the pcDNA3.1-*podxl* construct, so new constructs were generated using the pIRES2-EGFP expression vector. Chicken *podxl* cDNA was amplified from the pcDNA3.1-*podxl* construct using primers 5'-CCACTGCTTACTGGCTTATCG-3' and 5'-ACAACAGATGGCTGGCAAC-3'. The PCR product was ligated into the pCR2.1-TOPO cloning vector, excised with EcoRI, and ligated into pIRES2-EGFP. This was then sequenced after first checking the insert's orientation.

2.1.5 Chicken Podocalyxin Mutants

Ala, Asp, and ΔDTHL chicken *podxl* mutants were generated by site-directed mutagenesis using the Transformer Site-Directed Mutagenesis Kit (Clontech, Mountain View CA), according to manufacturer's instructions. Dr. Regis Doyonnas generated the alanine mutants by site-directed mutagenesis of the 4D1 pBluescript-chicken *podxl* construct. I generated the other mutants as described below. The pcDNA3.1-chicken *podxl* vector was initially used as a template for creation of the aspartic acid mutants using primers 5'-CACCAACGCTTCGACCAAAAGAAG-3' (putative PKC site) and 5'-AGGTGATGGAAGACGGCTCTGAAAT-3' (putative CKII site), as well as 5'-CCTGAAACACTAGTGGGCCCGT-3' for mutation of the XbaI site to SpeI for

selection purposes. Red lettering denotes codons of interest. However, conversion of the threonine residue in the putative CKII site to an aspartic acid residue involved mutagenesis of all three residues in the codon, which proved difficult. Instead, the previously mutated pcDNA3.1-chicken *podxl* CKII-alanine mutant was eventually used as a template because conversion of the alanine residue to aspartic acid required mutagenesis of just two residues.

Similarly, 5'-GAGGACCTAGAGGAATAGGATACGCATT-3' was used to insert a premature stop codon immediately preceding the C-terminal DTHL sequence by mutating the last glutamic acid residue to a stop codon, thereby creating the ΔDTHL mutant.

The Δtail mutant was created by using the 4D1 pBluescript-chicken *podxl* construct as a PCR template and the primers 5'-TCTCTCACTTTCCAGTCATCGTCC-3' and 5'-GCTCTAGAGCCTAGAAGCGTTGGTGACAGCAGC-3' to insert a premature stop codon after the juxtamembrane CCHQRF sequence. The PCR product was then ligated into pCR2.1-TOPO, excised with BamHI and XbaI, and ligated into the pcDNA3.1-N-terminal *podxl* construct.

The alternatively spliced form of chicken *podxl* was generated by amplification of the C'terminal portion of *podxl* from a pBluescript cloning vector containing *podxl's* splice variant (4B3 (McNagny et al., 1997)). The PCR product created with the primers 5'-TCTCTCACTTTCCAGTCATCGTCC-3' and 5'-

GCTCTAGACGGGAAATAGGTTCTCCTTCTGC-3' was ligated into pCR2.1-TOPO, excised with BamHI and XbaI, and inserted into the pcDNA3.1-N-terminal *podxl* construct.

In addition, the above variants were all later cloned into the pIRES2-EGFP expression vector. The primers 5'-CCACTGCTTACTGGCTTATCG-3' and 5'-ACAACAGATGGCTGGCAAC-3' were used to amplify all forms of chicken *podxl* from their respective pcDNA3.1 constructs. The PCR products were inserted into pCR2.1-TOPO, excised with EcoRI, and ligated into the multiple cloning site of pIRES2-EGFP.

The last mutant, the flag-tagged *podxl* construct lacking the majority of the extracellular domain, was generated in several steps. The first step involved isolation of *podxl's* cytoplasmic tail, transmembrane region, and a small portion of its extracellular domain from the pIRES2-EGFP-wt *podxl* construct using the HindIII restriction sites, and insertion into the empty pIRES2-EGFP vector. Secondly, the signal peptide and flag-tag were amplified, using the primers 5'-TAGCTAGCGAGATGGCCTTGCACCTTCT-3' and 5'-TACTCGAGGATGCCGCCCTTATCGTC-3', from a pMX-pie expression construct containing flag-tagged *cd43*, generously provided by Dr. Hermann Ziltener and Wooseok Seo. This PCR step also involved addition of NheI and XhoI restriction sites to the ends of the PCR product. This product was then ligated into pCR2.1-TOPO and sequenced. The final step required isolation of the signal peptide and flag-tag from pCR2.1-TOPO using the NheI and XhoI sites, and ligation into the same sites in the

pIRES2-EGFP construct containing *podxl's* transmembrane region and cytoplasmic tail. All constructs were sequenced after completion.

2.2 Cell Culture

2.2.1 Culture Conditions

MCF-7 and MDCK cells were routinely maintained in Advanced D-MEM/F12 medium (Invitrogen #12634-010, Burlington ON) supplemented with 5 % fetal bovine serum (FBS), HEPES, glutamine, penicillin, and streptomycin.

NS0 mouse myeloma cells (generously provided by Dr. Gary McLean and Dr. John Schrader) were routinely maintained in D-MEM supplemented with 10 % FBS, sodium pyruvate, non-essential amino acids, glutamine, penicillin, and streptomycin. CHO cells were cultured in D-MEM supplemented with 10 % FBS, glutamine, β-mercaptoethanol, penicillin, and streptomycin. FDCP-1 cells were maintained in RPMI supplemented with 10 % FBS, 2 % WEHI-3B conditioned medium as a source of IL-3, glutamine, β-mercaptoethanol, penicillin, and streptomycin.

R1 embryonic stem cells (ESCs) were routinely maintained in D-MEM supplemented with 15 % FBS, 10 ng/ml LIF, sodium pyruvate, non-essential amino acids, glutamine, β-mercaptoethanol, penicillin, and streptomycin. ESCs were cultured on mouse embryonic fibroblast feeder layers or gelatin coated plates.

2.2.2 Transfection Techniques

MCF-7 and MDCK cells were transfected with 30 μg DNA using the DMRIE-C transfection reagent (Invitrogen) according to manufacturer's instructions. DNA was diluted in 300 μl serum-free DMEM/F12 and slowly added to 15 μl DMRIE-C, also previously diluted in 300 μl DMEM/F12. This was mixed gently, incubated for 15 minutes, and diluted with 2.4 ml DMEM/F12. The transfection mixture was transferred to 10 cm dishes containing 30-50 % confluent cells and incubated for 6-8 hours. Media was replaced with DMEM/F12 containing 5 % FBS and glutamine, and stable clones were selected based on resistance to 400 μg/ml G418.

15 x 10^6 NS0 cells were electroporated in 500 μl cold PBS with 20 μg DNA in 0.4 cm cuvettes (200 V, 950 μF, time constant ~ 30 msec). Cells were then washed with culture media, and replated as bulk cultures and in 96 well plates for clonal selection. Culture in 1.5 mg/ml G418 was used to generate stable transfectants.

CHO cells were transfected in 60 mm dishes with the Lipofectamine Plus reagent (Invitrogen) according to manufacturer's instructions. Plus reagent (8 μl) was added to 2 μg DNA, previously diluted in 250 μl media lacking serum and antibiotics. This mixture was incubated for 15 minutes at room temperature while 12 μl of Lipofectamine reagent was diluted in 250 μl serum-free media. These components were combined and incubated for 15 minutes before they were transferred to plates of cells containing 2 ml of fresh serum-free media. After 3-hour incubation at 37°C, 3 ml of media containing serum was added, and expression was assessed 2 days later.

5.6×10^6 ESCs were electroporated in 0.8 ml PBS containing 20 μg DNA (240 V, 500 μF, time constant ~ 7 msec). Cells were then allowed to recover on ice for 20 minutes, washed with culture media, and transferred to 2 10-cm plates. Stable clones were obtained by culturing in 150 μg/ml G418.

2.3 Expression Analysis

2.3.1 Antibodies

The antibodies used in this thesis are shown in (Table 2-1 and Table 2-2).

Antibody	Type	Concentration	Procedure	Source
α-ezrin	ms IgG_1	25 μg/ml	Confocal	3C12: Abcam (Cambridge MA)
α-ezrin (biotin)	ms IgG_1	25 μg/ml	Confocal	3C12: NeoMarkers (Fremont CA)
α-flag	ms IgG_1	1 μg/ml	Western	M2: Sigma-Aldrich (Oakville ON)
α-flag (biotin)	ms IgG_1	1 μg/ml	Confocal, FC	M2: Sigma-Aldrich
α–GFP	rb IgG (pAb)	10 μg/ml	IF	Invitrogen
α-NHERF1	rb (pAb)	3 μg/ml	Confocal	Abcam
α-PECAM-1	rat IgG_{2a}	5 μg/ml	IHC	BD Biosciences
α-ch podocalyxin	ms IgG_1	neat	Confocal, FC	(McNagny et al., 1992)
α-ch podocalyxin	ms IgG_1	1:5	Western	(McNagny et al., 1992)
α-ms podocalyxin	rat IgG_1	5 μg/ml	Confocal, FC	MBL (Woburn MA)
α-ms podocalyxin	rat IgG_1	5 μg/ml	IHC	(Hara et al., 1999)
ms IgG_1	Isotype control	25 μg/ml	Confocal	DAKO (Mississauga ON)

Antibody	Type	Concentration	Procedure	Source
ms IgG_1 (biotin)	Isotype control	25 μg/ml	Confocal	R&D Systems (Minneapolis MN)
rat IgG_1	Isotype control	5 μg/ml	FC, IHC	Cedarlane (Hornby ON)
rb IgG	Isotype control	3 μg/ml	Confocal	Jackson ImmunoResearch Laboratories (Westgrove PA)
phalloidin	Alexa Fluor-568	25 units/ml	Confocal	Invitrogen

Table 2-1: Primary Antibodies used in this Thesis.

pAb: polyclonal antibody

ch: chicken, rb: rabbit, ms: mouse

FC: flow cytometry, IF: immunofluorescence, IHC: immunohistochemistry.

Antibody	Label	Concentration	Procedure	Source
α-ms Ig	APC	2 μg/ml	FC	BD Biosciences
α-ms Ig	HRP	0.1 μg/ml	Western	DAKO
α-ms IgG_1	Alexa Fluor 488	8- 20 μg/ml	Confocal	Invitrogen
α-ms IgG_1	Alexa Fluor 568	20 μg/ml	Confocal	Invitrogen
α-rat IgG	biotin	1 μg/ml	FC	Southern Biotech (Birmingham AL)
α-rat IgG	biotin	5 μg/ml	IHC	Vector Laboratories (Burlington ON)
α-rb IgG	Alexa Fluor 488	20 μg/ml	Confocal	Invitrogen
α-rb IgG	Alexa Fluor 488	4 μg/ml	IF	Invitrogen
streptavidin	Alexa Fluor 568	5 μg/ml	Confocal	Invitrogen
streptavidin	APC	0.5 μg/ml	FC	BD Biosciences
streptavidin	PE	1.25 μg/ml	FC	BD Biosciences

Table 2-2: Secondary Antibodies used in this Thesis.

APC: allophycocyanin, HRP: horse radish peroxidase, PE: phycoerythrin

rb: rabbit, ms: mouse

FC: flow cytometry, IF: immunofluorescence, IHC: immunohistochemistry.

2.3.2 Flow Cytometry and Cell Sorting

Cells were washed with FACS buffer (10 % FBS and 0.05 % sodium azide in PBS), labelled with primary antibodies (Table 2-1) for 20-30 minutes on ice, washed with FACS buffer, incubated with secondary antibodies (Table 2-2) for 20 minutes on ice, washed again, and stained with the viability marker 7-amino-actinomycin D (7AAD) (BD Biosciences, Mississauga ON). In most experiments, 10 000 viable cells were collected with a FACS Calibur flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Tree Star Inc, Ashland OR). Cell sorting was performed by Andy Johnson with a BD FACS Vantage cell sorter in the Biomedical Research Centre's FACS facility.

MCF-7 cells transfected with murine *podxl* were assessed using rat anti-mouse podocalyxin, biotinylated anti-rat IgG_1, and phycoerythrin (PE)- or allophycocyanin (APC)-conjugated streptavidin (SA). MCF-7 cells transfected with chicken *podxl* mutants were assessed using mouse anti-chicken podocalyxin or biotinylated mouse anti-flag and APC-conjugated anti-mouse Ig or APC-conjugated SA. Cells transfected with the empty vector were used as negative controls for podocalyxin staining.

Transfected ESCs were assessed for murine podocalyxin expression using rat anti-mouse podocalyxin, biotinylated anti-rat IgG_1, and phycoerythrin (PE)-conjugated streptavidin (SA). Rat IgG_1 was used as a negative control for podocalyxin staining.

2.3.3 Western blotting

Cells were lysed with RIPA buffer plus protease inhibitors, including phenylmethanesulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma-Aldrich #P8340). RIPA buffer contained 10 mM phosphate buffer, 150 mM sodium chloride, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM sodium fluoride. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were blocked for 30 minutes with 5 % skim milk in Tris buffered saline (TBS) plus 0.05 % Tween 20 (TBS-T), followed by a 2-hour incubation in primary antibody (mouse anti-flag or mouse anti-chicken podocalyxin) diluted in TBS-T and 3 5-minute washes with TBS-T (Table 2-1). Membranes were then probed with secondary antibody (horse radish peroxidase-coupled anti-mouse IgG) diluted in 2-3 % BSA in TBS-T for 45 minutes and washed 3 x 5 minutes with TBS-T (Table 2-2). Western blots were developed after exposing film (Kodak) to membranes that were previously incubated with enhanced chemiluminescence (ECL) reagent (Perkin Elmer, Woodbridge ON) for 1 minute.

2.3.4 RT-PCR

mRNA was isolated using μMACS mRNA isolation kits (Miltenyi Biotec, Auburn CA) according to manufacturers instructions. cDNA was generated using Thermoscript RT-PCR kits (Invitrogen) according to manufacturer's instructions. Murine *podxl* cDNA was detected by PCR with primers 5'- GAGGATTTGTGCACTCTACATGTG-3' and 5'-

TACTCGAGTGGGTTGTCATGGTAACC-3'. GFP was detected with primers 5'-AAGTTCATCTGCACCACCG-3' and 5'-TCCTTGAAGAAGATGGTGCG-3'. Hypoxanthine phosphoribosyl transferase (HPRT) was used as a positive control, with 5'-CTCGAAGTGTTGGATACAGG-3'and 5'-TGGCCTATAGGCTCATAGTG 3' primers.

2.4 Additional Characterization of ESC Clones for Conditional Podocalyxin Overexpressing Transgenic

2.4.1 β-galactosidase Assay

In order to assess ESC clones for β-glactosidase expression, they were washed with PBS, and fixed for 5 minutes at room temperature in 0.2 % glutaraldehyde, 0.01 % sodium deoxycholate, 0.02 % NP-40, 100 mm magnesium chloride, and 5 mm ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) in PBS. Cells were then washed 3 times and stained overnight at 37 °C using 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM magnesium chloride in PBS.

2.4.2 PCR to Detect Transgene

Genomic transgenic murine *podxl* was detected by PCR with primers 5'-GAGGATTTGTGCACTCTACATGTG-3' and 5'-TACTCGAGTGGGTTGTCATGGTAACC-3'. GFP was detected with primers 5'-AAGTTCATCTGCACCACCG-3' and 5'-TCCTTGAAGAAGATGGTGCG-3'.

2.5 Adhesion Assays

MCF-7 cells were plated in triplicate in 6 well plates (5 x 10^5 cells per well) and cultured for 24 hours. Suspension cells included those in culture media as well as those obtained after washing with PBS for 1 minute. Adherent cells were removed with trypsin, diluted with media, and counted as well.

2.6 Confocal Microscopy

Cells were cultured on glass cover slips and fixed for 20 minutes with 37 °C preheated 4 % PFA. All subsequent steps were performed at room temperature. Cells were then rehydrated with PBS, permeabilized with 0.1 % Triton X-100 for 10-15 minutes (or 0.5 % Triton X-100 for 10 minutes for actin labelling), and washed 2 x 10 minutes with PBS before immunolabelling. Cover slips were blocked for 40 minutes with 10 % goat serum and 1 % BSA in PBS, and then rinsed briefly with PBS. Samples were incubated with primary antibodies (Table 2-1) in 1 % BSA for 1 hour, followed by 4 10-minute washes with PBS or 1 % BSA in PBS. Secondary antibodies (Table 2-2) were also diluted in 1 % BSA in PBS and incubated with samples for 1 hour, followed by a single 10-minute wash with PBS or 1 % BSA in PBS. For actin labelling, Alexa Fluor 568-conjugated phalloidin (in 1 % BSA) was added for 15 minutes at this stage, followed by a 10-minute wash with PBS. Nuclei were labelled with 0.5 μg/ml 4', 6'-diamidino-2-phenylindole (DAPI) for 2 minutes, and cells were washed with PBS or 1 % BSA in PBS 4 x 10 minutes. Cover slips were mounted with fluoromount-G (Southern Biotech). Cells transfected with the

empty vector were used as negative controls for podocalyxin staining. Rabbit IgG was used as a negative control for NHERF-1 staining.

Biotinylated ezrin and podocalyxin dual labelling required additional steps to prevent cross-reactivity and background from endogenous biotin. Streptavidin was added during the blocking step at 1 μg/ml to block cellular biotin, and this was followed by 3 10-minute washes with PBS to remove excess unbound streptavidin. In contrast to other dual labelling experiments, in this case antigens had to be labelled sequentially. Podocalyxin was labelled first, with the primary antibody against chicken podocalyxin followed by Alexa Fluor-488-conjugated anti-mIgG$_1$. Cover slips were then blocked with 10 % mouse serum and 1 % BSA in PBS for 40 minutes followed by two additional PBS washes. Ezrin was then labelled with biotinylated anti-ezrin followed by Alexa Fluor-568-conjugated streptavidin before DAPI labelling, as described above. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Mouse IgG$_1$ was used as a negative control for ezrin staining.

Slides were examined using an Olympus Fluoview FV1000 confocal microscope (100X oil immersion objective, NA: 1.35, zoom: 1.4 or 60X oil immersion objective, NA: 1.40). Triple-labelled images were collected sequentially, and confocal sections were acquired in 0.180 μm or 0.230 μm steps. Micrographs were generated with several merged confocal planes or vertical sections of confocal stacks using Olympus Fluoview FV1000 software (version1.3b). Photos were arranged with Adobe Photoshop and Adobe Illustrator software.

2.7 Scanning Electron Microscopy (SEM)

Cells were grown on glass cover slips and fixed using 2.5 % glutaraldehyde with 1 % tannic acid in 0.1 M cacodylate buffer. SEM processing was performed by Derrick Horne in the BioImaging Facility at the University of British Columbia: samples were post-fixed with buffered 1 % osmium tetroxide, dehydrated in a graded series of ethanols, and critical point dried. Images were then collected using a Hitachi S4700 FESEM, and photos were arranged using Adobe Photoshop and Adobe Illustrator software.

2.8 Transmission Electron Microscopy (TEM)

2.8.1 TEM of Cell Lines

Cells were grown on filters (1 μm pore size: Becton Dickinson) and then fixed for 1 hour in 1.5 % glutaraldehyde and 1.5 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The filters were washed with 0.1 M sodium cacodylate buffer, and then post-fixed for 30 minutes on ice in buffered 1 % osmium tetroxide. They were washed with distilled water and stained *en bloc* for 30 minutes with 1 % uranyl acetate. Samples were then dehydrated through a graded series of ethanols and infiltrated with propylene oxide and Polybed. After embedding in Polybed, the samples were polymerized for 24 hours at 60 °C. Thin sections were prepared, stained with uranyl acetate and lead citrate, and viewed and photographed on a Philips 300 electron microscope operated at 60 kV by A. Wayne Vogl at the University of British Columbia. Negatives were scanned into digital

format and contrast adjusted using the Image Adjustments tool. Figures were arranged using Adobe Photoshop and Adobe Illustrator software.

2.8.2 TEM of Kidneys

Kidneys were isolated from E18 mice, cut into 4-6 pieces, and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. Derrick Horne completed the processing and imaging at the University of British Columbia's BioImaging Facility. Samples were washed with cacodylate buffer, post-fixed with 1 % osmium tetroxide, washed with distilled water, stained *en bloc* with 2 % uranyl acetate, and rinsed with distilled water. Samples were then dehydrated using a graded series of ethanols and embedded in resin. Thin sections were imaged using a Hitachi H7600 TEM, and figures were arranged using Adobe Photoshop and Adobe Illustrator software.

2.9 Immunofluorescence of Tissue Sections

2.9.1 Sample Preparation

Mice were anesthetized with 25 mg/ml avertin intraperitoneally and perfused with 4 % paraformaldehyde (PFA). Tissues were removed and fixed in 2 % PFA for 2 hours at 4 °C, followed by incubation in 20 % sucrose overnight at 4 °C. Excess liquid was removed, samples were placed in Tissue Tek embedding medium (Sakura Finetek USA Inc, Torrance CA), frozen in liquid nitrogen, and stored at –80 °C. Tissues were

sectioned using a cryostat set at 6-12 μm, dried overnight, and rehydrated in PBS for 1 hour at 4 °C.

2.9.2 Tissue Staining

Tissue sections were blocked overnight with 25 % goat serum and 10 % BSA in 0.3 % Triton X-100 in PBS. Sections were then incubated with anti-GFP (Table 2-1) in 10 % goat serum, 10 % BSA, and 0.3 % Triton X-100 in PBS for 1.5-2 hours at 4 °C. Samples were washed 5 x 12 minutes with 0.3 % Triton X-100 in PBS at 4 °C. Sections were incubated with secondary antibody (Alexa Fluor-488-conjugated goat anti-rabbit IgG) (Table 2-2) in 10 % goat serum, 10 % BSA, and 0.3 % Triton X-100 in PBS for 1.5-2 hours at 4 °C and washed as above. Cover slips were mounted with fluoromount-G. Tissue sections from C57BL/6 (B6) mice were used as negative controls for GFP staining. Slides were examined using a Zeiss microscope with a 40X objective or a 63X oil immersion objective.

2.10 Immunohistochemistry

Tissues were embedded in Tissue Tek, frozen in liquid nitrogen, and stored at –80 °C. Frozen samples were sectioned, fixed in acetone, and rehydrated in PBS. Samples were stained using the Vectastain ABC Kit, according to manufacturer's instructions (Vector Laboratories). Slides were blocked with 10 % FBS in PBS, labelled with primary antibodies (anti-mouse podocalyxin or PECAM-1) for 30-45 minutes (Table 2-1), washed with PBS, labelled with secondary antibody (biotinylated anti-rat IgG) (Table 2-2) for 30-

45 minutes, and washed again. Rat IgG_1 was used as a negative control for podocalyxin and PECAM-1 staining. Slides were then incubated in 0.3 % hydrogen peroxide in methanol for 15-30 minutes to quench endogenous peroxidase. This was followed by 2 3-minute PBS washes, incubation in the ABC reagent for 30-45 minutes, and another PBS wash. Slides were developed with the diaminobenzidine (DAB) substrate, and washed in PBS. Nuclei were labelled with 1 % methyl green for 2-3 minutes and slides were washed with PBS and dilute ammonia. Samples were then dehydrated through a graded series of ethanols, followed by methanol and xylene, and cover slips were sealed with Permount. Slides were examined using a Zeiss microscope with a 20X objective or a 63X oil immersion objective.

2.11 Mice

All mice were maintained on a B6 background in the Biomedical Research Centre's mouse facility. Mice were genotyped for the wildtype or knockout allele of *podxl* by PCR using primers 5'-GAGGATTTGTGCACTCTACATGTG-3', 5'-TATCGCCTTCTTGACGAGTTCTT-3', and 5'-AGTGAGAGACACATTGGGTAACT-3'. The first primer is a common primer, with a sequence found in the fifth exon of *podxl*, the second primer's sequence is found in the *neomycin resistance* cassette of knockout mice, and the sequence of the third primer is found in the fifth intron of wildtype animals. The expected PCR products are 550 bp (knockout) and 760 bp (wildtype) in length. Podocyte-specific podocalyxin transgenic mice were genotyped for presence of the transgene using the GFP-specific primers 5'-AAGTTCATCTGCACCACCG-3' and 5'-TCCTTGAAGAAGATGGTGCG-3', which produced a 360 bp PCR product.

CHAPTER 3 : OVEREXPRESSION OF WILDTYPE AND MUTANT PODOCALYXIN IN EPITHELIAL CELLS

3.1 Rationale

At the outset of this study, there were only two previous publications based on *in vitro* analysis of podocalyxin's function. The first described podocalyxin as a ligand for L-selectin in HEV (Sassetti et al., 1998), while the second suggested that podocalyxin inhibited cell-cell adhesion and disrupted cell junctions (Takeda et al., 2000). It was clear that further analysis was required in order to decipher podocalyxin's function and to understand its roles mechanistically. Later papers provided biochemical evidence to suggest that podocalyxin could interact with the actin cytoskeleton through ezrin and members of the NHERF family of adapter proteins (Li et al., 2002; Orlando et al., 2001; Schmieder et al., 2004; Takeda et al., 2001), but the functional implications of these interactions were unclear. Podocalyxin was therefore expressed in numerous cell lines in order to clarify its function and mechanism of action.*

- Some data presented in this chapter can be found in the following articles:

1) Nielsen, J.S., McCoy, M.L., Chelliah, S., Vogl, A.W., Roskelley, C.D., and McNagny, K.M. The CD34-related molecule, Podocalyxin, is a potent inducer of microvillus formation. *Proc Natl Acad Sci U S A*, submitted.

2) Somasiri, A., Nielsen, J.S., Makretsov, N., McCoy, M.L., Prentice, L., Gilks, C.B., Chia, S.K., Gelmon, K.A., Kershaw, D.B., Huntsman, D.G., McNagny, K.M. and Roskelley, C.D. (2004) Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression. *Cancer Res*, **64**, 5068-5073.

In addition, a particularly interesting feature of podocalyxin's normal expression pattern is its tendency to be expressed in cells with unique and intricate cell surface morphologies. For example, podocalyxin is expressed by megakaryocytes, which extend long processes when generating platelets (McNagny et al., 1997; Miettinen et al., 1999). As discussed in section 1.6, it is also essential for formation of the elaborate foot processes associated with mature kidney podocytes. Assessing the possibility of podocalyxin having a more global role in regulating cell surface morphology was therefore another major goal of this study.

Kidney and breast epithelial cell lines were the main model systems used for our *in vitro* studies since in addition to podocalyxin's essential role in normal kidney development (Doyonnas et al., 2001), our early studies also described podocalyxin as a predictive marker of poor outcome in human breast cancer (reviewed in section 1.10.1) (Somasiri et al., 2004). Specifically, two cell lines were used for the majority of experiments: MDCK and MCF-7 cells. MDCK cells were chosen because they are a well-characterized kidney cell line that had been used in other podocalyxin-related publications (Li et al., 2002; Orlando et al., 2001). MCF-7 breast carcinoma cells were used since they are a weakly invasive epithelial cell line that expresses low levels of endogenous podocalyxin, in comparison to the more invasive, highly podocalyxin-positive MDA-231 cell line (Figure 3-1) (Somasiri et al., 2004). Of note, podocalyxin is absent from the non-invasive T47D breast cell line. Thus, MDCK and MCF-7 cell lines were appropriate for overexpression studies to address the function of podocalyxin.

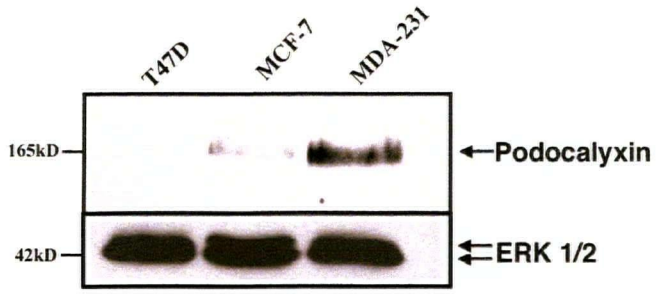

Figure 3-1: Endogenous Human Podocalyxin Expression in Breast Carcinoma Lines.

Western blot analysis demonstrates that metastatic MDA-231 cells express considerably higher levels of podocalyxin than the less invasive T47D and MCF-7 cell lines (Somasiri et al., 2004).

After finding a suitable assay system for assessing podocalyxin's function, various podocalyxin mutants were generated and tested using this system. These studies provided important mechanistic insights into podocalyxin's functions.

3.2 Expression of Full-Length Podocalyxin in MCF-7 and MDCK Cells

3.2.1 Expression Analysis

Murine podocalyxin was cloned into the pIRES2-EGFP expression vector to enable ectopic expression *in vitro*. This vector facilitates expression of any gene of interest, along with GFP via an internal ribosome entry site (IRES) sequence. In addition, a neomycin resistant cassette allows for selection of stably expressing transfectants.

MCF-7 and MDCK cells were transfected with the podocalyxin-containing plasmid, or the empty vector as a control. Stably transfected clones were isolated based on expression of podocalyxin or GFP in combination with the neomycin-resistance gene (Figure 3-2).

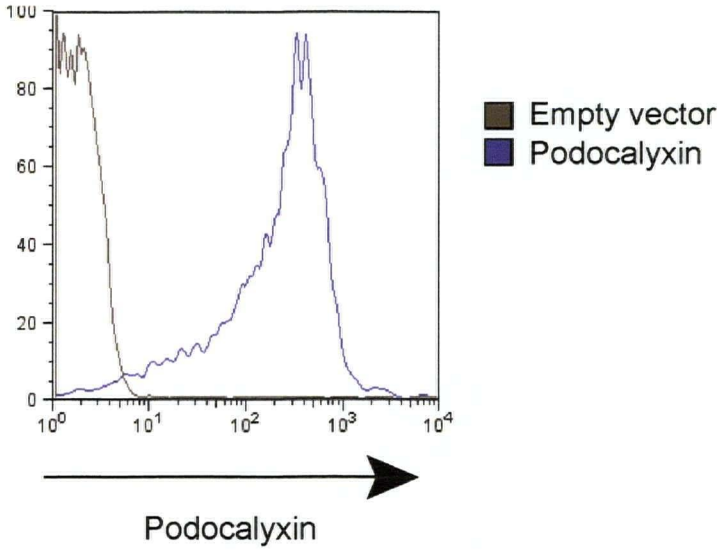

Figure 3-2: Ectopic Murine Podocalyxin Expression in Transfected MCF-7 Breast Epithelial Cells.

3.2.2 Podocalyxin Decreases Cell Adhesion in Epithelial Cells

Upon initial examination of transfected cells, the most obvious difference between those transfected with podocalyxin or empty vector was that cells ectopically expressing podocalyxin formed large clusters of suspension cells rather than confluent monolayers of adherent cells, as shown in Figure 3-3. Quantitative experiments revealed that there were close to four times as many cells in suspension in the podocalyxin-transfected population (Figure 3-4). These cells maintained viability, as assessed by flow cytometry and replating of disaggregated cells. These experiments did not explain, however, the mechanism leading to decreased cell-substrate adhesion. It is somewhat counterintuitive that a protein expressed on the apical surface of epithelial cells could lead to decreased adhesion at the basolateral surface. Further experiments (described below) provided some insights into this phenomenon.

Moreover, in support of previously published work (Takeda et al., 2000), there were also apparent alterations in cell-cell junctions in MCF-7 cells overexpressing podocalyxin (Figure 3-5) (Somasiri et al., 2004): instead of a well aligned apical bar of staining, as in the vector control, podocalyxin-positive populations displayed disorganized localization of the tight junction protein, ZO-1. Thus, the data confirmed that podocalyxin functions as an anti-adhesion molecule, affecting both cell-cell junctions, and cell-substrate interactions.

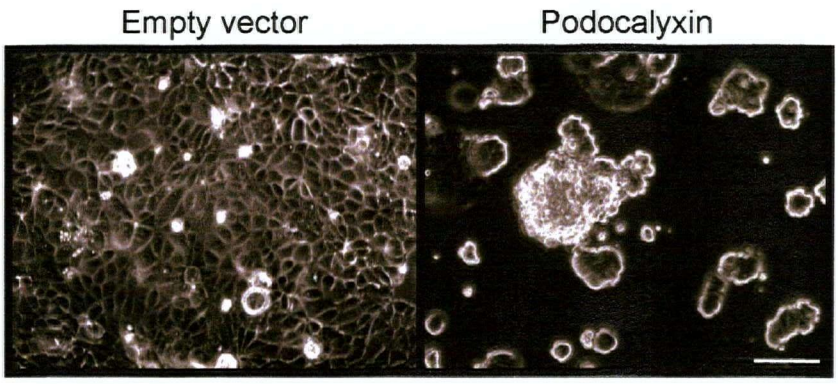

Figure 3-3: Transfected MCF-7 Cells Ectopically Expressing Murine Podocalyxin Exhibited Decreased Cell-Substratum Interactions.

Scale bar: 50 μm.

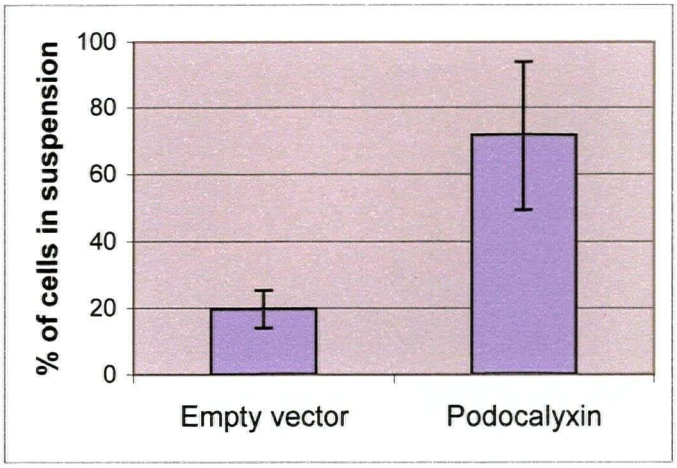

Figure 3-4: Podocalyxin Decreases Cell Substrate Adhesion.

After MCF-7 cells transfected with empty vector or vector encoding murine podocalyxin were sorted based on podocalyxin expression, 5 x 10^5 cells were plated per well in 6-well plates. One day later, suspension and adherent cells were counted in triplicate wells. Representative of three independent experiments.

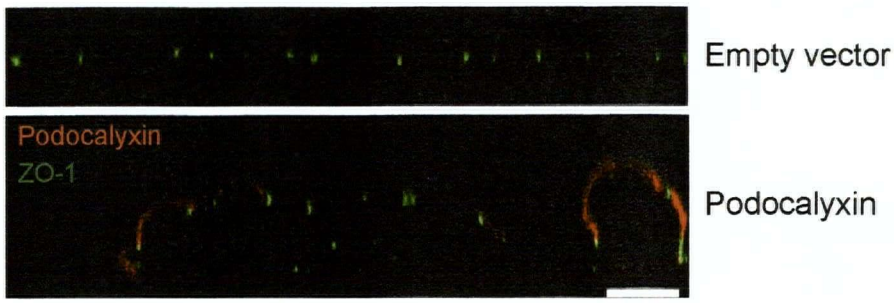

Figure 3-5: Podocalyxin-Induced Alteration of Cell-Cell Junctions.

MCF-7 cells transfected with empty vector or vector encoding murine podocalyxin and labelled with antibodies against ZO-1 (green) and podocalyxin (red). Cell junctions were abnormal and monolayers were disrupted upon ectopic expression of podocalyxin. Scale bar: 15 μm (Somasiri et al., 2004).

3.2.3 Podocalyxin Recruits NHERF1 to the Apical Surface of Cells

The previous experiments clearly supported the role of podocalyxin in disrupting cell adhesion, but how it accomplished this was not immediately obvious. Since the actin cytoskeleton plays an important role in cell adhesion, molecules that have been suggested to link podocalyxin to actin were examined. Although biochemical evidence suggested that podocalyxin could interact with both NHERF1 and NHERF2, podocalyxin had only been shown to colocalize with NHERF2 (Li et al., 2002; Takeda et al., 2001). Taking advantage of the fact that MCF-7 cells express NHERF1, its localization with respect to podocalyxin was assessed by confocal microscopy.

In cells expressing both molecules, podocalyxin and NHERF1 were clearly apically colocalized (Figure 3-6). In cells transfected with empty vector, however, NHERF1 was consistently expressed throughout the cytoplasm (Figure 3-6C, left panel). Thus, podocalyxin expression led to a dramatic increase in apically localized NHERF1 with a concomitant decrease in cytoplasmic staining (Figure 3-6C, right panel). Since NHERF family proteins function as scaffolding proteins and interact with actin, it had been suggested that they could play a role in proper localization of podocalyxin (Cheng et al., 2005; Li et al., 2002; Meder et al., 2005; Schmieder et al., 2004). It was therefore very intriguing that podocalyxin, not NHERF1, was responsible for apical localization of both molecules (Figure 3-6). Regardless, the interaction of podocalyxin with this scaffolding protein implied a link to the cytoskeleton and a potential clue to podocalyxin's role in blocking cell adhesion.

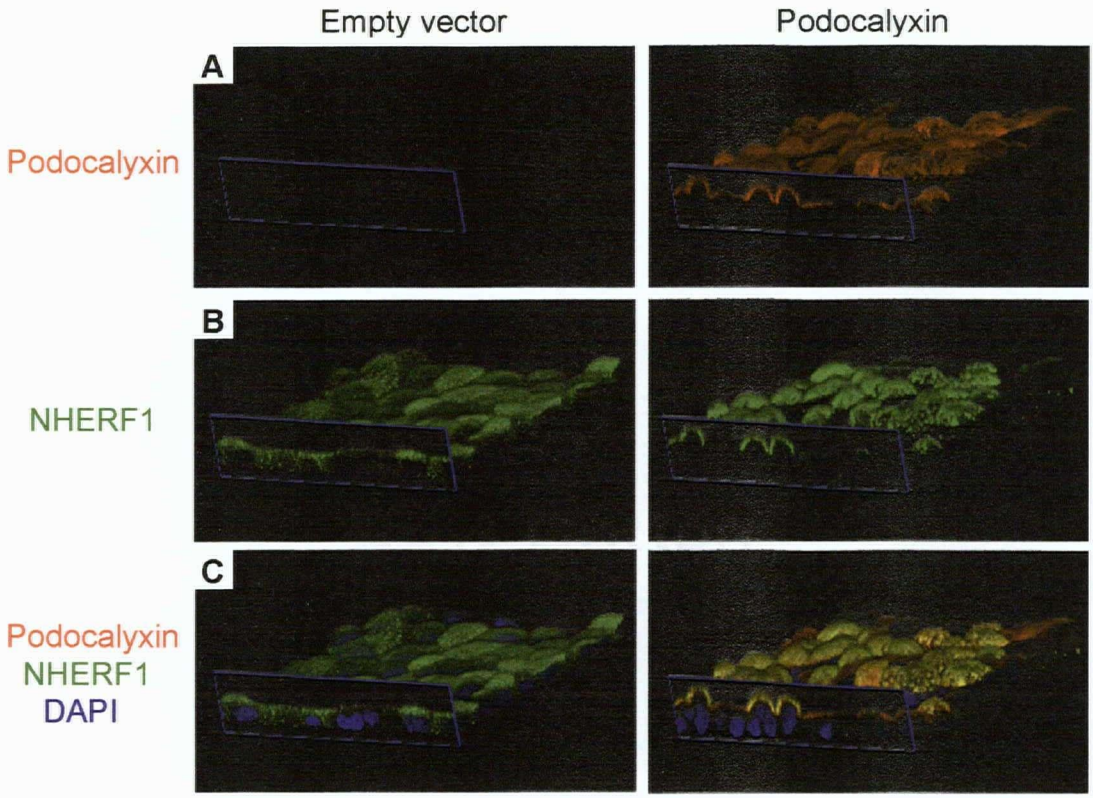

Figure 3-6: Confocal Images Demonstrated Apical Recruitment of NHERF1 by Podocalyxin.

MCF-7 cells transfected with empty vector or vector encoding murine podocalyxin were labelled with DAPI (blue) and antibodies against podocalyxin (red) and NHERF1 (green). Yellow represents colocalization of podocalyxin and NHERF1. Imaging by Marcia L. McCoy, University of British Columbia.

3.2.4 Morphological Changes: Podocalyxin Induces Microvillus Formation in Epithelial Cells

After gaining some insights into podocalyxin's function as an anti-adhesion molecule, it was possible to concentrate on its potential role in influencing cell morphology. Electron microscopy was used to assess the cell surface of epithelial cells transfected with podocalyxin or empty vector. In particular, the formation of microvilli was assessed by electron microscopy because there is some evidence to suggest that the podocalyxin binding protein, NHERF1 may be involved in the generation of such structures. For example, the intestinal brush border microvilli of one strain of NHERF1-null mice are strikingly disorganized in comparison to those of wildtype littermates (Morales et al., 2004). Furthermore, when MCF-7 cells are treated with estradiol, they upregulate NHERF1 mRNA, and the number and length of microvilli on the cell surface increases (Ediger et al., 1999; Vic et al., 1982). While this certainly does not prove that NHERF is responsible for microvillus formation, in combination with the above data it is suggestive enough to warrant further investigation. Thus, microvillus formation was assessed in podocalyxin-transfected MCF-7 cells.

The difference between the two cell populations was striking: although control cells had some microvilli, podocalyxin-transfected cells were covered with these cell surface protrusions, as shown by scanning electron microscopy (SEM) (Figure 3-7). A very similar result was observed in transfected MDCK cells (Figure 3-8), demonstrating that this dramatic phenotype was not an artifact of one particular cell line. Both cell lines were

also examined by transmission electron microscopy (TEM), with consistent results (Figure 3-9).

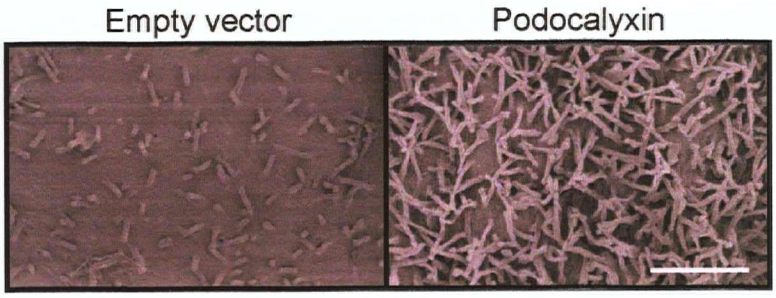

Figure 3-7: Podocalyxin Induced Microvillus Formation in MCF-7 Cells.

SEM's of MCF-7 cells transfected with empty vector or vector encoding murine podocalyxin. Scale bar: 2 μm. Representative of two independent experiments.

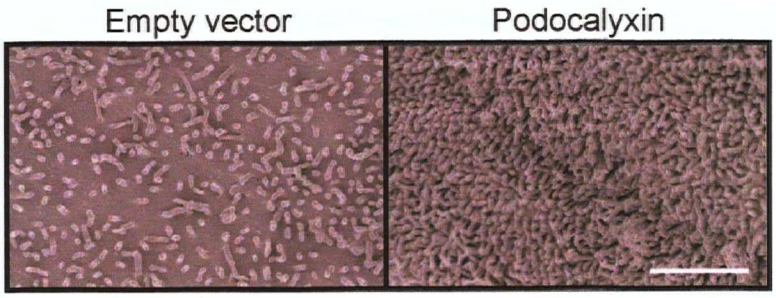

Figure 3-8: Podocalyxin Also Induced Microvillus Formation in MDCK Epithelial Cells.

SEM's of MDCK cells transfected with empty vector or vector encoding murine podocalyxin. Scale bar: 2 μm.

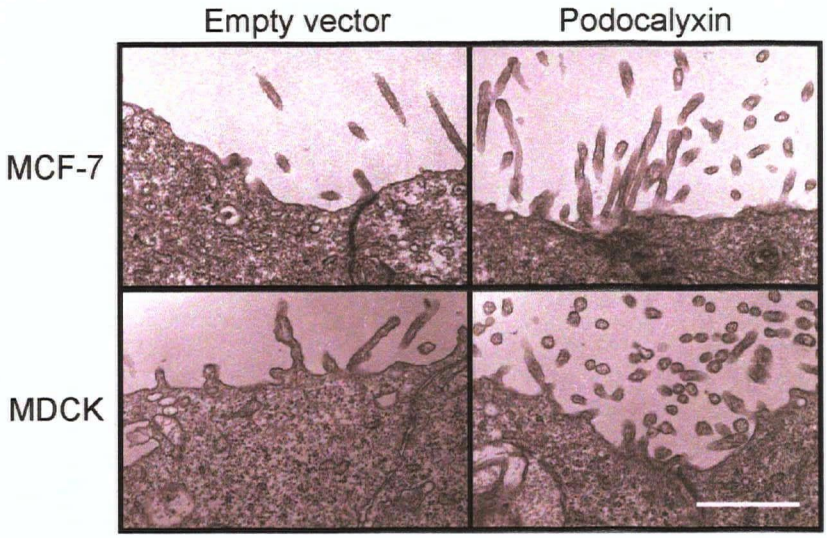

Figure 3-9: TEM's of Epithelial Cells Transfected with Empty Vector or Vector Encoding Murine Podocalyxin.

Scale bar: 1 μm. Representative of two independent experiments. Imaging by A. Wayne Vogl, University of British Columbia.

3.3 Generation and Analysis of Podocalyxin Mutants

In order to gain further insights into the mechanisms involved in podocalyxin-induced NHERF-recruitment and microvillus formation, a panel of chicken podocalyxin mutants was generated, as depicted in Figure 3-10. Most mutations were in the cytoplasmic tail as the high level of conservation in this region suggested functional importance, and mutations were expected to block or promote interaction with known and unknown binding partners. The mutants ranged from those lacking entire domains to those with single point mutations, as follows: 1) serine and threonine residues of two potential phosphorylation sites were mutated, alone or in combination, to alanine residues to block phosphorylation in the Ala mutants; 2) the same residues were converted to aspartic acid residues to mimic constitutive phosphorylation in the Asp constructs; 3) the ΔDTHL mutant lacked the C-terminal DTHL sequence essential for interaction with NHERF proteins; 4) Δtail lacked the entire cytoplasmic tail with the exception of the juxtamembrane sequence CCHQRF, which was retained as a membrane anchor (this mutant lacked all potential phosphorylation sites, the C-terminal NHERF-binding site, and a putative ezrin-binding site); 5) ΔEC lacked the majority of the extracellular domain (including the mucin domain and the cysteine-bonded globular domain) and instead encoded an extracellular flag-tag, as well as podocalyxin's transmembrane region and its full-length cytoplasmic tail; and 6) the final construct, Alt. splice, contained podocalyxin's naturally occurring spice variant, which lacks much of the cytoplasmic tail.

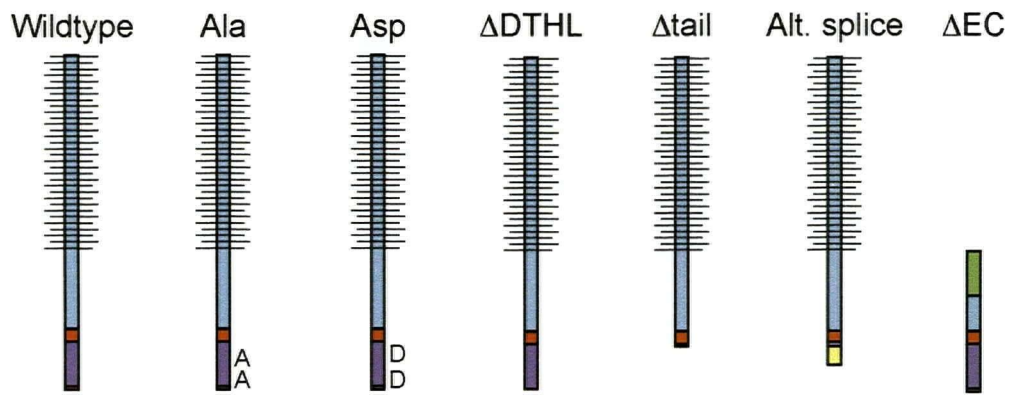

Figure 3-10: Schematic of Podocalyxin Mutants.

Light blue: extracellular domain, green: flag-tag, red: transmembrane domain, purple: cytoplasmic tail (including C-terminal DTHL in wildtype, Ala, Asp, and ΔEC), yellow: cytoplasmic tail of splice variant, horizontal lines: glycosylations, A: point mutation to alanine, and D: point mutation to aspartic acid.

Avian podocalyxin was chosen as the basis for these experiments for three reasons: 1) it is 85% identical to mammalian podocalyxin in its intracellular domain, 2) as with the murine protein used in section 3.2, wildtype avian podocalyxin also induced microvillus formation in MCF-7 cells, and 3) I could selectively detect its ectopic expression using a species-specific monoclonal antibody that reacts with native, fixed, and denatured forms of the molecule (McNagny et al., 1997).

3.3.1 Podocalyxin Mutant Expression Analysis

3.3.1.1 Podocalyxin-Positive Cells were Continuously Lost from Bulk Populations

The mutants described above were cloned into the pcDNA3.1 expression vector and transfected into MCF-7 cells. Unfortunately, there was a consistent problem with all podocalyxin overexpression studies: in bulk populations of transfected cells, cells expressing podocalyxin were continuously lost from cultures. Although this was partly due to loss of suspension cells when changing media, modification of the culture techniques to retain suspension cells did not completely solve the problem. Initially, this led to recloning of constructs into an alternative expression vector (pIRES2-EGFP). As this did not solve the problem either, FACS was used to serially sort cultures for podocalyxin expression. Although this enabled generation of populations containing higher proportions of podocalyxin-positive cells, expression was still not stable. Figure 3-11 shows FACS profiles of transfected cells before sorting, and after one, two, or three rounds of sorting. Although the percentage of podocalyxin-positive cells did increase

after each round, the cells that were cultured after each sort are shown in the white boxes, and this level of podocalyxin expression was clearly not maintained. Although not perfect either, the most successful strategy for generating stably expressing cultures was to isolate and expand single podocalyxin-positive clones. Using this strategy, at least three clones were isolated for each mutant. Most experiments were performed with these clones, and key results were confirmed with bulk populations.

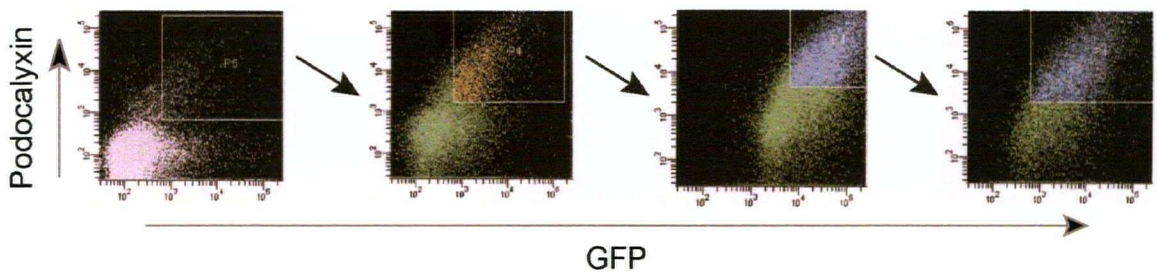

Figure 3-11: Podocalyxin Expression After Multiple Rounds of Cell Sorting.

Transfected MCF-7 cells were sorted based on podocalyxin and GFP expression. Cells in white boxes were expanded and serially sorted. Thus, cells in the first white box were sorted and expanded. These cells gave rise to the cells depicted in the second FACS plot. Similarly, only the cells in the second white box were retained; these gave rise to all of the cells depicted in the third FACS blot, and so on.

3.3.1.2 Podocalyxin Mutants were All Expressed and were of Expected Molecular Weights

Western blotting was used to assess the expression of each mutant in clonal populations (Figure 3-12). This also enabled confirmation that each form of podocalyxin was of the appropriate molecular weight. The lower band was non-specific, as it was also observed in empty vector transfected cells (Vector 6). Full-length podocalyxin was approximately 160 kDa, as expected (Wildtype 7 and Wildtype 13), as were mutants containing Ser/Thr→Ala or Ser/Thr→Asp point mutations (Asp 7, Ala 3, and Ala 6). Cytoplasmic tail deletions (Δtail 2 and Δtail 8) and the alternatively spliced form of podocalyxin (Alt. splice 5), which all lack most of podocalyxin's cytoplasmic tail, were noticeably shorter, as up to 71 amino acids had been deleted. Expectedly, truncation of the four C-terminal amino acids made little difference to the size of the protein (ΔDTHL 2 and ΔDTHL 15). The mutant lacking most of the extracellular domain was detected using an anti-flag antibody, which recognized the flag-tag inserted in place of the deleted portion of podocalyxin. As predicted, this mutant was considerably shorter than full-length podocalyxin due to the absence of the heavily glycosylated mucin domain (ΔEC 8).

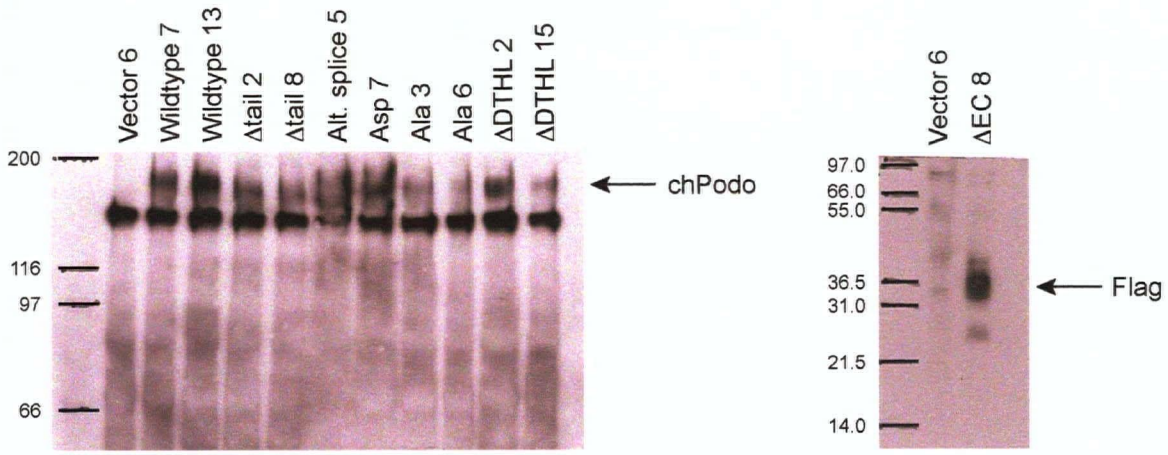

Figure 3-12: Western Blots Demonstrated Expression of Podocalyxin Mutants of the Expected Molecular Weights in Clonal Populations.

Note the slightly smaller size of podocalyxin mutants with shorter cytoplasmic tails. Cells transfected with the empty vector were used as negative controls. Size markers were measured in kDa. Representative of three independent experiments.

3.3.2 Mutation of Phosphorylation Sites and Analysis of Podocalyxin's Splice Variant did not Provide any Novel Insights into Podocalyxin's Function

Although many experiments were performed with all mutants, there was no detectable difference between wildtype podocalyxin and the phosphorylation mutants (Asp and Ala) or between podocalyxin's splice variant (Alt. splice) and the full cytoplasmic tail deletion (Δtail). For this reason, repetitive information has been omitted by including analysis of just five constructs in each figure: empty vector, wildtype podocalyxin, ΔDTHL, Δtail, and ΔEC. Western blotting was used to detect protein expression of the mutants described in the following experiments (Figure 3-13), and surface expression was confirmed by flow cytometry, as shown in Figure 3-14. Expression levels were comparable, with the exception of the extracellular deletion, ΔEC, which could not be accurately compared due to the use of an independent antibody for its detection.

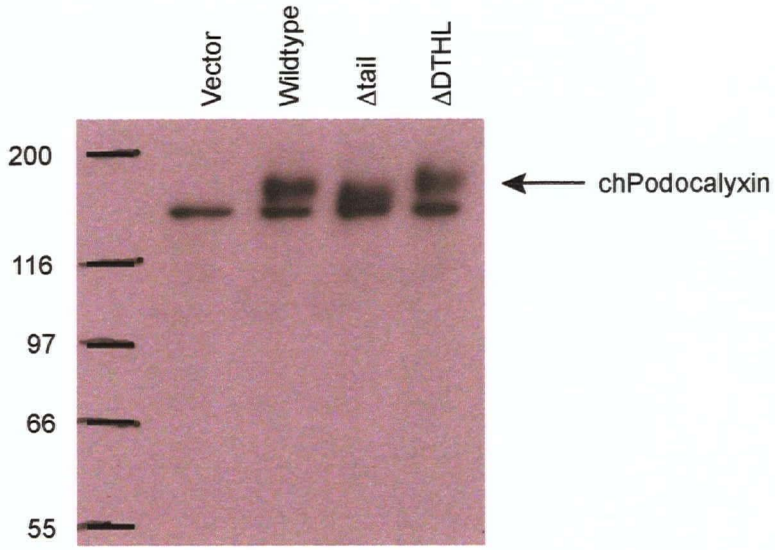

Figure 3-13: Western Blots Demonstrated Expression of Podocalyxin in Clonal Populations Used for Subsequent Experiments.

Note the slight decrease in size when the cytoplasmic tail was deleted. Size markers were measured in kDa.

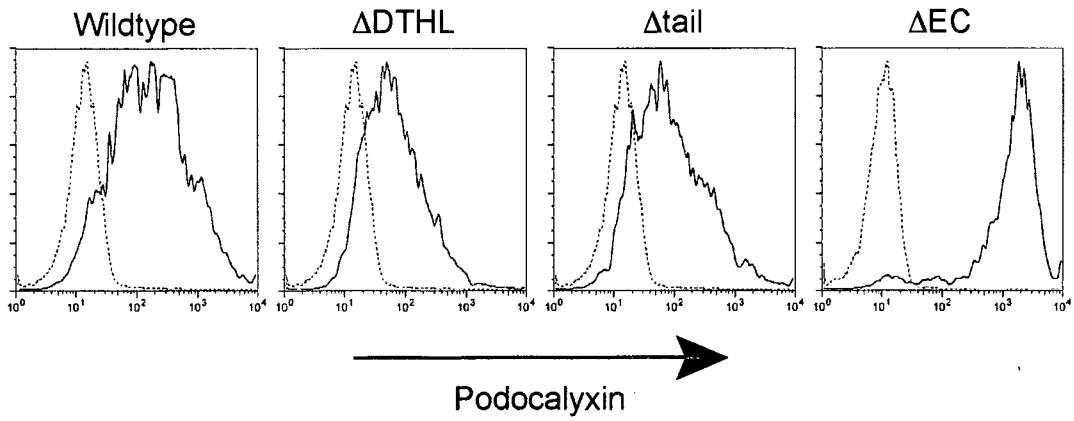

Figure 3-14: Podocalyxin was Expressed at Comparable Levels at the Cell Surface in Clonal Populations.

Transfected cells were clonally sorted, expanded, and labelled with antibodies against chicken podocalyxin or the flag-tag (ΔEC mutant). Dotted lines represent background staining measured on empty vector-transfected cells. Representative of three independent experiments.

3.3.3 Interaction of Podocalyxin with NHERF1

3.3.3.1 Podocalyxin's C-terminal DTHL Sequence is Required for Interaction with NHERF1

Previous experiments demonstrated that podocalyxin and NHERF1 were colocalized in MCF-7 cells (Figure 3-6), and it is known that podocalyxin's four C-terminal amino acids are the PDZ recognition site for NHERF proteins (Li et al., 2002; Takeda et al., 2001; Tan et al., 2006). If podocalyxin and NHERF1 were truly interacting in MCF-7 cells, then colocalization would be lost in cells expressing podocalyxin truncations lacking the PDZ binding site. Confocal microscopy was therefore used to assess this interaction. As shown in merged images taken at the apical surface of transfected cells, wildtype podocalyxin showed strong colocalization with NHERF1, while both C-terminal truncation mutants (Δtail and ΔDTHL) did not (Figure 3-15). In contrast, the ΔEC mutant lacking the extracellular domain but retaining the entire cytoplasmic tail of podocalyxin was found mainly apically localized and colocalized with NHERF1. This further demonstrates that podocalyxin recruits NHERF1 to the apical domain through its C-terminal DTHL motif.

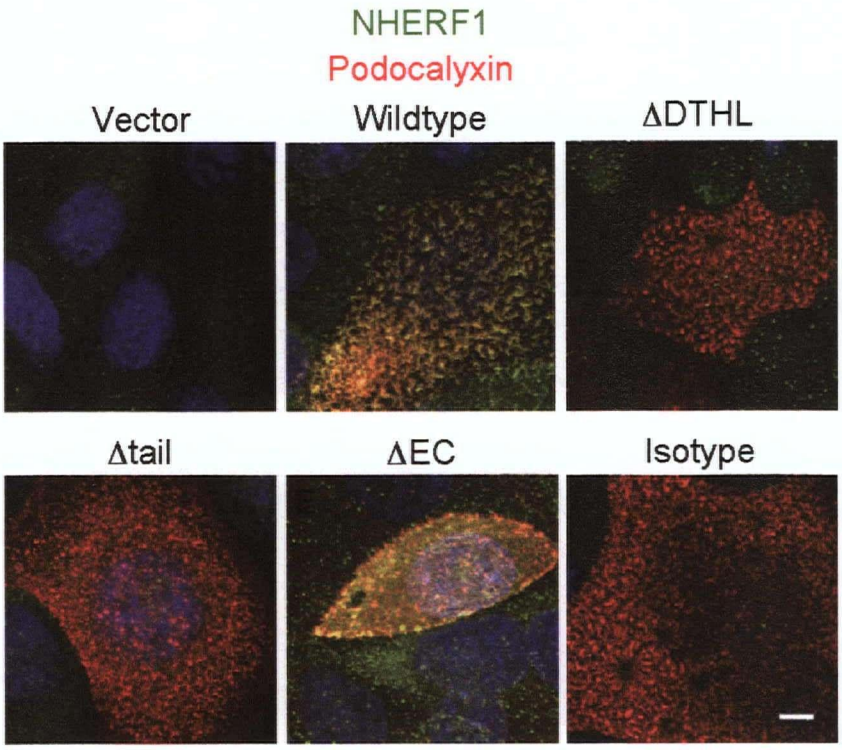

Figure 3-15: Confocal Analysis of NHERF1 and Podocalyxin at the Apical Surface of Transfected MCF-7 Cells.

Cells were labelled with DAPI (blue) and antibodies against NHERF1 (green) and podocalyxin (red). Yellow represents colocalization of NHERF1 and podocalyxin. The isotype control sample was labelled with DAPI (blue), anti-podocalyxin (red), and an isotype control for NHERF1 (green) to demonstrate the specificity of NHERF1 labelling. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

Apical recruitment of NHERF1 could be clearly visualized in vertical slices of confocal stacks (Figure 3-16). Cytoplasmic NHERF1 staining was visible in cells transfected with C-terminal deletions of podocalyxin, while an increase in apical NHERF1 staining was accompanied by a decrease in cytoplasmic staining in the wildtype and ΔEC samples. These experiments confirmed that podocalyxin and NHERF1 are *bona fide* interaction partners *in vivo*. Importantly, the podocalyxin-dependent recruitment of NHERF1 to the apical cell surface may have implications for functional regulation of NHERF family members.

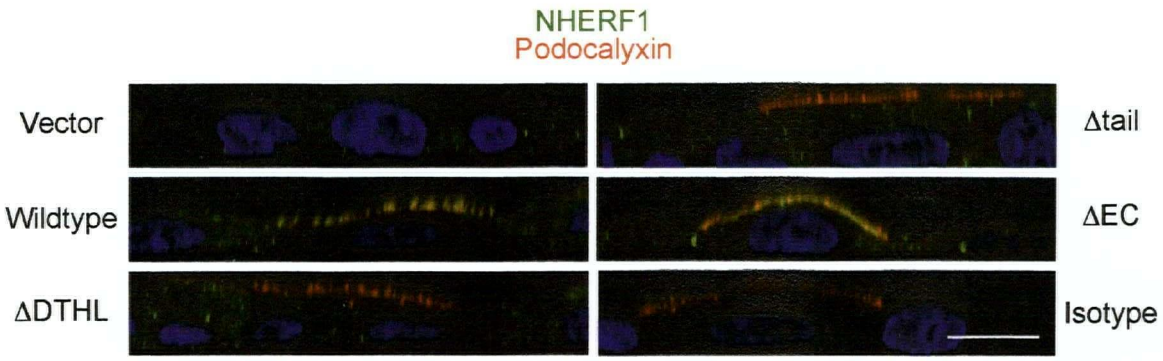

Figure 3-16: Analysis of NHERF1 and Podocalyxin in Vertical Slices of Confocal Stacks.

Transfected MCF-7 cells were labelled with DAPI (blue) and antibodies against NHERF1 (green) and podocalyxin (red). Yellow represents colocalization of NHERF1 and podocalyxin. The isotype control sample was labelled with DAPI (blue), anti-podocalyxin (red), and an isotype control for NHERF1 (green) to demonstrate the specificity of NHERF1 labelling. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

3.3.4 Analysis of Essential Sequences Required for Morphological Changes

The effect of loss of the podocalyxin/NHERF1 interaction on microvillus formation was then assessed by electron microscopy using clonally isolated MCF-7 cells transfected with podocalyxin mutant constructs (Figure 3-17A and Figure 3-18A).

3.3.4.1 Interaction with NHERF1 is Not Required for Formation of Microvilli

TEM demonstrated that, as expected, control cells had few microvilli while cells transfected with full-length podocalyxin displayed increased microvilli (Figure 3-17B). Strikingly, cells expressing ΔDTHL or Δtail mutants generated microvilli in similar numbers to full-length podocalyxin transfectants. SEM analysis confirmed these observations (Figure 3-17C). This demonstrated that direct interaction of podocalyxin with NHERF proteins was surprisingly <u>not</u> required for formation of microvilli.

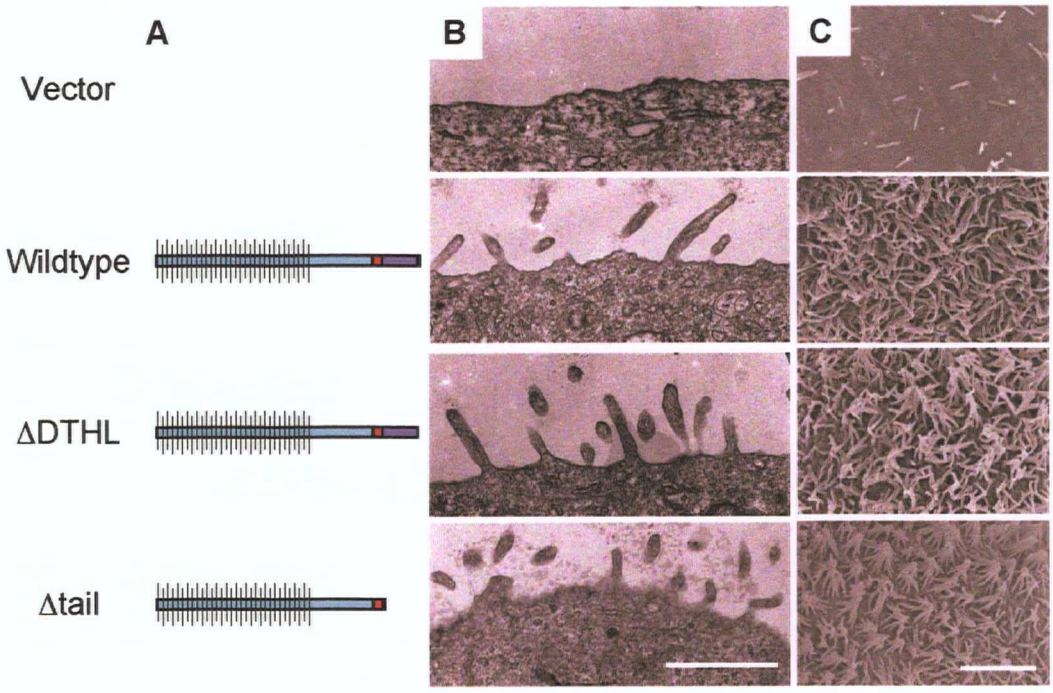

Figure 3-17: Podocalyxin Induced Microvillus Formation in a NHERF-Independent Manner.

(A) Schematic of podocalyxin and mutants, as described in Figure 3-10. (B) TEM's of transfected MCF-7 cells. Scale bar: 1 μm. (C) SEM's of transfected MCF-7 cells. Scale bar: 2 μm. Representative of two independent experiments. TEM imaging by A. Wayne Vogl, University of British Columbia.

3.3.4.2 Podocalyxin's Extracellular Domain is Required for Formation of Microvilli

Conversely, TEM revealed that the extracellular domain of podocalyxin was essential for microvillus formation: there was no increase in microvillus number in cells expressing the ΔEC mutant bearing only the flag-tagged transmembrane and cytoplasmic domain of podocalyxin (Figure 3-18B), even though this mutant was able to apically recruit NHERF1 (see Figure 3-15, above). This result was confirmed via SEM analysis (Figure 3-18C).

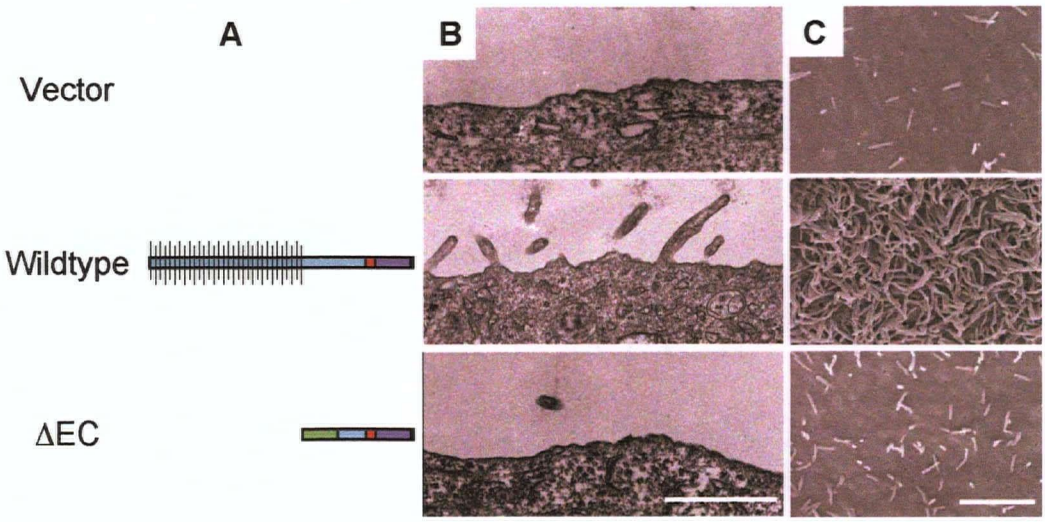

Figure 3-18: Deletion of the Majority of Podocalyxin's Extracellular Domain Abolished Microvillus Formation.

(A) Schematic of wildtype podocalyxin and ΔEC mutant. (B) TEM's of transfected MCF-7 cells. Scale bar: 1 μm. (C) SEM's of transfected MCF-7 cells. Scale bar: 2 μm. Representative of two independent experiments. TEM imaging by A. Wayne Vogl, University of British Columbia.

In order to quantitate this effect, all microvilli observed in six random 15 000X SEM fields were enumerated for each mutant (Figure 3-19). Full-length, ΔDTHL, and Δtail transfectants all had at least twice as many microvilli as vector-control and ΔEC transfectants. It is therefore possible to conclude that the extracellular domain, transmembrane region, and six amino acids of podocalyxin's cytoplasmic tail were sufficient to induce NHERF1-independent formation of microvilli.

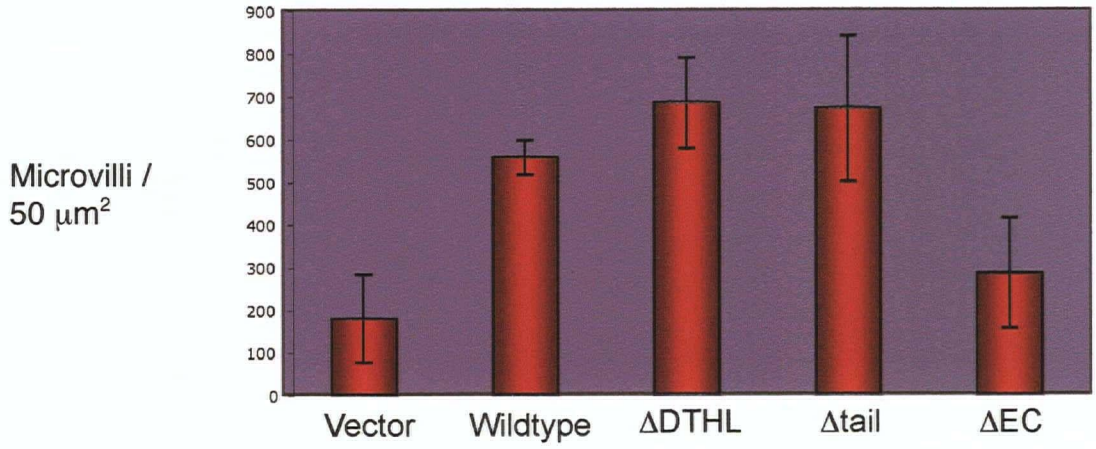

Figure 3-19: Microvillus Counts for Transfected Cells.

Microvilli in six random 50 μm^2 fields were enumerated for MCF-7 cells transfected with each podocalyxin construct. Error bars represent standard deviation. Representative of two independent experiments.

It has been shown that maintenance of podocyte foot process integrity is critically dependent on the negatively-charged glycosylations decorating podocalyxin's extracellular domain (Andrews, 1979; Kerjaschki et al., 1984; Seiler et al., 1975); it was therefore not particularly surprising that deletion of the entire mucin domain also prevented microvillus formation in MCF-7 cells. However, the fact that the highly conserved, NHERF-binding, cytoplasmic tail of podocalyxin was dispensable for microvillus formation was quite unexpected. It is known that the integrity of the actin cytoskeleton is essential for maintaining cell shape, and that linkage of actin to integral membrane proteins is important for generating and supporting cell surface protrusions, including microvilli (Revenu et al., 2004). Since NHERF1 and NHERF2 can connect podocalyxin to the actin cytoskeleton through ezrin (Morales et al., 2004; Takeda et al., 2001; Tan et al., 2006), it was initially assumed that podocalyxin's involvement in the formation of microvilli would require interaction with NHERF proteins. Moreover, NHERF2 interactions with podocalyxin have recently been shown to coincide closely with the formation of a "pre-apical domain" in MDCK cells (as discussed in section 1.5.4), and it was postulated that the association was a prerequisite for formation of this domain (Meder et al., 2005). However, the current data suggested that, although the C-terminal tail of podocalyxin was sufficient to actively recruit NHERF1 to apical plasma membrane domains, the formation of microvilli and, indeed, the apical targeting of podocalyxin to the plasma membrane occurred in the absence of any direct interaction with NHERF.

3.3.5 Interaction with NHERF is Unnecessary for Colocalization of Podocalyxin with Ezrin

Since microvilli formation coincides with dramatic reorganization of the apical membrane domain, polymerization of f-actin at the core of microvilli, and recruitment of members of the ERM family of proteins to the apical domain, presumably to act as linkers between the cytoskeleton and transmembrane proteins (reviewed in (Louvet-Vallee, 2000)), the localization of ezrin and f-actin were assessed in transfected cells. Interestingly, cells transfected with all forms of extracellular domain-containing podocalyxin, regardless of whether or not they contained a NHERF-binding sequence, displayed increased apical recruitment of ezrin and strong colocalization of podocalyxin with this protein (Figure 3-20). Increased apical recruitment of ezrin was especially evident in vertical sections of confocal stacks, where strong ezrin staining corresponded to cells expressing ectopic podocalyxin (Figure 3-21). Thus, recruitment of ezrin to the apical membrane occurred in the absence of any direct interaction of podocalyxin with NHERF. In contrast, recruitment of ezrin appeared to be somewhat less pronounced in the ΔEC mutant, again demonstrating the importance of the extracellular domain in podocalyxin function.

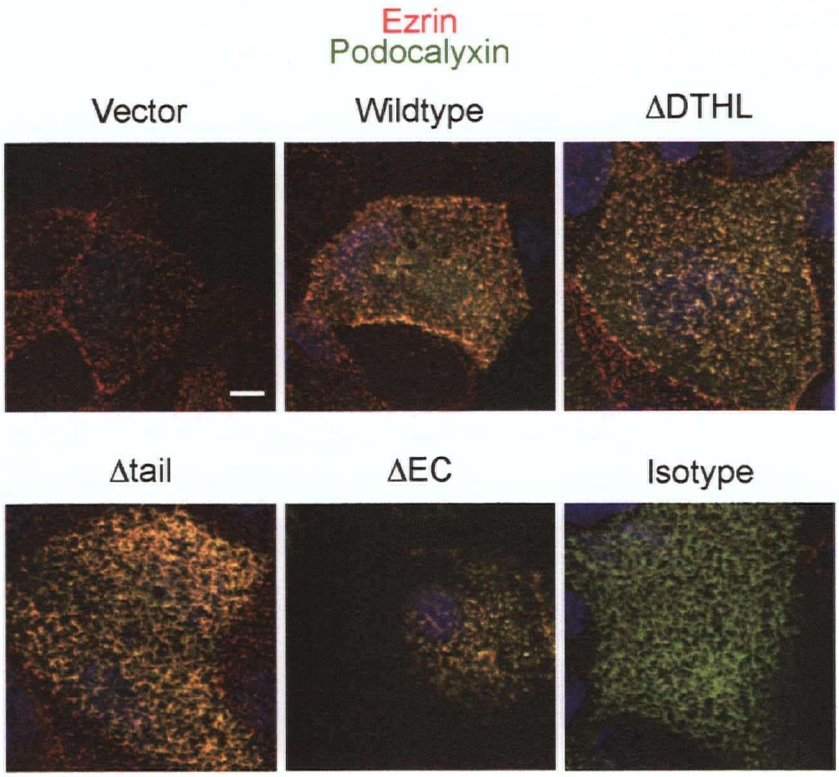

Figure 3-20: Confocal Analysis of Podocalyxin and Ezrin at the Apical Surface of Transfected MCF-7 Cells.

Transfected cells were labelled with DAPI (blue) and antibodies against ezrin (red) and podocalyxin (green). Yellow represents colocalization of ezrin and podocalyxin. The isotype control sample was labelled with DAPI (blue), anti-podocalyxin (green), and an isotype control for ezrin (red) to demonstrate the specificity of ezrin labelling. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

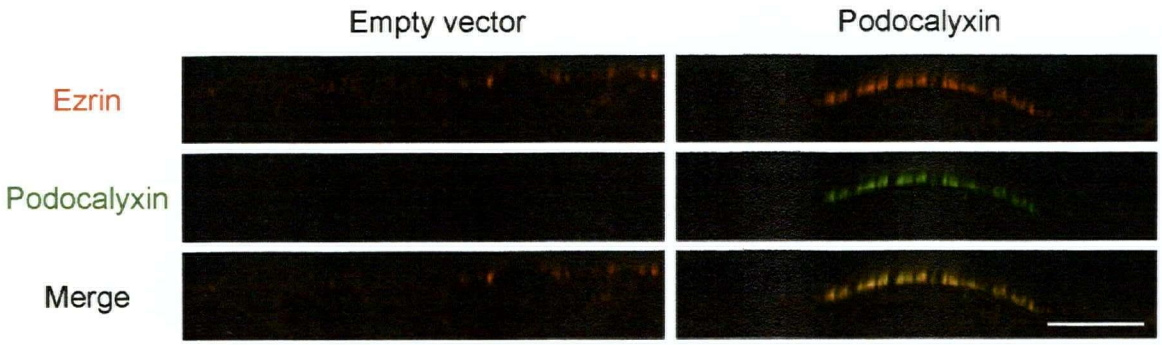

Figure 3-21: Vertical Sections of Confocal Stacks Demonstrated Increased Apical Recruitment of Ezrin in Cells Transfected with Podocalyxin.

Cells transfected with podocalyxin or empty vector were assessed for ezrin (red) and podocalyxin (green) in vertical sections of confocal stacks. Increased apical recruitment of ezrin was especially evident when staining was compared between neighbouring cells with and without ezrin. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

3.3.6 Recruitment of f-actin Occurs in the Absence of NHERF Binding

Furthermore, apical recruitment of f-actin was also independent of NHERF-binding. All forms of podocalyxin demonstrated apical colocalization with f-actin, but apical recruitment was notably less robust in the absence of the extracellular domain (Figure 3-22 and Figure 3-23). Consistent with electron microscopy results, cells expressing extracellular domain-containing podocalyxin mutants exhibited a clear punctate staining pattern on the apical surface of cells in protruding structures indicative of microvilli. To confirm that these structures bear all the structural hallmarks typical of microvilli, they were examined at high magnification by TEM. As with full-length podocalyxin, cells expressing ΔDTHL and Δtail mutants each clearly demonstrated the presence of actin filaments in the microvillar core, as shown both in longitudinal and cross-sections of individual microvilli (Figure 3-24). In summary, podocalyxin was able to recruit actin to microvilli in the absence of the bulk of its cytoplasmic domain, but the extracellular domain was essential for microvillus formation.

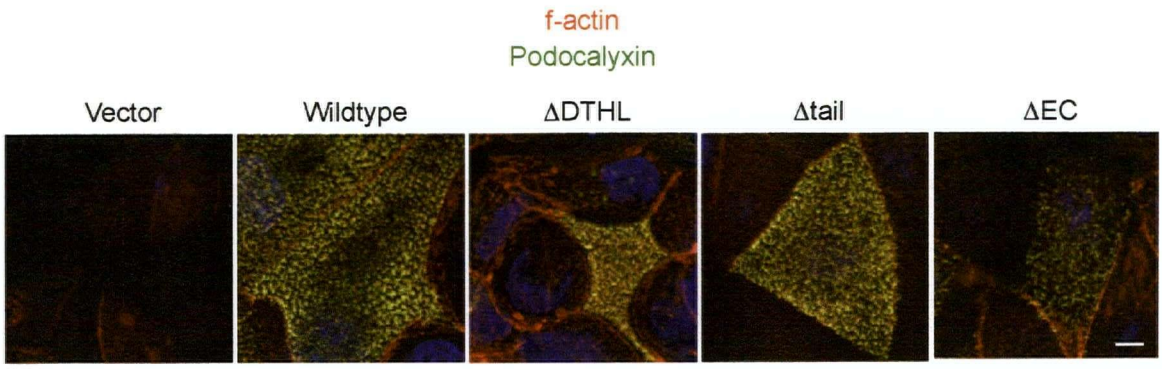

Figure 3-22: Confocal Images of the Apical Surface of Cells Show that f-Actin was Recruited in Podocalyxin-Transfected Cells.

MCF-7 cells transfected with wildtype and mutant podocalyxin were assessed for f-actin (red), podocalyxin (green), and nuclear (blue) labelling at the apical surface. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

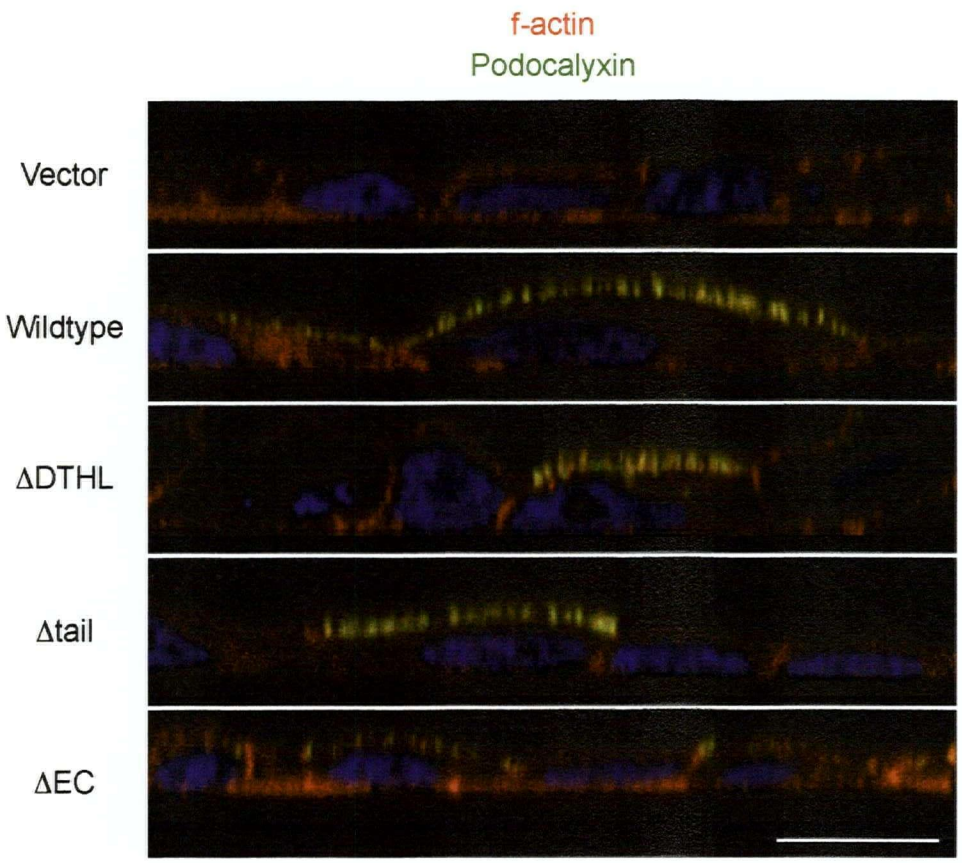

Figure 3-23: Vertical Sections of Confocal Stacks Demonstrated Apical Recruitment of f-Actin in Podocalyxin-Transfected Cells.

MCF-7 cells transfected with wildtype and mutant podocalyxin were assessed for f-actin (red), podocalyxin (green), and nuclear (blue) labelling in vertical sections of confocal stacks. Cells transfected with the empty vector were used as negative controls for podocalyxin staining. Scale bar: 5 μm. Representative of two independent experiments.

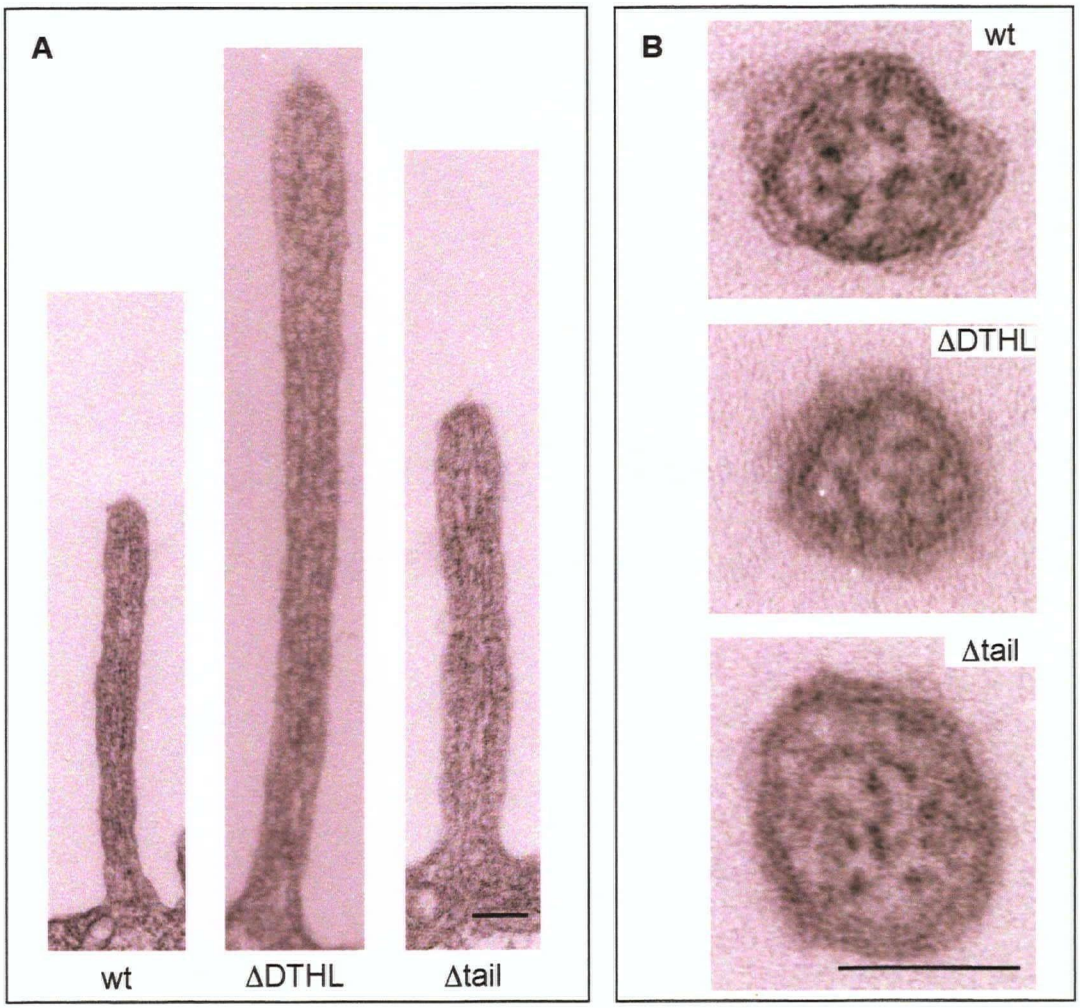

Figure 3-24: Actin Filaments were Visible in Individual Microvilli.

High magnification TEM's demonstrated the presence of actin filaments in microvilli of cells transfected with wildtype podocalyxin as well as ΔDTHL and Δtail mutants. Scale bars: 0.1 μm. (A) Longitudinal sections. (B) Cross sections. Imaging by A. Wayne Vogl, University of British Columbia.

3.4 Discussion

3.4.1 Summary

Podocalyxin was overexpressed in two epithelial cell lines in order to assess its effects on cell adhesion and morphology. In addition to decreasing cell-substrate adhesion and causing alterations in cell-cell junctions, podocalyxin was found to recruit NHERF1 and induce microvillus formation. Expression of podocalyxin mutants in epithelial cells provided some insights into podocalyxin's mechanism of action. The podocalyxin-dependent apical recruitment of NHERF1 was unexpected, but the requirement for the C-terminal DTHL sequence was not surprising. In contrast, the well-conserved cytoplasmic tail was found to be dispensable for microvillus formation, while the extracellular domain was essential.

3.4.2 Podocalyxin's Role in Determining Cell Morphology

Experimental evidence from the 1970's suggested that highly-glycosylated and heavily-sialylated glycoproteins play a key role in maintaining the integrity of the foot processes of kidney podocytes, as discussed in section 1.6.3 (Andrews, 1979; Seiler et al., 1977; Seiler et al., 1975). With the subsequent identification of podocalyxin as the major component of the podocyte glycocalyx and the tight correlation between its expression and podocyte foot process morphogenesis *in vivo*, this molecule became the prime candidate as a regulator of foot process formation (Dekan et al., 1991; Kerjaschki et al., 1984; Sawada et al., 1986; Schnabel et al., 1989). Gene targeting studies allowed us to

confirm that this molecule is indeed required for the generation of foot processes: although deficient animals generate podocyte precursors, these fail to undergo morphogenesis (described in section 1.6.4) (Doyonnas et al., 2001). Moreover, this defect is rescued by kidney-specific ectopic expression of podocalyxin (see chapter 5). With the current experiments, these observations can be generalized: rather than being a podocyte-specific phenomenon, expression of podocalyxin was also sufficient for induction of morphogenesis, as measured through microvillus formation, in two different epithelial cell lines, suggesting that it is a master-regulator of this process. Importantly, endogenous podocalyxin is expressed and apically targeted in normal mammary epithelium *in vivo* (Somasiri et al., 2004) and may be required for the extensive remodeling that occurs in this tissue.

Although the exact mechanism by which podocalyxin induces microvillus formation is unclear, my experiments suggest that an essential component of podocalyxin's activity as a cell morphogen is its extracellular domain: mutants lacking this domain, though apically targeted, were unable to induce microvillus formation. Several previous experiments *in vivo*, though indirect, support this notion: treatment of kidney podocytes to neutralize the negatively charged sialic acid residues on the podocyte surface leads to a dramatic loss of podocyte interdigitating foot processes (Andrews, 1979; Gelberg et al., 1996; Seiler et al., 1977; Seiler et al., 1975). This is believed to be due to a direct effect on podocalyxin for several reasons: podocalyxin is the most highly expressed sialoglycoprotein on these cells, its expression during embryogenesis perfectly coincides with formation of these structures, and disruption of the *podxl* gene leads to failure to

produce foot processes during embryogenesis (Doyonnas et al., 2001; Kerjaschki et al., 1984; Schnabel et al., 1989). It was therefore not surprising that deletion of the entire mucin domain also prevented microvillus formation. Although the precise mechanism by which this effect occurs is elusive, we propose two models. The first model provides a biophysical explanation for the induction of microvillus formation by podocalyxin. In this model, generation of additional plasma membrane may simply serve to evenly disperse the bulky, negatively charged mucin domains (Figure 3-25). This model is consistent with the previous reports of a dose-dependent weakening of cell junctions induced by ectopic expression of podocalyxin and the tight correlation between podocalyxin overexpression and breast cancer metastatic index (Somasiri et al., 2004; Takeda et al., 2000). It is also consistent with recent reports of podocalyxin as a "pre-apical" domain-forming protein (Meder et al., 2005).

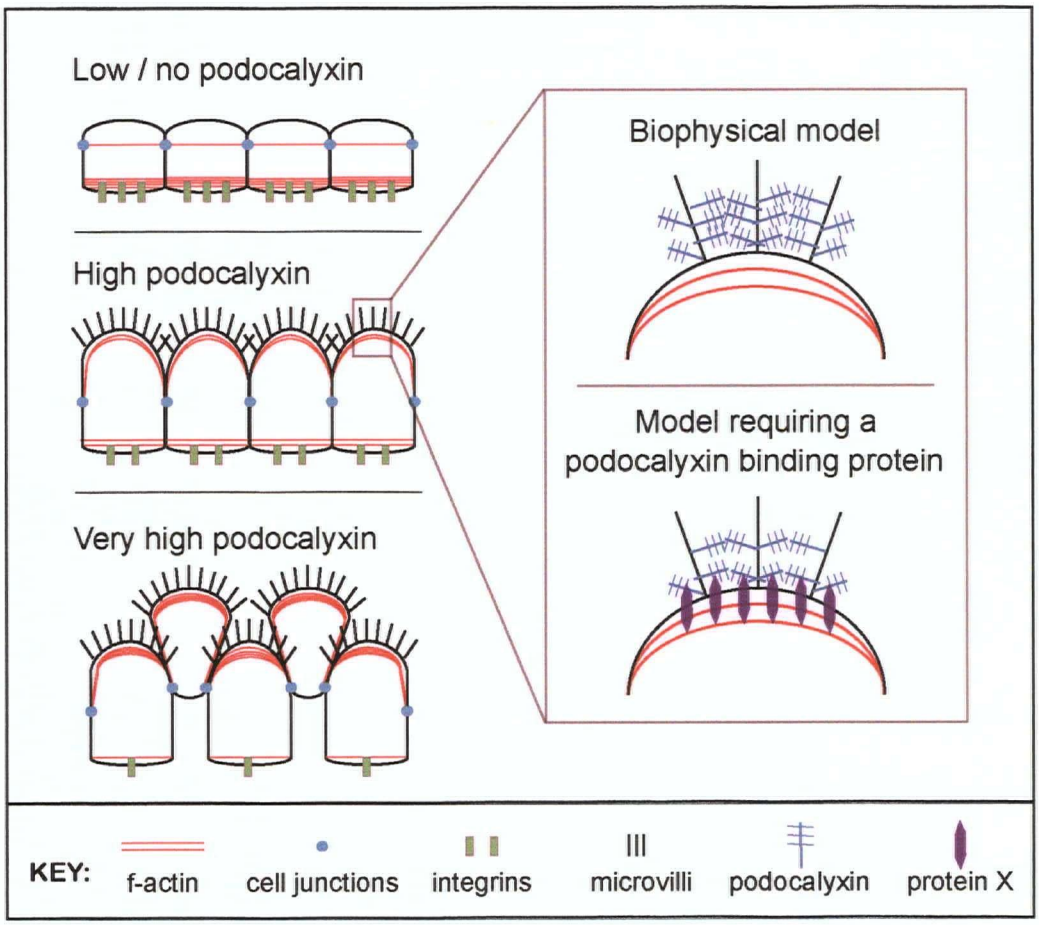

Figure 3-25: Two Models of Podocalyxin-Induced Microvillus Formation.

Ectopic expression of podocalyxin recruited f-actin to the apical surface and led to formation of microvilli. This may lead to a decrease in basolateral actin available for stabilizing integrin-mediated cell-substratum interactions. Two models are proposed to explain podocalyxin's mechanism of action.

On the other hand, a surprising result of my studies is the apparent dispensability of podocalyxin's cytoplasmic domain, including all potential phosphorylation sites and motifs with the capacity for directly binding ezrin or NHERF family proteins (Li et al., 2002; Meder et al., 2005; Schmieder et al., 2004; Serrador et al., 2002; Takeda et al., 2001). The formation of microvilli is tightly linked to the recruitment of f-actin and ERM proteins to the apical membrane domain. With the discovery that podocalyxin binds the NHERF family of ezrin-binding proteins, several groups have suggested that this family of adaptors is required for podocalyxin function (Li et al., 2002; Meder et al., 2005; Schmieder et al., 2004; Takeda et al., 2001; Weinman, 2001). Unexpectedly, however, my results demonstrated that formation of microvilli does not require direct interaction of podocalyxin with NHERF proteins. In support of this finding, although one strain of NHERF1-null mice implicated this adapter protein in intestinal microvillus formation or organization (Morales et al., 2004), a second strain failed to support the observation (Shenolikar et al., 2002). Notably, podocalyxin is not expressed in the intestinal brush border, so if NHERF1 is indeed involved in microvillus formation in this tissue, it would have to be independent of podocalyxin (McNagny et al., 1997). Regardless, my data suggest that podocalyxin may be able to recruit ezrin and f-actin by interacting with another molecule via its extracellular domain. This interaction may then transduce the signal to initiate microvillus formation (Figure 3-25). This provides an alternative hypothesis to the biophysical model proposed above and depends upon loss of a specific binding site in podocalyxin's extracellular domain.

3.4.3 Significance of Recruitment of NHERF by Podocalyxin

Although my results preclude a direct functional role for NHERF/podocalyxin interactions in formation of microvilli and establishing the apical cell domain, apical recruitment of NHERF1 by podocalyxin is likely to be very important for other aspects of podocalyxin and NHERF function. NHERF proteins have been shown to be involved in a variety of processes including ion transport, signal transduction, growth control, and receptor internalization, and they have also been shown to bind a wide variety of ligands and undergo oligomerization (reviewed in section 1.4.2) (Shenolikar et al., 2004; Thelin et al., 2005; Voltz et al., 2001; Weinman, 2001; Weinman et al., 2005). The fact that podocalyxin is a potent inducer of NHERF recruitment to microvilli may suggest a new link between the formation of these specialized structures and numerous biological processes (Figure 3-26). Regardless of microvillus formation, the podocalyxin-dependent localization of NHERF proteins could be quite important in their regulation.

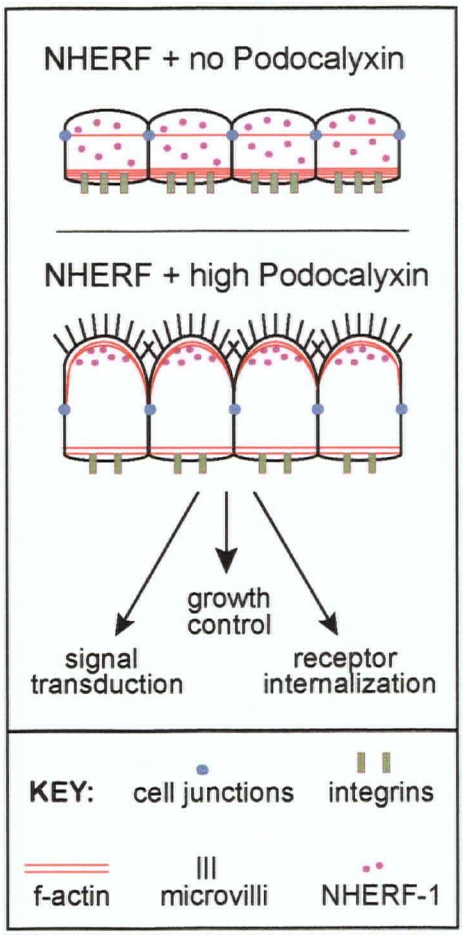

Figure 3-26: Model of Podocalyxin's Role in the Function of NHERF Family Members.

Although speculative, podocalyxin may regulate NHERF-dependent activities by altering its subcellular localization.

3.4.4 Podocalyxin and Microvilli in Adhesion / Anti-Adhesion

Podocalyxin has been shown to act as a pro- or anti-adhesin depending upon its cellular context, as discussed in sections 1.5.2 and 1.5.3. For example, when expressed in HEV podocalyxin acts as a ligand for L-selectin on lymphocytes, but it is likely that this pro-adhesive function is a special exception rather than the general rule. To act as a selectin ligand podocalyxin must be decorated with an HEV-specific carbohydrate modification that is not found on most other cells (Michie et al., 1993; Sassetti et al., 1998; Segawa et al., 1997). In contrast, when overexpressed in MDCK cells, which lack the appropriate enzymes for modifying podocalyxin for selectin-binding, the molecule was found to block cell-cell aggregation and modify cell junctions (Takeda et al., 2000). Likewise, we have previously shown that podocalyxin-deficient mice have various developmental defects (omphalocele and anuria) that are consistent with excessive cell adhesion in its absence (reviewed in sections 1.6.4.1 and 1.9) (Doyonnas et al., 2001). Finally, we have found that podocalyxin expression is upregulated in metastatic breast cancer cells and decreases cell-substrate adhesion when overexpressed *in vitro* ((Somasiri et al., 2004) and Figure 3-3). Our current results suggest that the ability to act as either a pro- or anti-adhesin may be closely linked to podocalyxin's ability to generate microvilli. Podocalyxin expression on microvilli of HEV, which are known to express adhesion molecules at their tips, could facilitate the reported L-selectin-dependent leukocyte rolling and transendothelial migration (Girard et al., 1999; Picker et al., 1991; von Andrian et al., 1995). Conversely, in most other cell types the podocalyxin-coated, microvilli-rich, apical domain may protect cells from non-specific adhesion. Thus, podocalyxin overexpression in breast may promote tumour cell dissemination by

initiating a general disruption of cell adhesion, particularly under conditions where apical membrane domains are expanded due to breakdown in polarity. This would help initiate metastatic spread by a different, although not necessarily mutually exclusive, mechanism from the well described epithelial-mesenchymal transition which specifically downregulates cell-cell junctions (Kang and Massague, 2004).

It is also tempting to speculate that the recruitment of f-actin to the apical membrane of cells to generate microvilli might deplete actin from the basal surface of these cells (Schmieder et al., 2004), thereby preventing stable interactions of integrins with the extracellular matrix. This hypothesis is supported by the observation that cells transfected with full-length podocalyxin or mutants lacking its cytoplasmic tail generate abundant microvilli and exhibit decreased cell-substratum adhesion, while cells transfected with podocalyxin lacking the extracellular domain do not generate microvilli and remain adherent ((Somasiri et al., 2004), Figure 3-25, and unpublished observations). Further studies delineating the mechanisms by which apical podocalyxin and basolateral integrins compete for actin may clarify this process.

CHAPTER 4 : GENERATION OF TRANSGENIC MICE CONDITIONALLY OVEREXPRESSING PODOCALYXIN

4.1 Rationale

There were four major reasons for attempting to generate a mouse overexpressing podocalyxin:

a) Loss-of-function and gain-of-function experiments are two very powerful approaches for elucidating the normal function of a poorly understood protein. The podocalyxin knockout mouse has a dramatic phenotype in that it dies within 24 hours of birth, but this prevents us from fully understanding podocalyxin's functions, especially in adult animals (reviewed in section 1.6.4) (Doyonnas et al., 2001). Much information has been obtained from *in vitro* podocalyxin overexpression experiments (see chapter 3), but there are many questions remaining, which may be answered using *in vivo* overexpression studies.

b) *In vitro* experiments suggest that podocalyxin mainly functions to block inappropriate cell adhesion. By overexpressing podocalyxin in tissues where adhesion is important, numerous biological processes could be altered in an attempt to clarify their importance and gain mechanistic insights. For example, blocking adhesion of leukocytes may mimic diseases such as leukocyte adhesion deficiency syndromes, by preventing leukocyte rolling and firm arrest (Bunting et al., 2002). Overexpression of podocalyxin in vascular endothelia may mimic

some characteristics of other disorders, like arthritis, by increasing vascular permeability (Doria et al., 2006).

c) Podocalyxin knockout mice die within one day of birth, presumably as a result of a major kidney defect. This precludes analysis of defects in other tissues in adult mice. Therefore, by crossing an inducible podocalyxin overexpressor mouse to a podocyte-specific Cre mouse, it would be possible to generate mice specifically expressing podocalyxin in the area where it is thought to be absolutely essential. This may enable the mice to overcome the lethal phenotype, thereby allowing analysis of other podocalyxin-deficient tissues in adult.

d) We have recently shown that podocalyxin is an excellent predictor of poor outcome in human breast cancer (reviewed in section 1.10.1) (Somasiri et al., 2004). It is possible that the anti-adhesive function of podocalyxin allows for more rapid dissemination of tumour cells. This possibility, as well as other potential roles for podocalyxin in breast cancer progression could be addressed by specifically overexpressing podocalyxin in mammary tissue. After inducing the formation of tumours using the mouse mammary tumour virus, disease progression could be followed in these mice and compared to non-transgenics.

There are therefore a number of interesting questions that could be addressed using conditional podocalyxin overexpressing mice.

4.2 The Cre-loxP System

The Cre-loxP system is a versatile tool allowing inducible expression, or deletion, of genes of interest (reviewed in (Lobe and Nagy, 1998)). Cre is a bacteriophage

recombinase that selectively induces site-specific recombination between 34 bp loxP consensus sequences (Figure 4-1). Thus, after inserting a gene in between two loxP sequences, Cre can be used to delete the intervening sequences, leaving behind a single loxP site. It is possible to make numerous tissue-specific knockouts by generating just a single mouse line with a transgene consisting of the gene of interest flanked by loxP sites and then crossing it with any of the many mice available that express Cre in a tissue-specific or temporally regulated manner. Likewise, this system can also be used to conditionally overexpress a gene. In this case, the loxP sites flank a spacer that separates the promoter from the gene of interest and includes a stop codon and poly-adenylation (polyA) sequences. This element can subsequently be deleted, thereby facilitating gene expression from a common promoter. Thus, the Cre-loxP system was deemed suitable for inducible expression of podocalyxin in mice.

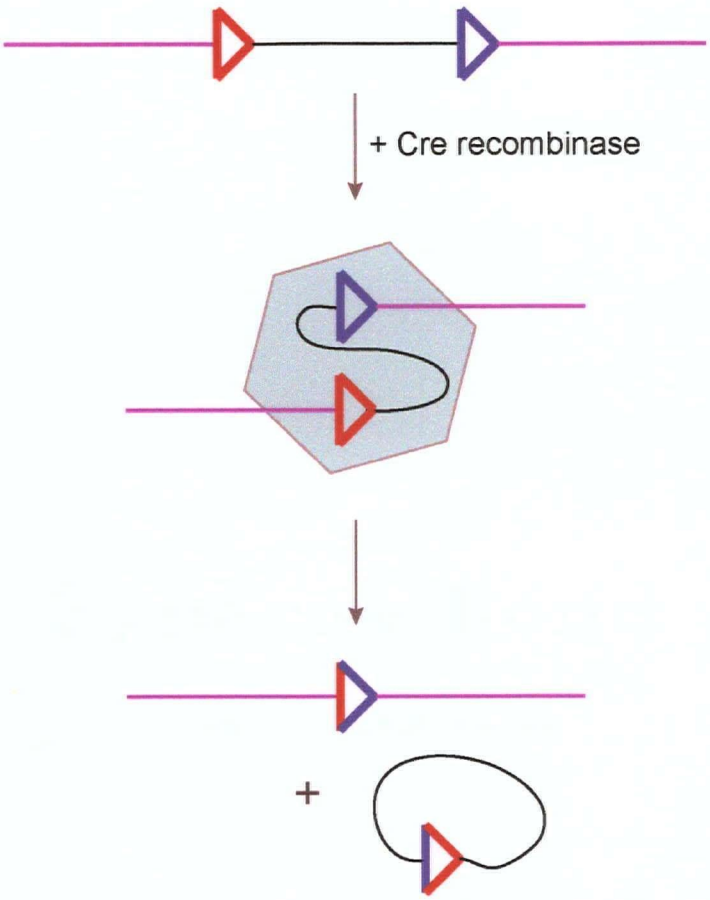

Figure 4-1: Schematic of Cre-Mediated Recombination.

Cre recombinase (hexagon) mediates homologous recombination between loxP sites (triangles), thereby excising the intervening (black) sequence and connecting the flanking (purple) sequences.

4.3 Transgenic Construct

There are several basic loxP-containing constructs available for generating transgenic mice; the one that I used to generate the conditional podocalyxin overexpressing transgenic is called Z/EG, and it encodes several useful features (Figure 4-2 and (Lobe et al., 1999; Novak et al., 2000)). Initially, podocalyxin and GFP expression were blocked by a *lacZ*/neomycin resistance (βgeo) fusion spacer cassette, which included a stop codon and three polyA sites. This was flanked by loxP sites, and was therefore excisable by Cre-mediated site-specific recombination. The CMV enhancer/β-actin promoter was used to drive expression of the gene of interest (*podxl*, in this case), which was inserted upstream of an IRES sequence and *GFP*, enabling expression of the reporter in concert with podocalyxin. Thus, the promoter initially drove ubiquitous expression of β-galactosidase (β-gal), but Cre-excision led to expression, instead, of podocalyxin and GFP (Figure 4-2).

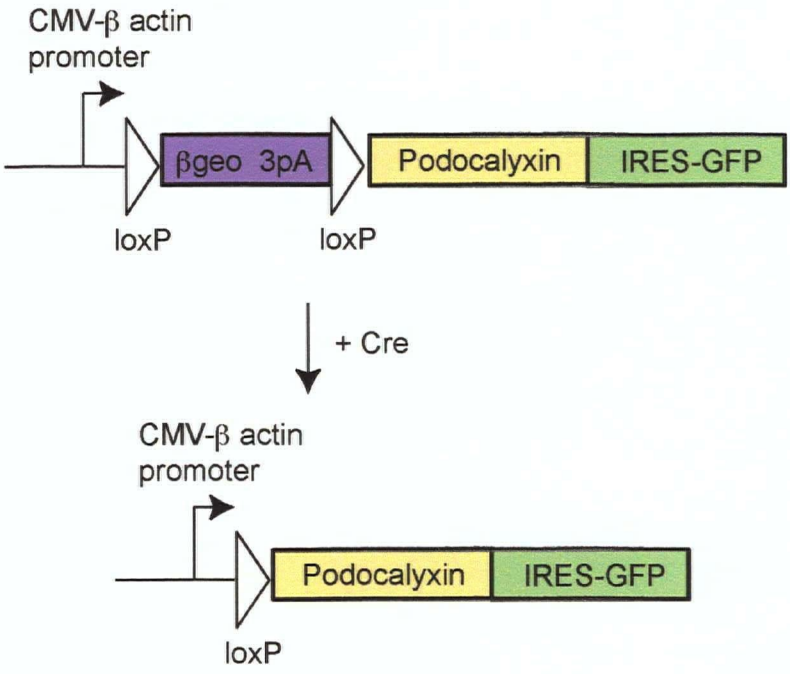

Figure 4-2: Schematic of Transgenic Construct Before and After Cre-Mediated Recombination.

The original construct encoded the βgeo fusion gene followed by polyA (pA) signals and flanked by loxP sites. Podocalyxin and GFP were encoded downstream and were therefore not expressed. Cre-mediated recombination deleted the spacer element and induced expression of podocalyxin and GFP.

4.4 In Vitro *Validation of Transgenic Construct*

Prior to using the construct to generate transgenic mice, a similar precursor plasmid lacking GFP was first tested *in vitro* by transfecting the murine myeloma cell line, NS0, which was chosen for ease of transfection (see section 2.1.1). After producing stable neomycin resistant lines containing the construct, the cells were transiently transfected with the pCI-Cre expression plasmid (generously provided by Dr. Fabio Rossi). Flow cytometry was then used to assess podocalyxin expression (Figure 4-3). As expected, due to the transient nature of the Cre transfection, some cells expressed podocalyxin, while many did not; the efficiency would be expected to increase with stable Cre transfection. Thus, this construct was found to function appropriately and the Z/EG-*podxl* plasmid was used to generate stable embryonic stem cell (ESC) lines.

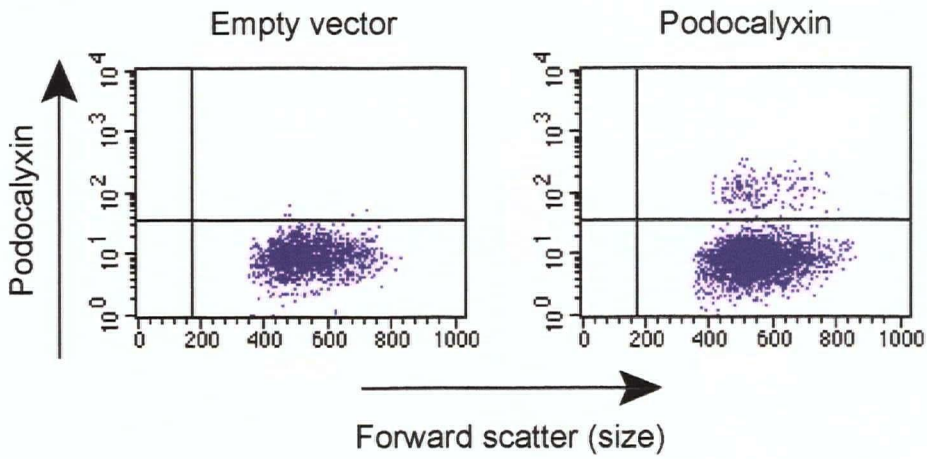

Figure 4-3: Cre-Mediated Recombination Induced Podocalyxin Expression in NS0 Cells Transfected with the Construct.

4.5 Selection of Transgene Positive ES Cells

The linearized construct was introduced into murine R1 ESCs by electroporation. Cells were then allowed to recover, after which time they were selected for neomycin resistance. Approximately 400 drug-resistant ESC colonies were picked and transferred to 96 well plates. Those colonies that continued to grow were replica plated onto feeder layers for continued expansion and preparation of stocks and onto gelatin-coated plates for analysis (Figure 4-4). Assessment of clones included confirmation of transgene presence by PCR (Figure 4-5) and screening cells, before and after differentiation, for β-galactosidase expression. This ensured that the transgene had integrated into a site that would facilitate expression in many cell types. Five clones met these criteria and were maintained for further screening: 2A7, 2B6, 2G12, 3E3, and 3H7.

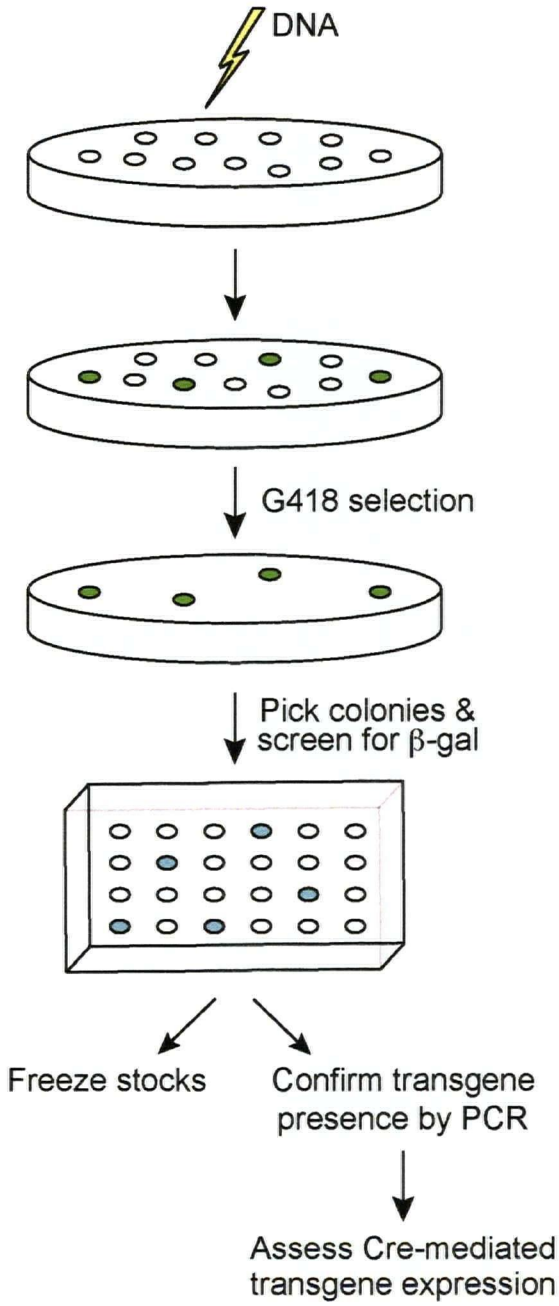

Figure 4-4: Overview of Selection Process.

RI ESCs were electroporated, and cells that had incorporated the construct (green circles) were selected based on resistance to neomycin (G418). These colonies were then screened for β-galactosidase expression, and positive clones (blue) were further assessed after generating frozen stocks.

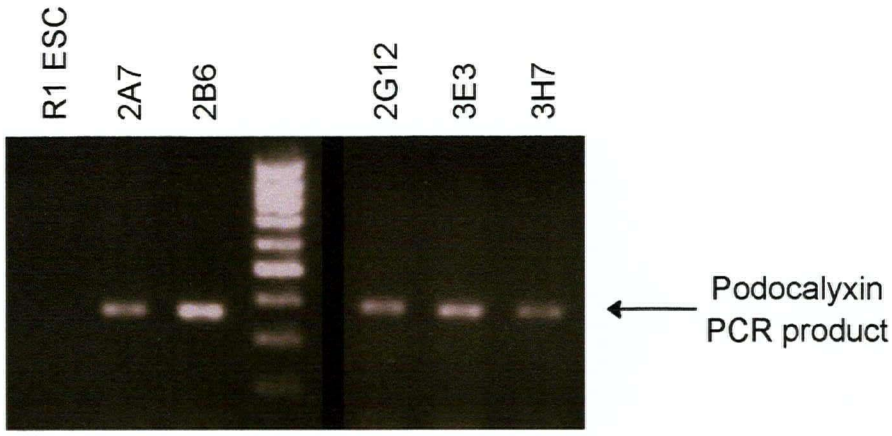

Figure 4-5: PCR-Based Detection of Transgene in Genomic DNA of ESCs.

Podocalyxin cDNA, encoded by the transgenic construct, was detected in β-glactosidase-positive clones, but not in parental R1 ESCs.

4.6 Transgene Expression Analysis After Cre-Mediated Recombination in ESCs

The next step in the selection process required testing the five clones for proper Cre-mediated excision of the spacer and subsequent expression of podocalyxin. RT-PCR was used to confirm the presence of podocalyxin mRNA after Cre-transfection (Figure 4-6). It was also noted that parental R1 ESCs expressed a low level of endogenous podocalyxin. This was not unexpected because it has been shown that human ESCs also express podocalyxin (Wei et al., 2005). Flow cytometry confirmed surface expression of exogenous podocalyxin (above background levels) in all five clones (Figure 4-7). Again, Cre was transfected transiently, and the efficiency would be improved upon sustained Cre expression *in vivo*. Furthermore, GFP expression was detected in all clones, both by RT-PCR (Figure 4-8), and by fluorescence microscopy. Based on this expression data, as well as colony morphology, two clones were chosen for generation of chimeric mice (2G12 and 2B6).

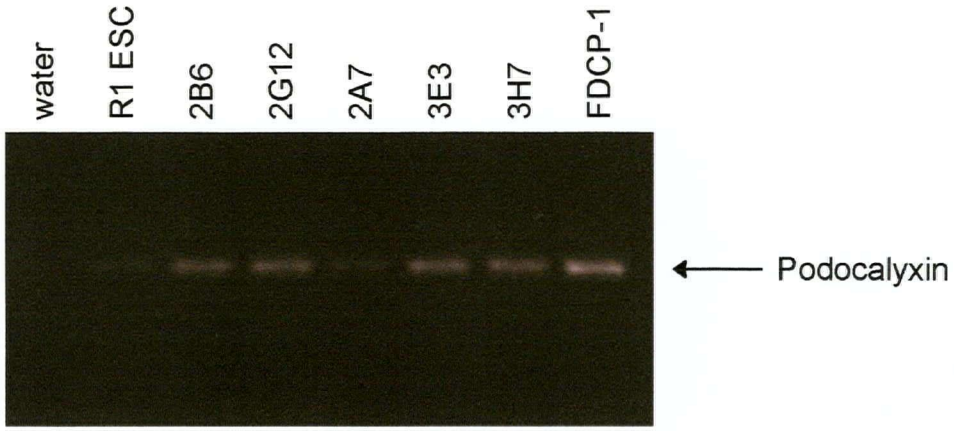

Figure 4-6: Expression of Podocalyxin mRNA in ESCs.

RT-PCR was used to detect podocalyxin mRNA in ESCs after transient transfection with Cre, and in positive control (FDCP-1) cells.

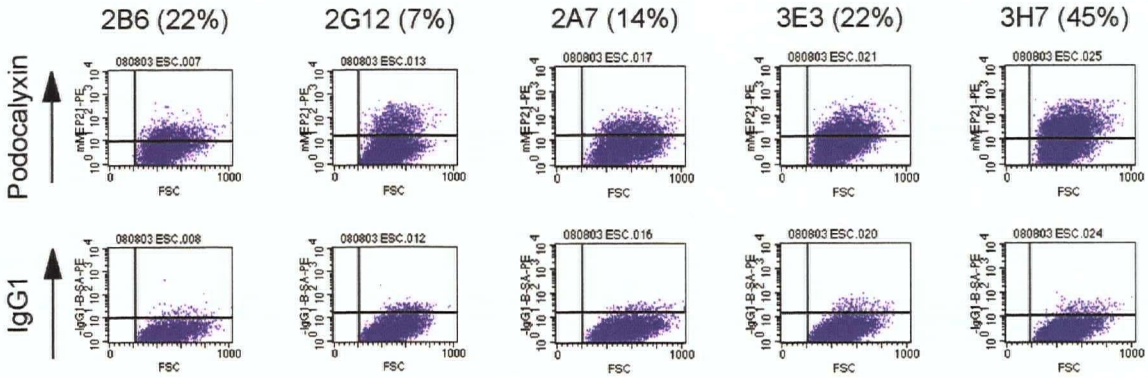

Figure 4-7: Analysis of Podocalyxin Expression by Flow Cytometry.

The five clones expressed various levels of podocalyxin after Cre transfection. Rat IgG_1 was used as a negative control for podocalyxin staining.

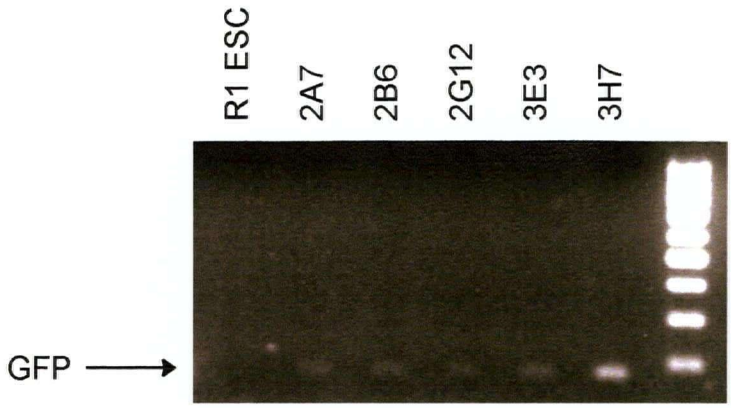

Figure 4-8: Expression of GFP mRNA in transfected ESCs.

Cre-induced expression of GFP was detected by RT-PCR in all transfected clones, but not in parental R1 ESCs.

4.7 Production of Chimeric Mice

Chimeric mice were generated with the 2B6 clone by Corrinne Lobe's laboratory using the technique of ESC-morula aggregation (Lobe et al., 1999). Although aggregations were performed repeatedly with several clones, only one experiment led to production of chimeric mice, and unfortunately none of the experiments resulted in germline transmission of the transgene.

4.8 Explanation for Lack of Germline Transmission in Transgenic Mice

The ESC clones were then sent to Jill Lahti's laboratory for an additional attempt at generating chimeric mice. Karyotyping is often completed before generating transgenic mice with cultured ESCs, so two clones (2G12 and 3E3) were karyotyped before proceeding. Using this technique, it was determined that both clones were trisomic for chromosome eight, through creation of what appeared to be isochromosomes (Figure 4-9). Trisomy eight is a fairly common chromosomal aberration noticed in cultured ESCs, as it gives cells a growth advantage (Liu et al., 1997). Unfortunately, although this genetic defect does not inhibit production of chimeric mice, it does prevent germline transmission of the transgene because the abnormal cells cannot form an entire viable animal. Since both tested clones had the same structural chromosomal aberration, it is likely that the original R1 ESCs were also trisomic for chromosome eight. This precluded further use of these cells for generating transgenic animals.

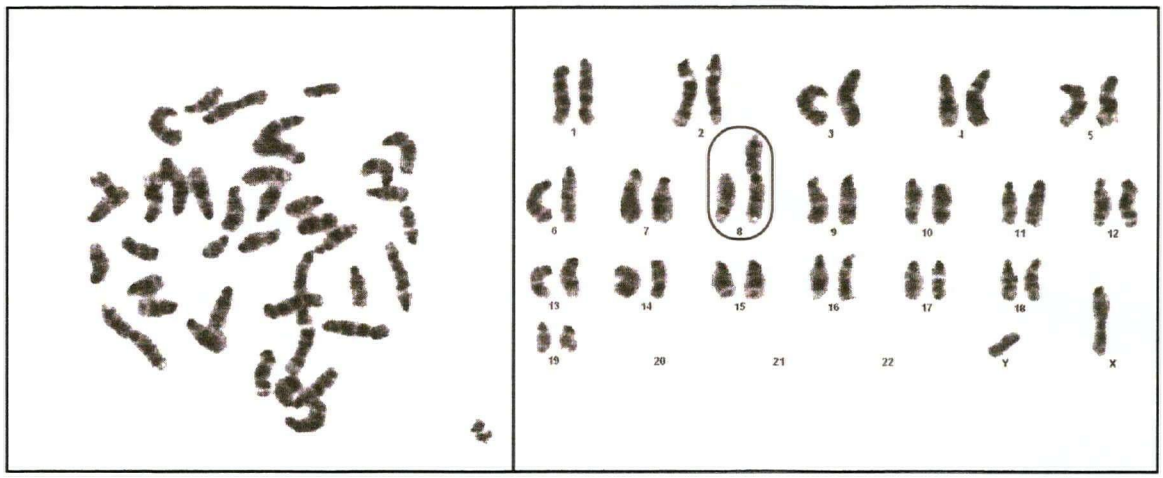

Figure 4-9: Karyotyping Uncovered the Presence of Trisomy Eight in Tested Clones.

4.9 Summary and Conclusions

We were interested in generating transgenic mice conditionally expressing podocalyxin as a tool for assessing podocalyxin's role in breast cancer, for investigating the effect of a general loss of adhesion in numerous cell types, and in order to rescue podocalyxin knockout mice by specifically expressing podocalyxin in kidneys, where it is presumed to be essential for viability of mice. A transgenic construct was generated which enabled expression of podocalyxin from a ubiquitous promoter, only after Cre-mediated excision of a spacer element. This construct was used to generate ESC clones that exhibited excellent expression of the transgene before and after differentiation, as assayed by β-galactosidase expression. Several clones were found to be acceptable for generation of transgenic mice based on morphology as well as efficient Cre-mediated inducible expression of podocalyxin and GFP. However, initial attempts at generating chimeric mice that propagated the transgene in the germline failed, and the ESCs were therefore examined for chromosomal aberrations. Two clones were tested, and both were found to possess an additional copy of chromosome eight. This explains the lack of germline transmission, and necessitates the generation of new ESC clones, beginning with new, untransfected ESCs. In the meantime, a more direct strategy was used to address one goal of this study: the repair of podocalyxin-knockout kidneys was attempted using a kidney-specific podocalyxin transgenic mouse (chapter 5).

CHAPTER 5 : REPAIR OF KIDNEY DEFECT IN PODOCALYXIN-NULL MICE

5.1 Rationale

Loss-of-function experiments can provide considerable insights into protein function, so podocalyxin-null mice had been generated previously (Doyonnas et al., 2001). Defects were expected in tissues where podocalyxin is expressed: podocytes of the kidney, hematopoietic progenitor cells, megakaryocytes, vascular endothelia, mesothelial cells lining organs, and a subset of neuronal cells (Doyonnas et al., 2001; Doyonnas et al., 2005; Garcia-Frigola et al., 2004; Hara et al., 1999; Horvat et al., 1986; Kerjaschki et al., 1984; McNagny et al., 1997; Miettinen et al., 1990; Miettinen et al., 1999; Vitureira et al., 2005). However, deletion of the *podxl* gene resulted in perinatal lethality, preventing analysis of defects in adult mice. Further analysis suggested that loss of podocalyxin in podocytes of the kidney was the cause of this dramatic phenotype. Podocalyxin-null mice were anuric, exhibited increased blood pressure, and were found to have drastic morphological abnormalities in kidney glomeruli upon analysis by electron microscopy (Doyonnas et al., 2001). In order to study defects in adult mice lacking podocalyxin, we attempted to rescue podocalyxin-null mice by ectopically expressing podocalyxin specifically in podocytes. The first strategy involved generating podocalyxin overexpressing mice using the inducible system described in the previous chapter. However, due to delays in that strategy, it was decided that a more direct strategy was required. The new strategy involved generating a podocyte-specific podocalyxin transgene.

5.2 Transgenic Construct

The promoter for the *NPHS1* gene, which encodes nephrin (discussed in section 1.6.5.1, and reviewed in (Salant and Topham, 2003; Tryggvason, 1999)), was chosen to drive ectopic expression of podocalyxin in podocytes. In the glomerulus, nephrin is found exclusively in the slit diaphragm region of podocytes, and nephrin and podocalyxin are initially expressed at a similar time point in development (Kawachi et al., 2002; Schnabel et al., 1989). The pIRES2-EGFP expression vector was chosen as the backbone for the transgenic construct. This vector included a neomycin resistance cassette, as well as a multiple cloning site downstream of a ubiquitous promoter. Murine podocalyxin cDNA was cloned into the multiple cloning site, upstream of *GFP*, which was expressed using an IRES sequence. Before generating transgenic mice, the *NPHS1* promoter was inserted upstream of *podxl*, and the ubiquitous CMV promoter was deleted (Figure 5-1). This enabled podocalyxin and GFP expression specifically in podocytes.

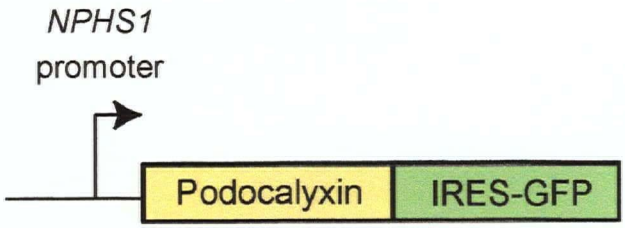

Figure 5-1: Schematic of Transgenic Construct.

The transgenic construct enabled tissue-specific expression of podocalyxin and GFP from the podocyte-specific *NPHS1* promoter.

5.3 In Vitro *Expression Analysis*

Before deletion of the ubiquitious promoter, the transgenic construct was tested by transfection into CHO cells. Transient transfection resulted in expression of both podocalyxin and GFP, as detected by flow cytometry (Figure 5-2).

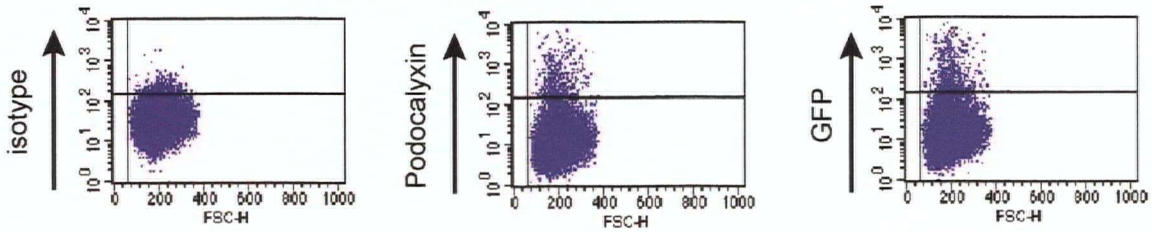

Figure 5-2: Transiently Transfected CHO Cells Expressed Podocalyxin and GFP from the Transgenic Construct.

Cells were labelled with anti-podocalyxin antibodies and assessed for podocalyxin and GFP expression by flow cytometry.

5.4 Generation of Transgenic Founders

The transgenic construct was linearized after successfully testing it *in vitro*; the final construct contained only the *NPHS1* promoter upstream of *podxl* and *GFP*. This DNA fragment was injected into the pronuclei of fertilized eggs in the Biomedical Research Centre's transgenic facility. Approximately 400 injections were performed, resulting in 51 pups. Genotyping results suggested that five mice contained the transgene, but only one founder produced consistently strong signals for the transgene by PCR.

5.5 Transgene Expression Analysis

Generation of transgenic mice by oocyte microinjection often results in insertion of multiple copies of the transgene into a single site. Traditionally, transgene copy number has therefore been assessed in order to approximate expression levels. However, expression can also be affected by many other factors. For example, the transgene may insert into a region of heterochromatin that could prevent its expression. Alternatively, it may be located next to a strong promoter, which could alter expression. In contrast, insertional mutagenesis could lead to disruption of another gene's normal expression pattern or even creation of a fusion protein. Therefore, when generating transgenic mice, several founders should be assessed to ensure that any phenotypes are the result of the transgene itself, and not the result of the transgene's insertion site. All five founders were therefore initially assessed for transgene expression.

5.5.1 Transgene Expression in Glomeruli of Founders' Kidneys

In that podocytes were the transgene's target cells, the founders' kidneys were assessed for expression, after first insuring that the transgene had been transmitted to subsequent pups. It is important to remember that the founders were otherwise normal animals with additional, ectopic expression of podocalyxin (as well as GFP) from the transgene. Thus, podocalyxin expression could not be assessed in these animals; the foreign GFP protein was therefore used as a marker. GFP expression was very faint, and could only be convincingly detected after immunolabelling using an anti-GFP antibody. The founder with the strongest transgene signal by PCR also had the brightest GFP staining in kidney glomeruli (Figure 5-3). This founder was known as 3-3 as it was the third pup from the third set of injections. Two other founders (1-9 and 3-10) also had reasonable levels of GFP expression, while the last two (1-6 and 1-8) were extremely weak. Analysis at higher magnification showed a distinctive staining pattern, as shown in the image of a single glomerulus in Figure 5-4. This pattern is typical of apical membrane labelling of glomerular podocytes.

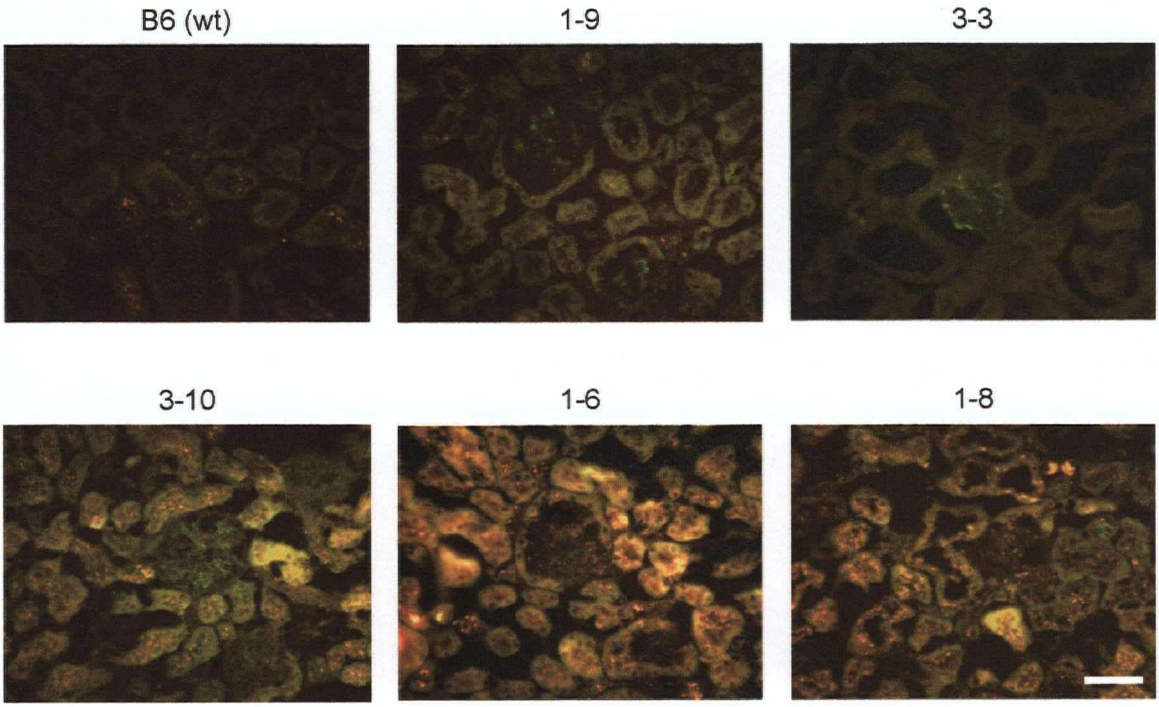

Figure 5-3: GFP Expression in Glomeruli of Transgenic Founders.

After perfusion with paraformaldehyde (PFA), kidneys were frozen, sectioned, and immunolabelled with anti-GFP antibodies, as described in chapter 2. Bright green staining represents GFP in glomeruli, while yellow staining is background immunofluorescence. Tissues from wildtype (B6) mice were used as negative controls for GFP staining. Scale bar: 50 μm.

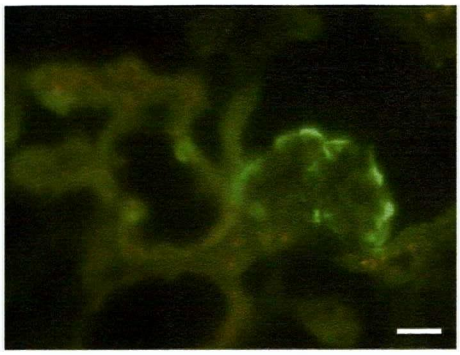

Figure 5-4: Glomerulus from Founder with Highest GFP Expression Levels.

After PFA perfusion, kidneys were frozen, sectioned, and immunolabelled with antibodies against GFP. Positive staining can be seen in the glomerulus. Tissues from wildtype (B6) mice were used as negative controls for GFP staining. Scale bar: 20 μm.

5.5.2 Other Tissues Examined by Immunofluorescence for GFP Expression Demonstrate Lack of Non-Specific Expression

After assessing transgene expression in kidney, it was also necessary to identify sites of inappropriate expression. Other areas where podocalyxin is normally expressed, such as hematopoietic cells and vascular tissues, were important to examine because ectopic expression in these areas would prevent analysis of defects in cells lacking podocalyxin when crossed with podocalyxin-null animals. No expression was detected in the liver or lung, where vascular expression would be noticeable (Figure 5-5), or in the peripheral blood (Figure 5-6). As podocalyxin and nephrin can both be found in a subset of cells in the brain (Garcia-Frigola et al., 2004; Putaala et al., 2000; Putaala et al., 2001; Vitureira et al., 2005), this organ was also examined, but GFP expression was not detected (Figure 5-7). In summary, although all five founders demonstrated some degree of GFP expression in kidney, only three had reasonable levels. Inappropriate expression of the transgene was not detected (Table 5-1).

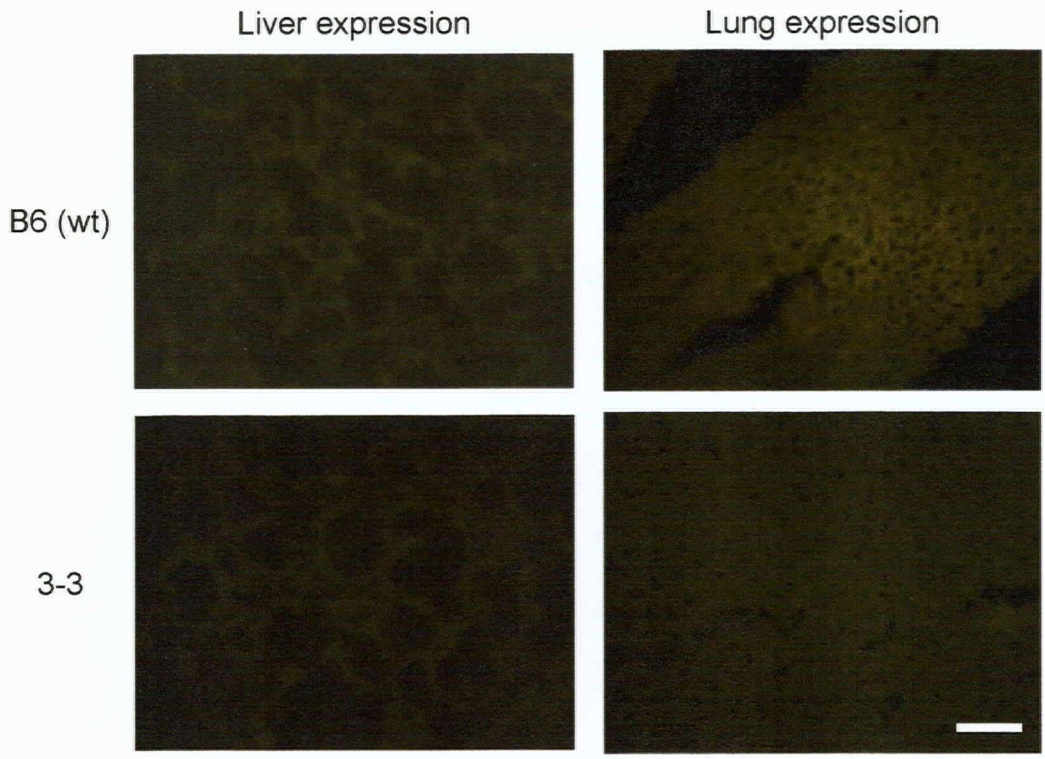

Figure 5-5: Lack of Non-Specific Expression in Liver and Lung.

After PFA perfusion, tissues were frozen, sectioned, and immunolabelled with antibodies against GFP. Transgene expression was not detected in vascular endothelia. Tissues from wildtype (B6) mice were used as negative controls for GFP staining. Kidney sections from founder 3-3 were used as positive controls for GFP staining. Scale bar: 50 μm.

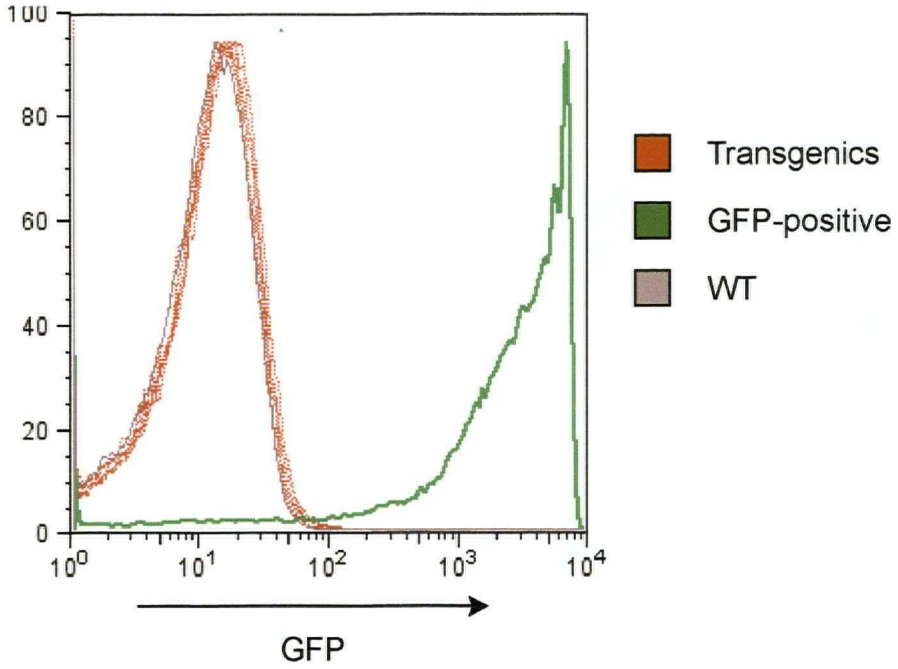

Figure 5-6: Transgene Expression was Undetectable in Peripheral Blood.

Peripheral blood was isolated from progeny of original founders and assessed for GFP expression by flow cytometry. GFP expression was compared to expression in peripheral blood isolated from wildtype (B6) and ubiquitous GFP-expressing transgenics.

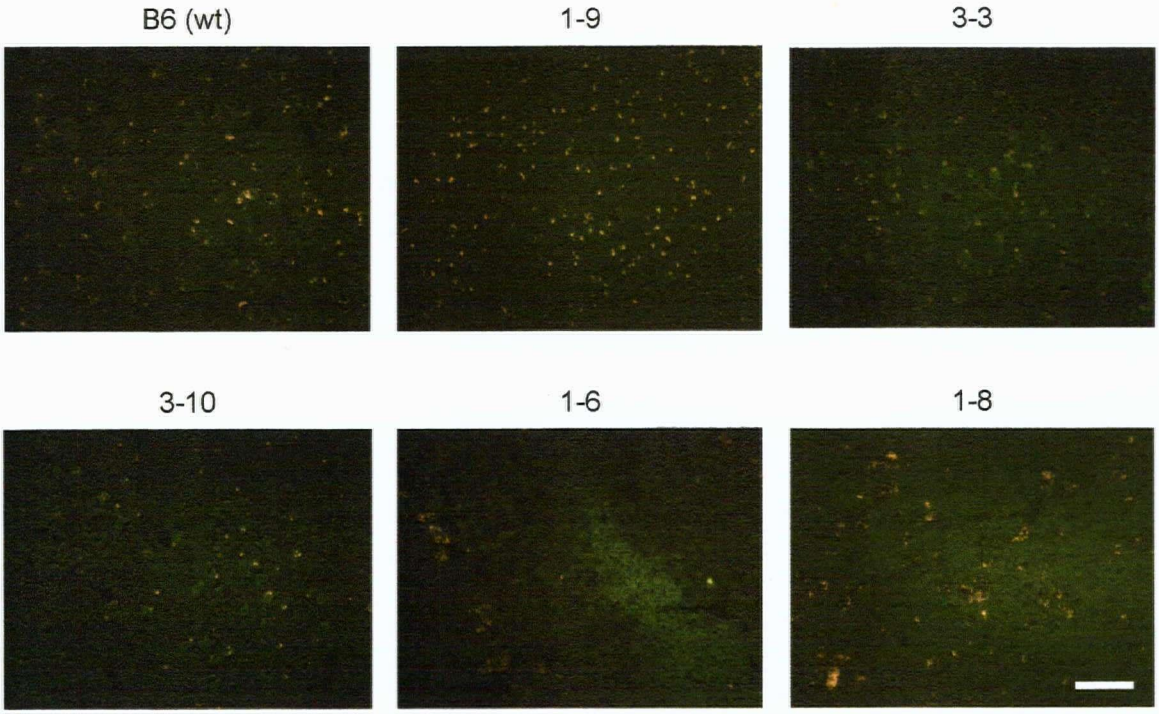

Figure 5-7: GFP Expression was Not Detected in Brains of Founders.

After PFA perfusion, brains were frozen, sectioned, and immunolabelled for GFP. Although there appeared to be some background fluorescence, there was no specific expression in brain. Tissues from wildtype (B6) mice were used as negative controls for GFP staining. Kidney sections from founder 3-3 were used as positive controls for GFP staining. Scale bar: 50 μm.

Founder	Expression Pattern				
	Kidney	Brain	Blood	Liver	Lung
3-3	++	-	-	-	-
1-9	++	-	-	nd	nd
3-10	+	-	-	nd	nd
1-8	weak	-	-	nd	nd
1-6	weak	-	-	nd	nd

Table 5-1: Summary of Transgene Expression.

(nd: not determined)

5.6 Breeding Scheme and Expected Numbers of Rescued Mice

Individual colonies were expanded from the three most suitable founders (3-3, 1-9, and 3-10) in order to obtain mice for further breeding experiments. *Podxl*$^{+/+}$*transgene*$^{+/-}$ progeny were then crossed with *podxl*$^{+/-}$*(transgene*$^{-/-}$*)* mice to obtain mice heterozygous for both the *podxl* knockout allele and the new, randomly integrated *podxl* transgene (Figure 5-8). Obviously, it was necessary to use mice heterozygous for the *podxl* knockout allele because of the demonstrated lethality of homozygotes. After obtaining *podxl*$^{+/-}$ *transgene*$^{+/-}$ animals, these were interbred to obtain transgene-containing mice homozygous for the *podxl* knockout allele. The expected frequency of each potential genotype from these breedings is shown in Table 5-2. Mice of interest were those expressing no endogenous podocalyxin while also harbouring at least one copy of the new transgene. The expected frequency of these rescued mice was 3/16, as shown in blue in Table 5-2.

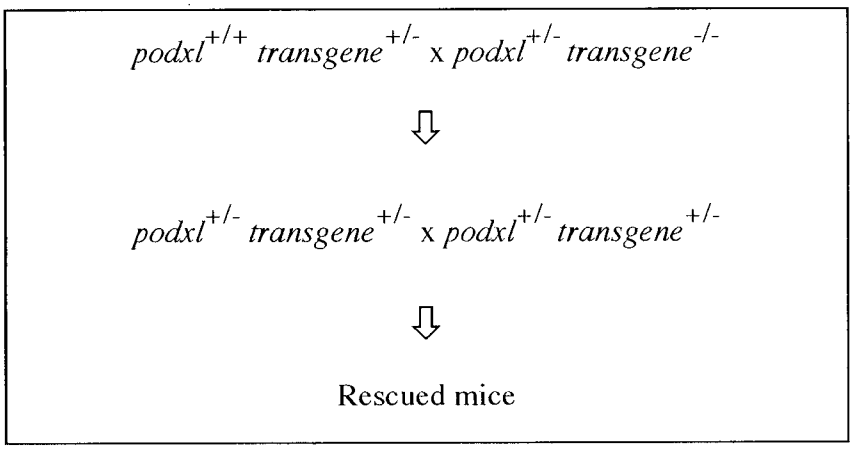

Figure 5-8: Breeding Scheme used to Generate Mice Expressing Podocalyxin only in Kidney.

	podxl $^{+/+}$	*podxl* $^{+/-}$	*podxl* $^{-/-}$
transgene $^{+/+}$	1/16	2/16	**1/16**
transgene $^{+/-}$	2/16	4/16	**2/16**
transgene $^{-/-}$	1/16	2/16	1/16

Table 5-2: Expected Numbers of All Genotypes Resulting from *Podxl*$^{+/-}$*Transgene*$^{+/-}$ Crosses.

Blue represents the frequency of potentially rescued mice.

5.7 Transgenic Mice Still Die within 24 Hours of Birth

After intercrossing *podxl*$^{+/-}$*transgene*$^{+/-}$ mice, all resulting pups that survived beyond the first 24 hours were genotyped. Although normal Mendelian ratios were seen for *podxl*$^{+/+}$ and *podxl*$^{+/-}$ transgene positive animals, there was not a single surviving *podxl*$^{-/-}$ *transgene*$^{+}$ mouse (Table 5-3). Although only the first five litters are shown in Table 5-3, all subsequent litters were also devoid of rescued pups. Notably, there was no evidence of insertional mutagenesis, as mice with the transgene were found in similar numbers to those lacking it when mice heterozygous for endogenous *podxl* and the transgene were crossed with *podxl*$^{+/-}$ animals (Table 5-4).

Litter	Genotype		
	podxl $^{+/+}$	*podxl* $^{+/-}$	***podxl*** $^{-/-}$
1	0	6	**0**
2	3	3	**0**
3	1	2	**0**
4	3	4	**0**
5	2	4	**0**
TOTAL	9	19	**0**

Table 5-3: Actual Numbers of Transgene-Positive Mice of Each Genotype in the First Five Litters Resulting from *Podxl*$^{+/-}$*Transgene*$^{+/-}$ Crosses Demonstrated a Lack of Rescue of *Podxl*$^{-/-}$ Mice.

Litter	*transgene* $^{+/-}$			*transgene* $^{-/-}$		
	podxl $^{+/+}$	*podxl* $^{+/-}$	*podxl* $^{-/-}$	*podxl* $^{+/+}$	*podxl* $^{+/-}$	*podxl* $^{-/-}$
1	0	4	0	0	3	0
2	2	3	0	2	0	0
3	1	1	0	0	4	0
4	1	3	0	0	3	0
5	1	3	0	0	3	0
6	1	3	0	1	3	0
7	0	2	0	0	2	0
8	0	2	0	3	1	0
TOTAL	**6**	**21**	**0**	**6**	**19**	**0**

Table 5-4: Actual Numbers of Transgene-Positive and Transgene-Negative Mice in Eight Litters Resulting from Breeding of *Podxl*$^{+/-}$*Transgene*$^{+/-}$ Mice with *Podxl*$^{+/-}$ *Transgene*$^{-/-}$ Mice Demonstrated that Transgene-Integration was not Detrimental.

5.8 Potential Reasons for Lack of Rescue

The lack of surviving pups was clearly disappointing, and it was also somewhat surprising. There were, however, several possible explanations for this outcome:

a) Podocalyxin may not have been expressed from the transgene. Although unlikely, it was possible that the GFP expression observed in kidneys of transgenic animals did not correlate with ectopic podocalyxin expression.

b) Podocalyxin may have been expressed at the incorrect time during development, thereby preventing proper maturation of podocytes.

c) Podocalyxin may have been expressed at inadequate levels. This was also not particularly likely because *podxl*$^{+/-}$ mice have well-developed, functional kidneys (Doyonnas et al., 2001).

d) Even if podocalyxin was expressed in the correct cells, it may have been mislocalized.

e) There may be other lethal podocalyxin-related defects.

5.9 Transgenic Podocalyxin Expression Analysis

In order to address the possibilities outlined above, the expression of podocalyxin was first assessed. This was accomplished by isolating kidneys from mice just prior to birth. At this time point, all genotypes were present in expected ratios. Furthermore, ectopic podocalyxin expression could be analyzed by comparing *podxl*$^{-/-}$*transgene*$^{+}$ kidneys with *podxl*$^{-/-}$*transgene*$^{-}$ kidneys. RT-PCR was used to demonstrate the presence of podocalyxin mRNA expressed from the transgene (Figure 5-9). Kidneys isolated from wildtype and

podocalyxin-null mice lacking the transgene were used as positive and negative controls, respectively. Both podocalyxin-null mice containing the transgene were expressing podocalyxin mRNA, as shown in red in Figure 5-9. Immunohistochemistry was used to confirm protein expression, as shown in Figure 5-10. Although there was only a thin brown layer of podocalyxin staining in the *podxl*$^{-/-}$*transgene*$^{+}$ glomeruli (left panel) in comparison to the widespread expression observed in wildtype animals (middle panel), it is important to remember that podocalyxin is also normally expressed on all vascular endothelial cells. Typical vascular staining is shown in the right panel, where PECAM-1 staining has been used to indicate vasculature. In contrast to the podocalyxin staining seen in the transgenic kidney, apical staining was most notably absent from the outer layer of cells in the compact glomerulus labelled with antibodies against PECAM-1. At high magnification, it was also more evident that podocalyxin was not only expressed, but was correctly localized to the apical surface of podocytes in transgenic animals (Figure 5-11). Thus, a lack of appropriate podocalyxin expression was not the reason for the lack of rescue observed in breeding experiments.

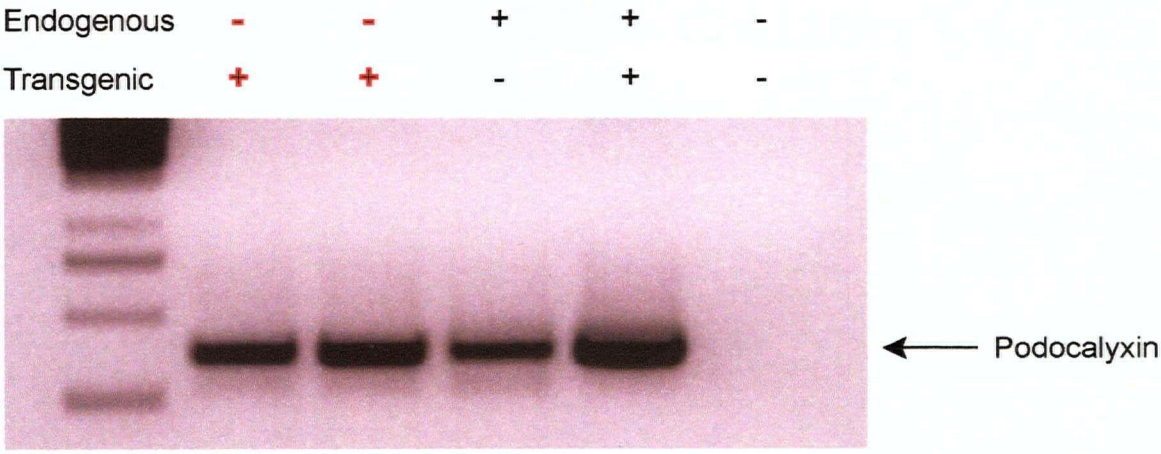

Figure 5-9: Podocalyxin mRNA was Expressed from the Transgene during Development in Kidneys of Transgenic Mice.

After isolation of mRNA from kidneys of E18 mice, RT-PCR was performed using *podxl*-specific primers. Kidneys from E18 podocalyxin-null mice were used as negative controls, and kidneys from E18 wildtype (B6) mice were used as positive controls for expression of podocalyxin mRNA.

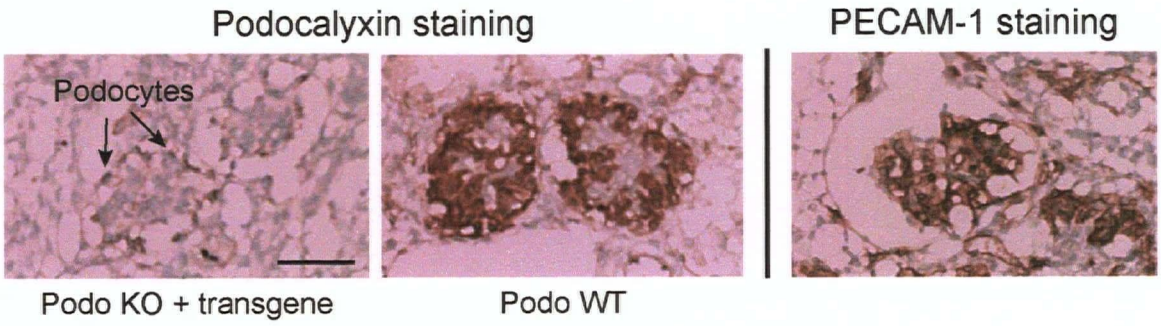

Figure 5-10: Immunohistochemistry was used to Detect Podocalyxin Expression in Kidney Glomeruli of Transgenic Mice during Development.

Kidneys were isolated from E18 mice, frozen, sectioned, fixed, and immunolabelled for podocalyxin or PECAM-1, as a vascular endothelial cell-specific marker. Note the wildtype sample displayed podocalyxin staining on blood vessels and podocytes, while glomeruli from *podxl*$^{l-}$ mice expressing the transgene displayed only a thin layer of podocalyxin staining, indicative of apical labelling of podocytes. Kidney sections from E18 podocalyxin-null mice were used as negative controls for podocalyxin staining, and rat IgG_1 was used as a negative control for PECAM-1 staining. Representative of two independent experiments. Scale bar: 50 μm.

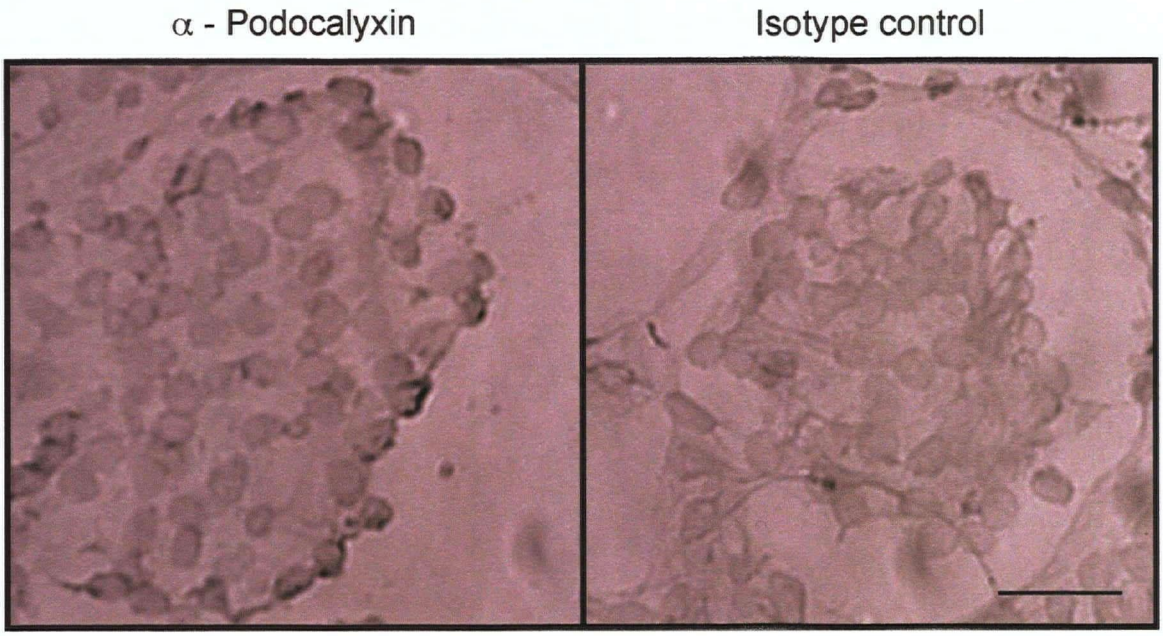

Figure 5-11: Apical Staining of Podocytes was Evident in High Magnification Images of Glomeruli from *Podxl*$^{+/-}$ Transgene-Positive Mice.

Kidneys were isolated from E18 mice, frozen, sectioned, fixed, and immunolabelled. A thin, dark layer of podocalyxin staining was most obvious around the outer edge of the glomerulus, while staining was absent in the isotype control sample. Scale bar: 10 μm.

5.10 Morphological Analysis of Kidneys

Although light microscopy was used to demonstrate that podocalyxin was expressed appropriately in glomerular podocytes, these images could not be used to assess cellular morphology. To address this issue, kidneys were again isolated from animals just before birth and analyzed by TEM.

Podocalyxin-null mice exhibit two major morphological abnormalities in podocytes (reviewed in section 1.6.4.1) (Doyonnas et al., 2001). They completely lack the interdigitating foot processes that normally surround capillaries in the glomerulus, and neighbouring podocytes maintain tight junctions between cell bodies. During normal maturation of podocytes, tight junctions are lost, and replaced by slit diaphragms between interdigitating foot processes (reviewed in section 1.6.1). These characteristics were therefore assessed by electron microscopy in kidneys of *podxl*$^{-/-}$*transgene*$^{+/-}$ animals. The morphologies previously observed for wildtype and podocalyxin-null animals were confirmed (Figure 5-12). Strikingly, podocalyxin knockout animals expressing the kidney-specific transgene had noticeably improved podocyte morphologies. There was an absence of tight junctions between podocytes, and interdigitating foot processes were very evident (Figure 5-12). Higher magnification images clearly showed the intricate foot processes (Figure 5-13). Slit diaphragms between foot processes were also detectable under close examination. This confirmed that podocalyxin is absolutely required for morphogenesis of podocytes, and that ectopic expression of podocalyxin in these cells was sufficient for repairing the defect.

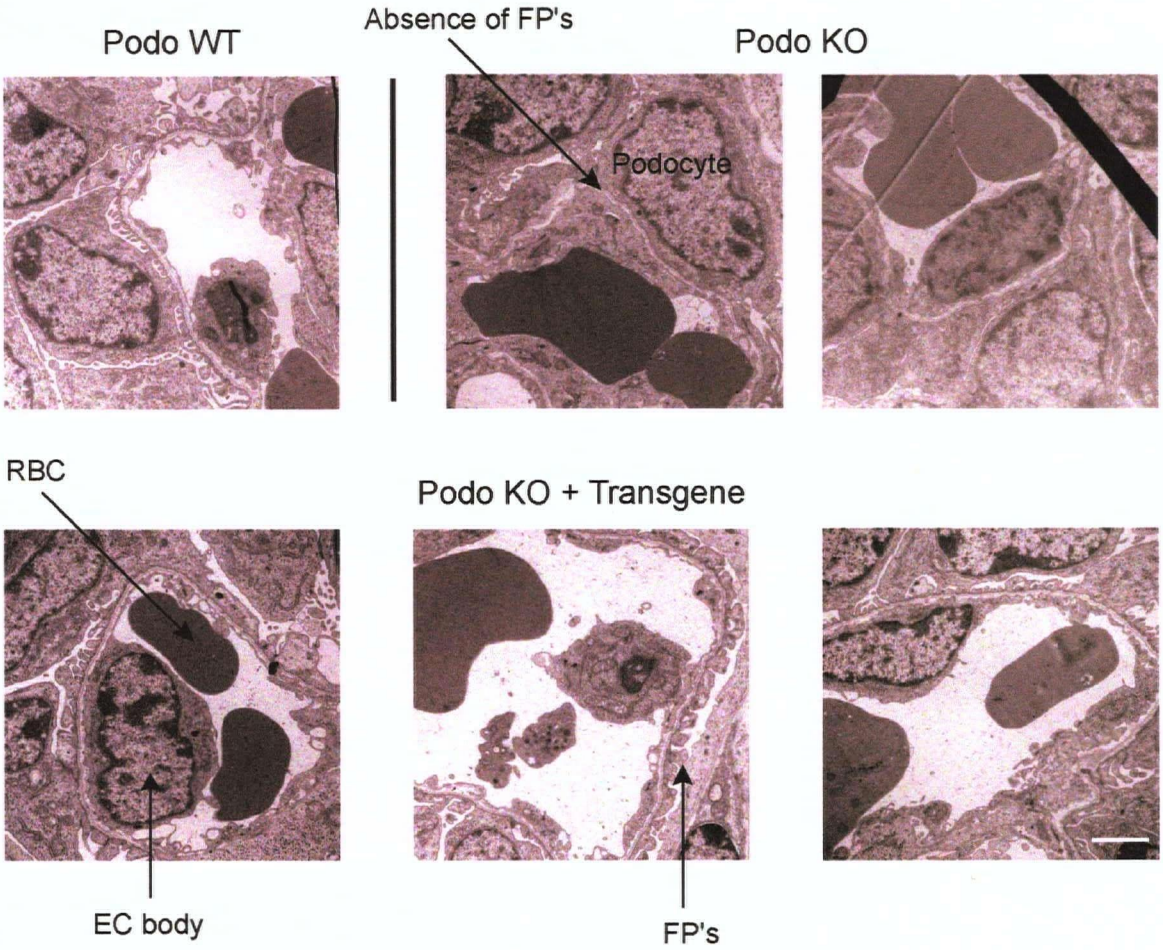

Figure 5-12: TEM's of Podocytes from E18 Wildtype, Podocalyxin-Null, and Podocalyxin-Null/Transgene-Positive Double Transgenic Mice.

Kidneys were isolated from E18 mice, fixed, sectioned, and processed for TEM. Each image shows a cross-section of a capillary, and most include circulating red blood cells (RBC). Podocytes were situated outside the capillary, extending interdigitating foot processes (FP's), which were visible in wildtype and podocalyxin knockout samples. Scale bar: 2 μm. Imaging by Derrick Horne, BioImaging Facility, University of British Columbia.

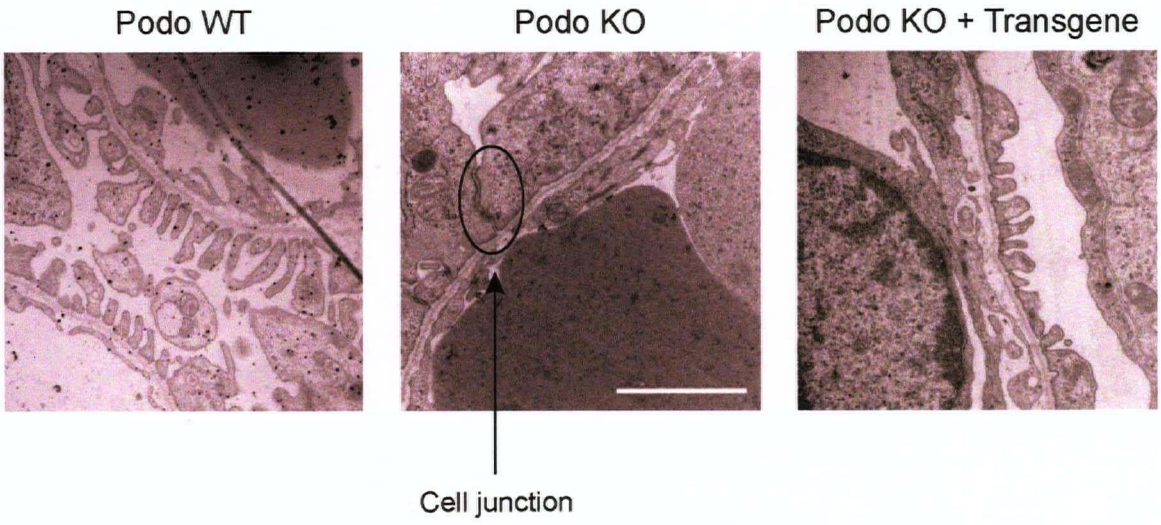

Figure 5-13: High Magnification TEM's of Podocytes from E18 Wildtype, Podocalyxin-Null, and Podocalyxin-Null/Transgene-Positive Double Transgenic Mice.

Note the intricate foot processes in wildtype and podocalyxin knockout/transgene-positive samples and the presence of cell junctions in the podocalyxin knockout sample. Scale bar: 3 μm. Imaging by Derrick Horne, BioImaging Facility, University of British Columbia.

5.11 Summary and Conclusions

Podocalyxin-null animals exhibit perinatal lethality. This phenotype was presumed to be due to a podocyte-related kidney defect since drastic morphological changes are observed in these cells in the absence of podocalyxin (Doyonnas et al., 2001). In an effort to rescue podocalyxin knockout mice to facilitate analysis of defects in adult animals, podocalyxin was ectopically expressed using the podocyte-specific *NPHS1* promoter. Expression analysis demonstrated appropriate expression of the transgene in several founder animals, with substantial expression in one founder.

Ectopic kidney-specific expression of podocalyxin did not prevent the perinatal lethality observed in knockout animals, but it did repair the morphological defects in the podocytes. It should be noted, however, that although there was a dramatic difference in podocyte morphology between podocalyxin knockout animals with and without the transgene, it is possible that the kidney defect was not adequately repaired due to somewhat aberrant expression. Careful examination suggests that foot processes may have been slightly flatter and slit pores marginally narrower in rescued mice compared to wildtype controls. It may be that ectopic expression levels were slightly lower, or that expression was initiated a little later in glomerular development than would be expected from the endogenous *podxl* promoter. However, nephrin and podocalyxin are expressed at almost identical times during development (Putaala et al., 2000; Putaala et al., 2001; Schnabel et al., 1989). Furthermore, the striking morphological difference between podocalyxin knockout mice expressing the transgene and those lacking it, in combination with the consistent lethality within 24 hours of birth, suggests that podocalyxin-null

animals may actually die as a result of other podocalyxin-related defects. This hypothesis is supported by the fact that some transgenic mice lack kidneys entirely but die within 48 hours of birth, rather than 24 hours (Guo et al., 2002). Thus, although kidney failure would eventually lead to death, podocalyxin-null animals may actually die earlier because of an additional defect. This is a very exciting possibility and will require further investigation.

CHAPTER 6 : CONCLUDING REMARKS

6.1 Summary and Discussion

Podocalyxin is a sialomucin closely related to CD34 and endoglycan (Nielsen et al., 2002). CD34 family members have been proposed to function in enhancing proliferation, blocking differentiation, establishing polarity, and regulating cell adhesion positively and negatively (Baumhueter et al., 1993; Cheng et al., 1996; Fackler et al., 1995; Fieger et al., 2003; Meder et al., 2005; Sassetti et al., 1998; Takeda et al., 2000). All three family members have C-terminal PDZ binding motifs that, in the case of podocalyxin and endoglycan, facilitate interaction with NHERF adapter proteins (He et al., 1992; Kershaw et al., 1997a; Li et al., 2002; McNagny et al., 1997; Nielsen et al., 2002; Sassetti et al., 2000; Simmons et al., 1992; Takeda et al., 2001; Tan et al., 2006). This association is known to connect podocalyxin with the actin cytoskeleton via ezrin, a linkage that is disrupted in several disease models (Orlando et al., 2001; Takeda et al., 2001).

Podocalyxin is widely distributed on vascular endothelial cells (Horvat et al., 1986; Kershaw et al., 1997a). It is also expressed by hematopoietic progenitors, platelets and megakaryocytes, a subset of neuronal cells, and mesothelial cells lining many organs (Doyonnas et al., 2001; Doyonnas et al., 2005; McNagny et al., 1997; Miettinen et al., 1999; Vitureira et al., 2005). Strikingly, it is most highly expressed on the elaborate glomerular podocytes of kidney, where it is vital for proper formation of elaborate foot process extensions (Doyonnas et al., 2001; Kerjaschki et al., 1984). Importantly,

abnormal podocalyxin expression is associated with a number of pathological conditions. Although it has not yet been linked to kidney dysfunction in human, mice lacking podocalyxin have poorly developed podocytes, exhibit anuria, and die within 24 hours of birth (Doyonnas et al., 2001). In human, increased podocalyxin expression is an independent predictor of poor outcome in breast cancer, while mutations in the podocalyxin gene, *podxl*, are found in patients with highly aggressive prostate cancer (Casey et al., 2006; Somasiri et al., 2004). Podocalyxin is also linked with several other types of malignancies.

6.1.1 Podocalyxin's Role in Cell Morphogenesis

In order to gain insights into podocalyxin's role in disease and in normal development, it was overexpressed in MCF-7 breast carcinoma cells (chapter 3). This blocked formation of confluent monolayers, and cells instead exhibited decreased cell-substratum interactions. Ectopic expression also induced a dramatic increase in microvillus formation. Podocalyxin expression on cells with complex membrane extensions is a common theme under physiological conditions. Most noticeably, it is essential for podocyte foot process formation (Doyonnas et al., 2001; Schnabel et al., 1989). However, it is also expressed on a subset of neurons, and formation of cellular extensions by podocytes and neurons display many similarities (reviewed in (Kobayashi et al., 2004)). Although these two cell types are functionally very different, the activities of both are critically dependent on intricate architectures. These highly branched cells require an extensive network of cytoskeletal constituents. Microtubules and intermediate filaments support major processes of podocytes and neuronal axons and dendrites, while actin

filaments form the backbone of foot processes and dendritic spines. Furthermore, many of the same cytoskeletal proteins are found in podocytes and neurons, implying that similar mechanisms underlie the formation of cellular extensions in both cell types.

Moreover, podocalyxin is also expressed by megakaryocytes, which extend long processes when differentiating into platelets (Miettinen et al., 1999; Vitureira et al., 2005). Over a period of 4-10 hours, the entire megakaryocytic cytoplasm is converted into microtubule-based proplatelet extensions (reviewed in (Patel et al., 2005)). In addition to formation of these long extensions, the ends of proplatelets bend and bifurcate in an actin-dependent process termed end amplification; this facilitates generation of thousands of platelets from each megakaryocyte. The exact mechanisms involved in formation of podocyte foot processes and dendritic spines, as well as the bifurcation of proplatelets are unknown, but they are all actin-based processes and they all occur in cells expressing the actin-associated protein, podocalyxin. Thus, I believe that a global function of podocalyxin is to facilitate the generation of morphologically distinct structures.

In support of this hypothesis, Cheng *et al* recently published that podocalyxin is required for tubulogenesis *in vitro* (Cheng et al., 2005). MDCK cells are derived from kidney tubular cells and can be induced to form tubules *in vitro*. Depletion of endogenous podocalyxin by siRNA blocks tubulogenesis. Although this system is somewhat artificial since kidney tubules do not normally express podocalyxin, the results may be relevant to other tissues where podocalyxin is typically expressed (Dekan et al., 1990; Takeda et al.,

2001). For example, podocalyxin in mammary epithelial cells (Somasiri et al., 2004) and vascular endothelia (Horvat et al., 1986) may facilitate remodelling and angiogenesis, respectively.

Podocalyxin is comprised of an extensive mucin-like extracellular region as well as a well-conserved cytoplasmic tail, which interacts with the actin cytoskeleton through NHERF proteins and ezrin. Various podocalyxin mutants were generated and expressed *in vitro* in order to study podocalyxin's mechanism of action. Surprisingly, although interaction with the actin cytoskeleton is likely involved in formation of podocalyxin-induced cellular extensions, deletion of the majority of podocalyxin's cytoplasmic tail did not affect microvillus formation. In contrast, and more expectedly, tubulogenesis in MDCK cells does require podocalyxin's intracellular domain (Cheng et al., 2005). The apparently contradictory evidence suggests that alternative mechanisms may be utilized under certain conditions, or that the two types of morphogenetic alterations occur via somewhat different pathways.

The requirement for the mucin-like domain, however, is supported by all available data. Podocalyxin-dependent microvillus formation was completely ablated upon deletion of the extracellular domain, foot process architecture is drastically affected by neutralization of podocalyxin's negative charge, and tubulogenesis is much less extensive when the mucin domain is removed, whether genetically or by treatment with inhibitors of O-linked glycosylation (see chapter 3) (Andrews, 1979; Cheng et al., 2005; Seiler et al., 1977). Podocalyxin may stimulate the formation of cellular extensions in an attempt to

separate its bulky, negatively charged mucin domains from each other. Once generated, however, the stability of such protrusions would likely be dependent on reorganization of the actin cytoskeleton.

Although it is clear that podocalyxin can induce actin reorganization (Figure 3-23 and (Schmieder et al., 2004)), it is surprising that direct interaction with NHERF1 or ezrin was not required in MCF-7 cells. The most plausible explanation is that podocalyxin can associate with another integral membrane protein (via its extracellular domain) that itself interacts with the actin cytoskeleton or transduces a signal to instigate actin reorganization (Figure 3-25). Although this has not been proven, there is also no evidence to definitively refute it. For example, in rats treated with protamine sulfate or sialidase to neutralize podocalyxin's negative charge, there is a decrease in the amount of ezrin and NHERF2 that co-immunoprecipitate with podocalyxin in glomerular extracts (Takeda et al., 2001). This has been explained by postulating that reduction of podocalyxin's extracellular negative charge leads to an intracellular conformational change, which disrupts the interaction between NHERF2 and podocalyxin. However, it is also possible that an additional integral membrane protein that interacts with podocalyxin's extracellular domain is normally found in this protein complex and that protamine sulfate and sialidase treatments remove an extracellular protein recognition site, thereby preventing the interaction of podocalyxin with the rest of the complex. Thus, the idea of an extracellular binding partner for podocalyxin is an intriguing possibility that I would like to investigate.

6.1.2 Podocalyxin's Role in Cell Adhesion

Along with its role in cell morphology, there is clearly mounting evidence to implicate podocalyxin, along with CD34, in decreasing cellular adhesion. Interestingly, it appears that CD34 and podocalyxin may employ several mechanisms to act as global anti-adhesins. They have been shown to decrease cell-substratum adhesion, as well as cell-cell adhesion, both by interfering with cell–cell junctions and by blocking aggregation of cells in suspension (chapter 3) (Doyonnas et al., 2001; Drew et al., 2005; Economou et al., 2004; Somasiri et al., 2004; Takeda et al., 2000). Decreased cell-cell adhesion between multiple podocalyxin or CD34 expressing cells in suspension can easily be explained by steric hindrance or charge repulsion between sialomucins on nearby cells. Expression of podocalyxin at the tips of microvilli, also generated in a podocalyxin-dependent manner, would further increase the anti-adhesion phenotype, as microvilli are the first point of contact between cells.

Similarly, cell-cell junctions may simply be affected by relocalization of podocalyxin or CD34 toward the junctions, thereby forcing neighbouring cells further and further apart. In support of this model, loss of cell-cell junctions between adjacent kidney podocytes during development coincides precisely with podocalyxin expression and migration toward the basal cell surface (Schnabel et al., 1989). Again, microvillus formation near the apical surface, between these cells would further serve to increase the spacing between cells.

Decreased cell-substratum interactions are more difficult to explain because CD34 and podocalyxin are generally apical membrane proteins. In the unusual HGEC culture system, where some podocalyxin is aberrantly localized to the basolateral surface, decreased cell-substratum interactions can be explained by steric hindrance or charge repulsion, where the negatively charged mucin-like domain of podocalyxin is expressed in the vicinity of the similarly charged laminin or collagen extracellular matrix components. In most cells, though, this is not the case. How, then, does an apical protein affect adhesion at the basal surface? An intriguing possibility is that podocalyxin-induced actin recruitment for generation of apical cell surface structures may deplete actin from the basal surface, thereby decreasing the stability of integrin-mediated adhesion complexes at the basal surface (Figure 6-1). In support of this idea, cells transfected with podocalyxin mutants that induced microvillus formation also displayed decreased cell adhesion, whereas the extracellular deletion mutant did not induce microvillus formation or decrease cell adhesion. Similarly, podocalyxin may weaken cell-cell junctions by titrating actin away from these junctional proteins as well. Moreover, since podocyte foot processes contain an extensive network of actin filaments, podocalyxin may affect multiple aspects of podocyte architecture by inducing actin reorganization. Thus, loss of podocalyxin may indirectly affect cytoskeletal interactions with proteins of the slit diaphragm complex in addition to those on the basal cell surface. This will require further investigation of the interplay between actin recruitment by apical and basolateral proteins.

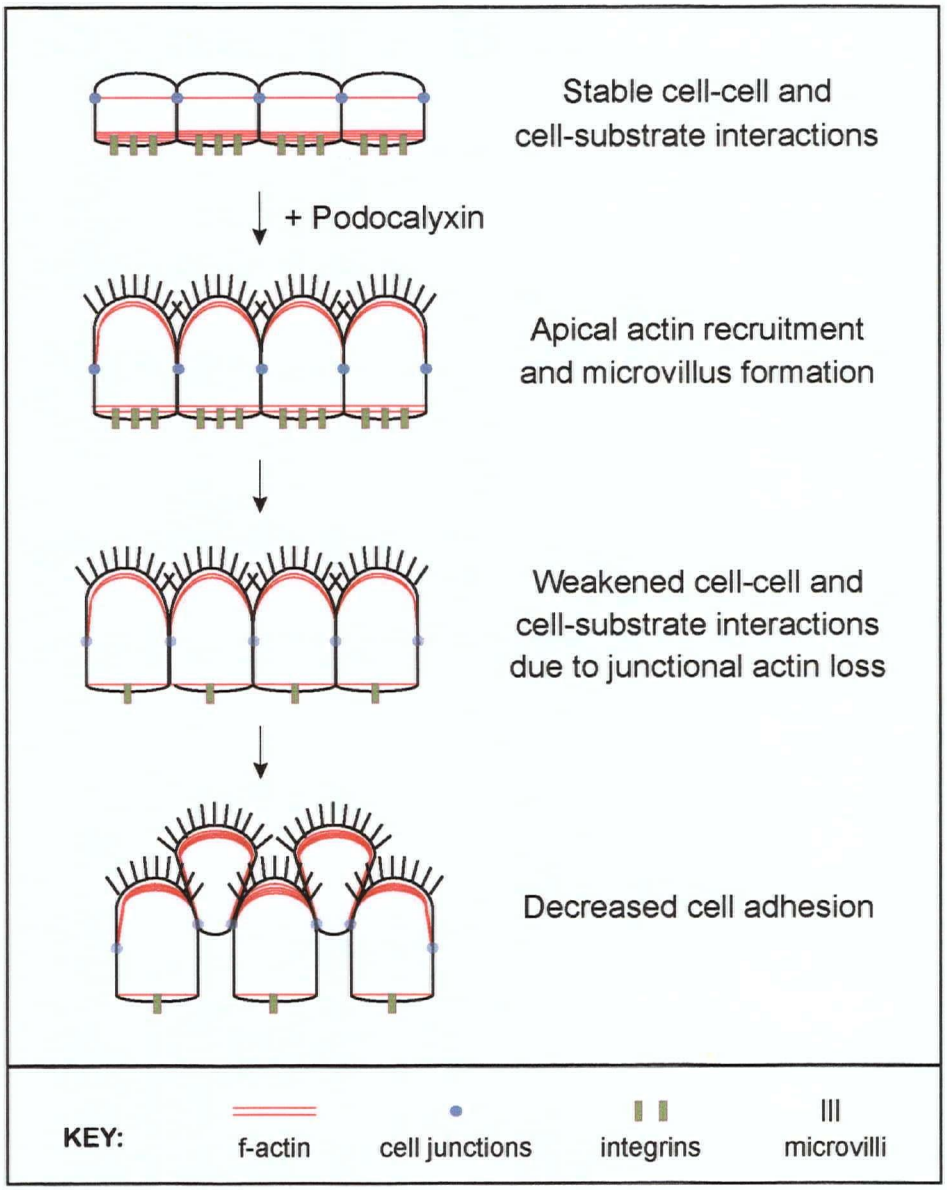

Figure 6-1: Model Demonstrating a Possible Mechanism for Decreased Cell-Substrate Adhesion Induced by Apical Podocalyxin Expression.

Apical recruitment of f-actin by podocalyxin may titrate actin away from basal integrins and cell-cell junctional proteins, thereby weakening these interactions.

6.1.3 Podocalyxin-Dependent NHERF Localization

Podocalyxin's role in apical recruitment of NHERF1 is another significant result of this work. Podocalyxin is known to interact with the NHERF family of adapter proteins through its C-terminal PDZ recognition sequence (Li et al., 2002; Takeda et al., 2001; Tan et al., 2006). Since NHERF family members can link proteins indirectly to the actin cytoskeleton through ERM domain-dependent interactions with cytoskeletal linker molecules, it had been proposed that NHERF proteins are responsible for apical localization of podocalyxin. In support of this, podocalyxin mutants lacking the PDZ binding site are subtly mislocalized (Cheng et al., 2005; Li et al., 2002; Meder et al., 2005; Schmieder et al., 2004). However, more intriguingly, I have now shown that podocalyxin is actually responsible for localization of NHERF1 in MCF-7 cells (see chapter 3). While only about 20 % of podocalyxin is mislocalized in the absence of interaction with NHERF, virtually <u>all</u> NHERF1 is dispersed throughout the cytoplasm, rather than at the apical surface, in cells ectopically expressing podocalyxin mutants incapable of interacting with NHERF proteins. This, in combination with the apical localization of both podocalyxin and NHERF early in cell polarization, suggests that podocalyxin, not NHERF1, is the major player in the process of apical domain establishment and targeting.

Apical recruitment of NHERF proteins by podocalyxin also has important implications for the numerous activities of these scaffolding proteins. NHERF1 and NHERF2 are capable of interacting with a wide variety of molecules, including ion transporters, signalling proteins, and receptors, such as epidermal growth factor receptors and β_2-

adrenergic receptors ((Lazar et al., 2004), and reviewed in (Shenolikar et al., 2004)). Thus, podocalyxin-dependent localization may play an essential role in regulation of many cellular processes.

6.1.4 Ectopic Podocyte-Specific Podocalyxin Expression Repairs Foot Process Architecture but Fails to Rescue Podocalyxin-Null Mice

Although podocalyxin overexpression has been assessed *in vitro* (chapter 3), it has yet to be investigated *in vivo*. Another major goal of my thesis was therefore to attempt to generate transgenic mice overexpressing podocalyxin in an inducible manner (chapter 4). I cloned *podxl* into a transgenic vector for use in the Cre-loxP inducible system, and produced ESCs that specifically expressed podocalyxin and GFP in a Cre recombinase-dependent manner. Unfortunately, although chimeric mice were generated, the transgene was never transmitted in the germline. Karyotyping of two ESC clones revealed the existence of a chromosomal aberration, likely also present in the parental R1 ESCs, which precluded generation of viable, fully transgenic animals.

One of the main reasons for generating conditional podocalyxin overexpressing transgenic mice (chapter 4) was to generate animals specifically expressing podocalyxin in podocytes. These mice were then to be crossed with *podxl*$^{+/-}$ mice, with the intention of generating *podxl*$^{-/-}$ mice expressing transgenic podocalyxin specifically in podocytes. Since *podxl*$^{-/-}$ animals die within 24 hours of birth, apparently as a result of a kidney

defect, expression in podocytes was expected to rescue these mice, thereby enabling analysis of adult mice lacking podocalyxin in other tissues.

Due to delays in production of conditional podocalyxin expressing mice (chapter 4), a new strategy was initiated in order to express podocalyxin specifically in podocytes. *Podxl* was inserted into an expression vector downstream of the podocyte-specific *NPHS1* promoter, and transgenic mice were generated using this construct (chapter 5). The transgene was expressed specifically in podocytes, so *podxl*$^{+/+}$*transgene*$^{+/-}$ mice were crossed with *podxl*$^{+/-}$*transgene*$^{-/-}$ mice, and these were interbred to generate mice expressing podocalyxin only in kidney. Strikingly, podocytes in these mice displayed relatively normal foot processes and lacked aberrant cell-cell junctions, which are inappropriately retained in podocalyxin-deficient mice. This provided conclusive evidence that podocalyxin is both necessary and sufficient for podocyte morphogenesis, again demonstrating podocalyxin's role in generation of cell surface projections.

However, although podocyte architecture was repaired in these transgenic mice, they still suffered perinatal lethality, suggesting that podocalyxin may play additional essential roles in other tissues. Loss of podocalyxin may cause vascular defects, such as decreased permeability for transendothelial migration, increased global stickiness of vascular endothelial cells to hematopoietic cells, or collapse of blood vessels due to interactions between opposing membranes in their lumens. Platelets lacking podocalyxin may generate clots inappropriately, or they may be decreased in number due to megakaryocyte abnormalities. Loss of podocalyxin from the linings of body cavities may lead to

increased organ damage due to inappropriate adhesion of organs to one another. There may even be neuronal defects in podocalyxin-null animals. Thus, the absence of rescued mice is actually a more fascinating outcome than that which was predicted. It will be interesting to assess other developmental defects that may be caused by loss of podocalyxin.

6.2 Significance of Results

Expression of podocalyxin in a variety of tissues, including podocytes, vasculature, some hematopoietic cells, boundary elements separating organs, and a subset of neuronal cells, along with altered expression in numerous malignant situations, makes it a potentially important protein for further analysis. The absolute requirement for podocalyxin expression in order for mice to survive beyond the first day of life makes it even more intriguing.

6.2.1 Podocalyxin in Normal Development

Adhesion and morphogenesis are both extremely important activities throughout development. Adhesion, or lack thereof, regulates proper migration of cells, while morphogenesis is critical for the generation of many specialized cell types. Podocalyxin is clearly important in facilitating formation of intricate podocyte foot processes, but it may also play a role in generation of neuronal processes and the megakaryocytic extensions involved in platelet production. In addition, it may be involved in global cell migration and movement of hematopoietic cells across endothelial barriers when

colonizing new niches. Furthermore, the coating of podocalyxin on mesothelial cells lining body cavities likely protects organs from damage. This is especially evident in podocalyxin knockout animals, which are inefficient at retracting physiologic omphaloceles late in development. Thus, podocalyxin expression throughout development and in the adult may regulate adhesion and cell morphogenesis in a variety of tissues.

6.2.2 Podocalyxin in Cancer Progression

Podocalyxin has also been associated with a wide variety of cancers. It is upregulated in hepatocellular carcinoma and a subset of testicular cancers, and it is also found in leukemias (Chen et al., 2004; Kelley et al., 2005; Schopperle et al., 2003). Importantly, it is upregulated or mutated in highly aggressive breast cancers, Wilms' tumours, and prostate cancers (Casey et al., 2006; Somasiri et al., 2004; Stanhope-Baker et al., 2004). Although the result of podocalyxin dysregulation in these malignant situations is not yet clear, its association with the most aggressive cases and its role in regulating cell adhesion suggest that it may facilitate metastasis. Moreover, solid tumours, which require increased nutrients, and therefore increased blood flow, may become more aggressive as a result of podocalyxin-induced angiogenesis. Determination of the underlying mechanisms behind increased podocalyxin expression and its effects in these disease conditions is therefore particularly important.

6.3 Future Directions

Although I have gained some important insights into the functions of podocalyxin, the mechanisms behind its activities, its roles in development, and the consequences of aberrant expression, this work has generated many more questions. In my opinion, the most interesting avenues to pursue include understanding where podocalyxin is absolutely essential throughout development, how it affects cancer progression, and how it can induce morphological changes in the absence of the majority of its cytoplasmic tail.

6.3.1 Understanding Podocalyxin Mechanistically

Finding an *in vitro* model system for assessing podocalyxin function (chapter 3) was a major step forward, and the induction of microvillus formation can now be used to elucidate mechanisms of podocalyxin-induced morphological changes. Deletion of all but six juxtamembrane amino acids of podocalyxin's intracellular domain was expected to completely block interactions with all known cytoplasmic interaction partners, including NHERF proteins and ezrin. While NHERF certainly does not interact with this deletion mutant (Figure 3-15), absence of podocalyxin/ezrin interactions must be confirmed. Although two of the three amino acids known to be involved in directly binding ezrin were retained in the podocalyxin Δtail mutant, ICAM-3, which also interacts directly with ezrin, fails to bind when only the first eight amino acids are retained in the cytoplasmic tail (Schmieder et al., 2004; Serrador et al., 2002). Thus, the REHQRSGS sequence of ICAM-3 is <u>insufficient</u> for interaction with ezrin, suggesting that the podocalyxin tail mutant that included only CCHQRF (Δtail) would also be unlikely to bind. However, to

formally prove this, GST-pull down experiments will be performed using GST-tagged N'terminal ezrin and lysates from cells expressing podocalyxin mutants.

Assuming that the Δtail mutant cannot interact with ezrin, it is possible that an additional protein interacts with the extracellular or transmembrane regions of podocalyxin in order to link it to the actin cytoskeleton, or at least to transduce the signal required for actin reorganization and microvillus formation. Although much of podocalyxin's extracellular domain is not conserved, the juxtamembrane stalk retains about 70 % similarity across species, so this is a likely candidate region for a protein-protein interaction domain. Although finding an extracellular binding partner for podocalyxin may be difficult, the question of whether or not there is one is an important one to address.

Specific deletions of podocalyxin's transmembrane domain, the juxtamembrane extracellular stalk, the cysteine-bonded globular domain, and portions of the mucin domain should be generated in an effort to pinpoint regions of importance. In addition, reagents could be used to modify podocalyxin's mucin domain *in vitro*. For example, sialic acid residues could be removed using neuraminidase, or O-glycosylation could be blocked entirely. Thus, many experiments could be performed in order to pinpoint the mechanism by which podocalyxin regulates cell morphology.

6.3.2 Podocalyxin's Role in Development

The unexpected lack of rescue of podocalyxin knockout mice expressing transgenic podocalyxin specifically in kidney was quite intriguing. If the kidney defect was really

the only cause of perinatal lethality in podocalyxin-deficient animals, reintroduction of the protein into podocytes should have repaired the defect and rescued the mice. Thus, either podocalyxin was expressed inadequately in transgenic animals, or the kidney defect is not the only major abnormality in podocalyxin-null mice. The transgene did seem to be expressed sufficiently to repair the defect, as dramatic morphological differences were noted between *podxl*$^{-/-}$ animals with and without the transgene, although it was possible that there were some slight differences between these mice and wildtype littermates (chapter 5). Thus, although podocyte-specific expression of podocalyxin did not enable analysis of defects in adult animals, with the appropriate assays it may still be possible to find other defects in podocalyxin-deficient animals shortly before birth.

6.3.3 Podocalyxin's Role in Cancer Progression

While podocalyxin has been associated with numerous types of cancer, confirmation that it is a causal factor in cancer progression has yet to be obtained. This will be investigated using two approaches. First, MCF-7 cells ectopically expressing podocalyxin (chapter 3) will be injected into immunocompromised mice. MCF-7 cells are known to generate tumours, and overexpression of podocalyxin may enhance metastasis of these cells. If differences are noted between cancer progression in mice injected with podocalyxin-transfected cells and vector control cells, then the experiment will be repeated using MCF-7 cells ectopically expressing podocalyxin mutants (chapter 3). This will allow us to gain insights into exactly how podocalyxin affects cancer progression, mechanistically. Thus, the critical domains responsible for cancer progression will be identified, and potential interacting proteins will be examined. Importantly, the podocalyxin binding

protein, ezrin is essential for numerous signalling pathways implicated in metastasis and poor outcome in various cancers (Casey et al., 2006).

The second strategy for assessing podocalyxin's involvement in cancer aggressiveness involves overexpression of podocalyxin in mammary tissue, and perhaps other sites, in transgenic mice. The Cre-loxP strategy discussed in chapter 4 is being repeated using fresh R1 ESCs. Podocalyxin expression will therefore be inducible in any tissue, or at any time point, as long as a Cre mouse is available. For example, crossing podocalyxin inducible transgenic mice to whey acidic protein (WAP)-Cre mice (Wagner et al., 1997) will specifically induce podocalyxin expression in the mammary gland. Tumour formation will then be induced in these mice using the mouse mammary tumour virus (MMTV), and cancer progression will be compared between these mice and those in which podocalyxin expression was not induced. Thus, these two models will be used to address podocalyxin's role in cancer progression. Future experiments will therefore build upon the insights gained in this thesis work in order to unravel podocalyxin's role in normal development and disease progression.

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