Abstract

Despite the widespread use of CD34-family sialomucins (CD34, podocalyxin (Podxl) and endoglycan) as vascular endothelial cell (EC) markers, there is remarkably little known of their role in vascular development and functions with the exception of vessel lumen formation in the developing mouse embryo (Podxl) and vessel patency during tumor angiogenesis and inflammation (CD34). Because germ-line deletion of Podxl in mice causes perinatal death, we generated mice that conditionally delete Podxl in vascular endothelial cells (Podxl^{ΔEC} mice) to study the role of podocalyxin in adult mouse vessels. Although Podxl^{ΔEC} adult mice are viable and thrive, we discovered increased basal and inflammation-induced pulmonary vascular permeability. Furthermore, Podxl^{ΔEC} mouse adult lungs display airspace enlargement with increased collagen deposition and exhibit a gene expression profile similar to regenerating lung. To study whether endothelial cell morphology influences the defective lung architecture and the vascular permeability phenotype in Podxl^{ΔEC} mice, we isolated primary vascular ECs from lung tissue. Podxl^{ΔEC} ECs display enhanced adhesion to fibronectin (FN) in a static adhesion assay. When plated on matrix-coated transwells, Podxl^{ΔEC} EC spread normally on FN but display defective spreading on laminin and collagen I. Thus, expression of Podxl in EC is required for normal lung architecture and function in adult mice and adhesion of EC to extracellular matrix components.

Although its expression has not been well characterized, in humans, endoglycan expression has been reported in vascularized tissues. In mouse blood vessels, we showed that vascular smooth muscle cells (vSMC), but not EC, express the highest levels of endoglycan. Using a mouse aortic smooth muscle line (MOVAS-1) we found that forced expression of endoglycan enhances basal but not platelet derived growth factor (PDGF)ββ-dependent vSMC migration in vitro. Further studies to understand the role of endoglycan in primary vSMC showed that endoglycan is upregulated with differentiation to a contractile phenotype, but is not influenced by inflammatory stimuli or mitogenic factors.
The findings of this thesis suggest that the CD34 family regulates vascular development and function with a role for podocalyxin in EC-matrix adhesion relevant to normal lung function and a role for endoglycan in SMC differentiation and migration.
Preface

I have performed and generated all of the experiments, data and figures presented in this thesis with the following exceptions:

For the generation of the $Podxl^{\text{flox}}$ mice described in Chapter 2, cloning and screening of ES cells were completed by Christina Sina, blastocyst injections were done under the supervision of Dr. Danny Chui, and breeding strategies from chimeras through established $Podxl^{\text{flox}}$ allele were completed by Dr. Michael R. Hughes and final crosses to $Cdhl5-Cre$ and Rosa26-YFP mice were maintained by Erin J. Debruin.

Figure 2.1 was generated by Dr. M. R. Hughes, figures 2.8 and 2.9 were generated with the assistance of Kay Jian and figure 2.16 was generated by Bernard Lo.

For the generation of the Endgl$^{\text{flox}}$ mice described in Chapter 3, cloning and screening of EC cells was completed by Dr. Sebastian G.B. Furness, blastocysts injections were done under the supervision of Dr. Danny Chui, breeding strategies from chimeras through established $Podxl^{\text{flox}}$ allele were completed by Dr. Michael R. Hughes and final crosses to $Tagln$-Cre mice were maintained by Erin J. Debruin.

Figures 3.6 A&B were generated by Dr. Sebastian G.B. Furness and 3.6C was generated by Dr. Michael R. Hughes.

This work was approved by the Animal Care Committee under Certificate Numbers: A06-1483, and A09-0222.
Table of Contents

Abstract.................................................................................................................................... ii

Preface..................................................................................................................................... iv

Table of Contents .................................................................................................................... v

List of Tables .......................................................................................................................... ix

List of Figures.......................................................................................................................... x

List of Symbols & Abbreviations ........................................................................................ xii

Acknowledgements ............................................................................................................... xv

Dedication ............................................................................................................................ xvii

Chapter 1 : Introduction ........................................................................................................ 1

1.1 The vasculature ................................................................................................................... 1

1.1.1 Endothelial cells ........................................................................................................... 2

1.1.1.1 Defining endothelial cells ...................................................................................... 3

1.1.1.2 Endothelial-specific transgenic mice ...................................................................... 3

1.1.1.3 Endothelial cell basement membrane components and interactions ................... 5

1.1.1.4 Endothelial cell function ....................................................................................... 7

1.1.1.4.1 Vascular permeability ..................................................................................... 8

1.1.1.4.2 Structural heterogeneity of endothelial cells required for barrier control ........... 8

1.1.1.4.3 Regulation of basal permeability ..................................................................... 9

The glycocalyx...................................................................................................................... 9

The transcellular pathway .................................................................................................. 11

The paracellular pathway ................................................................................................. 12

1.1.1.4.4 Inflammation-induced permeability ................................................................. 14

1.1.1.4.5 Acute lung injury ............................................................................................. 15

1.1.1.4.6 The study of barrier function and vascular permeability .................................. 16

1.1.2 Smooth muscle cells .................................................................................................. 18

1.1.2.1 Defining smooth muscle cells ............................................................................. 18

1.1.2.2 Smooth muscle cell plasticity ............................................................................. 19

1.2 Pulmonary development and disease .......................................................................... 23
1.2.1 Lung development ................................................................. 23
1.2.2 Lung pathologies ................................................................. 26
1.2.3 Factors that influence lung development ................................ 27
1.2.4 Maintenance of lung structure requires communication between the airways and vasculature ................................................................. 28
1.2.5 Lung regeneration ............................................................... 30
1.3 CD34 family ........................................................................... 33
  1.3.1 Protein structure, genomic organization and binding partners. 33
  1.3.2 Cellular distribution of CD34 family .................................. 37
    1.3.2.1 Vascular expression .................................................... 37
      1.3.2.1.1 Podocalyxin ......................................................... 37
      1.3.2.1.2 CD34 ................................................................. 38
      1.3.2.1.3 Endoglycan ......................................................... 39
    1.3.2.2 Hematopoietic and additional sites of expression .............. 40
      1.3.2.2.1 Podocalyxin ......................................................... 42
      1.3.2.2.2 CD34 ................................................................. 43
      1.3.2.2.3 Endoglycan ......................................................... 44
  1.3.3 Emerging role of the CD34 family in the vasculature ............... 45
  1.3.4 CD34 function in hematopoietic cells ................................ 48
  1.3.5 Podocalyxin function outside the vasculature ......................... 50
  1.3.6 Published function of endoglycan ..................................... 52
1.4 Aims and rationale of the study .............................................. 53

Chapter 2: Vascular inactivation of the Podxl gene leads to increased vascular permeability, altered lung structure and defective endothelial cell adhesion and spreading ................................................................. 55
  2.1 Introduction ........................................................................... 55
  2.2 Materials & methods ............................................................ 59
    2.2.1 Animals ........................................................................ 59
    2.2.2 Generation of conditional podocalyxin knockout ................. 60
    2.2.3 Cells ............................................................................ 61
    2.2.4 Genomic PCR and real-time qPCR .................................. 62
    2.2.5 Antibodies and reagents ............................................... 65
    2.2.6 Flow cytometry analysis ............................................... 66
2.2.7 Immunohistochemistry ................................................................. 67
  2.2.7.1 Podocalyxin staining ............................................................... 67
  2.2.7.2 Von Willebrand factor and Ki-67 staining ............................... 67
  2.2.8 Morphometric analysis ............................................................ 68
  2.2.9 Determination of vascular permeability and lung edema ......... 68
  2.2.10 Lung volumes and right ventricular hypertrophy .................. 69
  2.2.11 Lung function measurements .................................................. 69
  2.2.12 Multiphoton and second harmonic generation imaging ......... 70
  2.2.13 Cell viability assay ............................................................... 71
  2.2.14 Static adhesion assay ............................................................ 71
  2.2.15 Spreading assay ................................................................. 71
  2.2.16 Statistical analysis ............................................................... 72

2.3 Results ......................................................................................... 73
  2.3.1 Podocalyxin is ablated in PodxlEC mice .................................. 73
  2.3.2 Isolation and characterization of PodxlEC and PodxlEC/Cd34- lung endothelial cells .......................... 79
  2.3.3 PodxlEC mice have increased basal and inflammation-induced lung vascular permeability in vivo ............................................................. 81
  2.3.4 Deletion of vascular Podxl contributes to enlarged airspaces .......................... 84
  2.3.5 PodxlEC mice have increased lung volume and resistance in both the central airways and lungs ........................................................................ 86
  2.3.6 Lung maintenance is impaired in PodxlEC mice ....................... 90
  2.3.7 Gene profiling of adult lungs show changes in matrix components, and structural genes as a result of deletion of podocalyxin in endothelial cells ............................................................. 91
  2.3.8 Structural matrix components are mislocalized in lungs lacking podocalyxin .......... 93
  2.3.9 Isolation and characterization of cultured PodxlEC lung endothelial cells .......................... 96
  2.3.10 Loss of podocalyxin affects static adhesion and spreading of endothelial cells, likely through modulation of integrin expression ........................................................................ 98

2.4 Discussion ...................................................................................... 102
  2.4.1 Podocalyxin is required to maintain vascular permeability ....... 103
  2.4.2 Lung regeneration is activated but cannot be maintained as a result of endothelial cell specific loss of podocalyxin ........................................................................ 106
  2.4.3 Podocalyxin and CD34 have different functions in the endothelium ........................................ 107
  2.4.4 Endothelial cell adhesion to laminin and fibronectin is controlled by podocalyxin expression ........................................................................ 109
Chapter 3: Endoglycan in the mouse vasculature .......................................................... 111
  3.1 Introduction......................................................................................................................... 111
  3.2 Materials & methods........................................................................................................... 112
    3.2.1 Animals ......................................................................................................................... 112
    3.2.2 Generation of conditional endoglycan knockout, Endgl<sup>lox</sup> mice ....................... 113
    3.2.3 Cells............................................................................................................................. 114
    3.2.4 DNA constructs and endoglycan mutants ................................................................. 115
    3.2.5 Cell viability assay ....................................................................................................... 115
    3.2.6 Antibodies & reagents ................................................................................................. 116
    3.2.7 Wound migration assays ............................................................................................. 117
    3.2.8 Western blotting ........................................................................................................... 117
    3.2.9 Genomic PCR, RT-PCR and real-time qPCR ............................................................. 118
    3.2.10 Flow cytometry ........................................................................................................ 119
    3.2.11 Immunohistochemistry and immunocytochemistry ............................................... 119
    3.2.12 Statistical analysis ..................................................................................................... 119
  3.3 Results.............................................................................................................................. 120
    3.3.1 Endoglycan is expressed on mouse smooth muscle cells but not endothelial cells ..... 120
    3.3.2 Differentiation of smooth muscle results in upregulation of endoglycan expression ... 126
    3.3.3 “Conditional endoglycan knockout mice” fail to lose endoglycan protein expression . 130
    3.3.4 Endoglycan dampens PDGFββ-mediated smooth muscle cell migration ............... 136
  3.4 Discussion......................................................................................................................... 140

Chapter 4: Conclusions & discussion............................................................................... 145
  4.1 Podocalyxin summary and discussion .............................................................................. 145
    4.1.1 The role of podocalyxin in endothelial cell adhesion and spreading ....................... 146
    4.1.2 The elusive cause of the <i>Podxl<sup>−/−</sup></i> perinatal lethality........................................... 150
    4.1.3 <i>Podxl<sup>EC</sup></i> mice as a model for lung aging............................................................... 151
    4.1.4 Future directions........................................................................................................... 152
  4.2 Endoglycan summary and discussion .............................................................................. 155
    4.2.1 Endoglycan as a lineage marker for smooth muscle cells ....................................... 156
    4.2.2 Future directions.......................................................................................................... 157

References............................................................................................................................ 159
List of Tables

Table 1.1: Integrins expressed on endothelial cells and their extracellular ligands................. 6
Table 1.2: CD34, podocalyxin and endoglycan expression in vascular related cells.............. 40
Table 1.3: Expression patterns of podocalyxin, CD34 and endoglycan................................. 41
Table 2.1: Genotyping primers .............................................................................................. 62
Table 2.2: Real time qPCR primers ....................................................................................... 63
Table 2.3: Primary antibodies .............................................................................................. 65
Table 2.4: Secondary antibodies .......................................................................................... 66
Table 3.1: Genotyping primers for endoglycan conditional knockout mice ....................... 114
Table 3.2: Primary antibodies ............................................................................................ 116
Table 3.3: Secondary antibodies ........................................................................................ 117
Table 3.4: Mouse primer sequences for real-time quantitative PCR................................. 119
Table 3.5: Endoglycan antibodies ....................................................................................... 120
List of Figures

Figure 1.1: A schematic representation of the glycocalyx on the surface of endothelial cells. ................................................................................................................................................................................................. 10
Figure 1.2: Schematic of transport pathways in continuous endothelium ........................................ 12
Figure 1.3: Schematic representation of tight junctions and adherens junctions in endothelial cells ......................................................................................................................................................... 13
Figure 1.4: An integrated paradigm for functional and phenotypic plasticity of smooth muscle cells................................................................................................................................................................................................. 20
Figure 1.5: Model of how intussusceptive microvascular growth occurs ........................................... 25
Figure 1.6: Adult lung airway and alveolar progenitor cells ................................................................. 30
Figure 1.7: Proposed model for regenerative alveolarization mediated by proliferation of lung epithelial progenitors. ............................................................................................................... 32
Figure 1.8: Protein structure, genomic organization and splicing of the CD34 family ...................... 36
Figure 1.9: Proposed functions for CD34-type proteins based on published literature .................. 46
Figure 2.1: Targeted, conditional deletion of the Podxl locus ................................................................. 74
Figure 2.2: Analysis of podocalyxin expression in PodxlEC tissues ...................................................... 76
Figure 2.3: Histological analysis of podocalyxin expression in PodxlEC tissues ................................. 77
Figure 2.4: Gene expression and surface expression of endothelial cell markers in PodxlEC and PodxlEC Cd34−/− lungs .......................................................................................................................................................... 80
Figure 2.5: Basal and inflammatory induced vascular permeability in PodxlEC and PodxlEC Cd34−/− mice .................................................................................................................................................................................. 82
Figure 2.6: Alveolarization in PodxlEC and PodxlEC Cd34−/− mice. ......................................................... 85
Figure 2.7: Physiological analysis of PodxlEC and PodxlEC Cd34−/− mice ................................................... 87
Figure 2.8: Lung mechanical analysis of PodxlEC mice ........................................................................ 88
Figure 2.9: Lung mechanics of PodxlEC lungs .................................................................................... 89
Figure 2.10: MMP14 expression and lung proliferation by Ki67 staining ............................................. 91
Figure 2.11: Gene expression of matrix, adhesion and lung structure related genes in PodxlEC and PodxlEC Cd34−/− lungs ......................................................................................................................................................... 92
Figure 2.12: Second harmonic generation and multi-photon imaging .................................................. 94
Figure 2.13: \( Podxl^{EC} \) lung mEC have identical endothelial markers to \( Podxl^{\text{flox}} \) lung mEC 97
Figure 2.14: Adhesion and spreading of lung mECs on matrix ............................................. 99
Figure 2.15: Expression analysis of integrins involved in matrix binding ......................... 101
Figure 2.16: Histological analysis of CD34 expression in \( Cd34^{-/-} \) lung tissues .............. 108
Figure 3.1: Evaluation of endoglycan-reactive antibodies .................................................. 122
Figure 3.2: Analysis of endoglycan expression in tissue .......................................................... 124
Figure 3.3: Endoglycan is a marker of smooth muscle cells and not endothelial cells in the mouse .................................................................................................................................... 125
Figure 3.4: Immunocytochemistry demonstrated membrane localization of endoglycan on smooth muscle cells .............................................................................................................. 126
Figure 3.5: Endoglycan is upregulated with differentiation of SMC to a contractile phenotype and not activation by PDGF\( \beta \beta \) or LPS ............................................................... 128
Figure 3.6: Targeted deletion strategy for the endoglycan locus .......................................... 131
Figure 3.7: Genotyping of \( \text{Endgl}^{\text{flox}}/\text{Tagln-Cre} (\text{Endgl}^{\text{SMC}}) \) smooth muscle cells .......... 134
Figure 3.8: Endoglycan overexpression results in a spindle like-morphology ...................... 136
Figure 3.9: Endoglycan expression dampens PDGF\( \beta \beta \) induced smooth muscle migration 138
Figure 4.1: Levels of podocalyxin may explain the contradictory roles in cell adhesion ... 147
Figure 4.2: Model demonstrating two possible mechanisms for \( Podxl^{EC} \) mEC defective spreading on laminin ......................................................................................................................... 149
## List of Symbols & Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>Alveolar capillary dysplasia</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>ARDS</td>
<td>Adult respiratory distress syndrome</td>
</tr>
<tr>
<td>B6</td>
<td>C57Bl/6J</td>
</tr>
<tr>
<td>BPD</td>
<td>Bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CArG</td>
<td>CC(AT-rich)$_n$GG motif</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary diseases</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DTHL</td>
<td>Aspartate-threonine-histidine-leucine</td>
</tr>
<tr>
<td>ECGS</td>
<td>Endothelial cell growth supplement</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>En$_n$th</td>
<td>Embryonic day, where $n$ is the number of days</td>
</tr>
<tr>
<td>EPC</td>
<td>Circulating endothelial progenitor cell</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin, moesin</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Frt</td>
<td>Flp-recombinase target sequences</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin bound epidermal growth factor</td>
</tr>
<tr>
<td>HDMEC</td>
<td>Human dermal microvascular endothelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venules</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatic growth factor</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>KOMP</td>
<td>Knock-out mouse project</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-N^G^-nitroarginine methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LV+S</td>
<td>Left ventricle with septum</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>Lymphatic vessel endothelial receptor-1</td>
</tr>
<tr>
<td>mEC</td>
<td>Mouse primary endothelial cells</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>miR</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLI</td>
<td>Mean linear intercept</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPEF</td>
<td>Multi-photon excitation fluorescent microscopy</td>
</tr>
<tr>
<td>mSMC</td>
<td>Mouse smooth muscle cells</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvessel density</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvessel density</td>
</tr>
<tr>
<td>Neo^R</td>
<td>Neomycin resistance cassette</td>
</tr>
<tr>
<td>NHERF</td>
<td>Solute carrier family 9 (Na+/H+ exchanger), member 3 regulator</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>PTS</td>
<td>Proline, serine and threonine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real time qPCR</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHG</td>
<td>Second harmonic generation</td>
</tr>
<tr>
<td>sLeX</td>
<td>Sulfated sialyl Lewis-X</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>Smooth muscle myosin heavy chain</td>
</tr>
<tr>
<td>SM22α</td>
<td>Smooth muscle 22a</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>TCE</td>
<td>Transforming growth factor control elements</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>VVO</td>
<td>Vesicular vacuolar organelles</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona occludins</td>
</tr>
<tr>
<td>αSMA</td>
<td>Smooth muscle actin</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank all the people who have been essential for my success during the course of my graduate studies. First of all, I would like to thank my supervisor, Dr. Kelly McNagny for welcoming me to the lab, for all the support and guidance through the ups and downs, and giving me the opportunity to develop into an independent researcher. I would also like to acknowledge past and present members of my lab, particularly Dr. Poh Tan, Dr. Jami Bennett, Dr. Julie Nielsen, Dr. Sebastian G. B. Furness, Helen Merkens and Kay Jian for all their assistance, guidance and instruction to combat the curse of podocalyxin, and wish all the luck in the world to our newest graduate students Kim Snyder and Bernard Lo. I would like to particularly thank Dr. Michael Hughes for all the instruction and support through the technically challenging, the unanswered questions, and the successes.

Thank you to my committee, Dr. Cal Roskelley, Dr. Jamie Piret and Dr. Darryl Knight for their support and direction as my projects continually shifted throughout the years. Special thanks to Dr. Jody Wright for who took the time to explain the methods for evaluating lung pathology. Thanks also to all the faculty and members of the other labs in the building that were happy to share reagents, advice and assistance through the years. I would also like to thank the members of The Biomedical Research Centre, including core staff Les Rollings, Michael Williams, Andy Johnson, and Taka Murakami who are always willing to help out to further our research.

My sincerest gratitude goes out to my friends and family who have supported me through the years, even if they didn’t always understand what I was doing. To Dr. Krystle Veerman, my friend and fellow student at the BRC the Timmies runs and the courage to continue when nothing ever seemed to go right. To Dr. Leslie So Alfaro and Dr. Bahareh Ajami thanks for the sympathetic ear and the knowledge that there always is a light at the end of the tunnel. To my Guiding friends Agnes d’Entremont, Tammy Tromba, Crystal Bewsa, Adele Cooshek we’ve all gone through a lot in the last few years, but it’s amazing what we can get through when we’ve got great friends who like to play in the wilderness.
To my wonderful husband, Doug Debruin who convinced me to become the first Dr. Debruin in the family. I know when you promised to stand with me regardless of how long it took me to complete my PhD, you hadn’t quite figured on this long. Now its time for us to grow up and move forward to whatever life brings us.

This research was made possible by grants from CIHR and the Heart and Stroke Foundation of BC and Yukon to Dr. Kelly McNagny, as well as personal fellowships received from NSERC, UBC, and the CIHR/MSFHR Transplant Research Training Program.
To my Dad,
Chapter 1: Introduction

1.1 The vasculature
The cardiovascular system is the first system to develop within the embryo in order to provide the transport of both the nutrients and waste required to maintain growth and differentiation. The process starts by vasculogenesis; the de novo formation of primitive blood vessels from the differentiation of progenitor cells into endothelial cells (Risau and Flamme, 1995; Jin and Patterson, 2009). There are two progenitors cells that produce committed endothelial cells, the angioblast, which can only differentiate into endothelial cells and the hemangioblast that is able to differentiate into both hematopoietic cells and endothelial cells (Jin and Patterson, 2009). Vessels created by vasculogenesis are typically primitive and require remodeling in order to become mature vessels. As a result, much of the remaining vascular network is formed by angiogenesis, the growth of blood vessels from pre-existing vessels. Vessels produced by angiogenesis are more stable and produce the mature vascular network necessary for optimum growth and development. These vessels specialize into vessel types and are stabilized by the addition of matrix and mural cells in order to develop into the robust vascular structure found in the adult organism.

The vascular network in mammals requires many types of vessels to transport the blood and lymph throughout the body. The primary function of the blood is to provide gas exchange, deliver nutrients, as well as maintain the appropriate ionic milieu to cells within the tissues. Starting from the right side of the heart, deoxygenated blood is pumped into the lungs through thick walled arteries that maintain flow through pulsation. Arteries, because of the high pressures, have a defined structure starting with the endothelial cells that line the innermost part of the intimal layer, and are supported by matrix and mesenchymal cells. This is surrounded by the media layer, which is formed by elastic fibers and smooth muscle cells. The adventitia is the outermost layer, forming a protective cover of disorganized collagen, fat and fibroblasts. Within the lung, the blood flows in to arterioles and then the microvascular capillaries within the lung. Capillaries are essentially a rolled sheet of endothelial cells that form thin walled vessels with limited support from mural cells, such as pericytes, or the extracellular matrix. The capillaries are the primary location for the exchange of gas, soluble
factors, nutrients, hormones and other signaling molecules between the blood and tissue. The capillaries also control fluid content in the tissue. The oxygenated blood is then returned through the venous system, first through the post capillary venules and then to the veins to the left side of the heart. Post-capillary venules have micro valves to maintain flow, and are a key site for leukocyte trafficking during an immune response. Veins are thin walled vessels that maintain flow with valves rather than pulsation. Within the systemic circulation, the oxygenated blood is circulated to the various organs throughout the body via the arteries and arterioles, delivering the necessary oxygen, nutrients and other factors necessary for normal function, and removes the various waste products including carbon dioxide factors, before the blood is returned to the heart via the venous system. Meanwhile, the lymphatic system is responsible for draining fluid from the extracellular space, absorbing lipids and providing the transport of immune cells between lymphoid organs (Oliver and Srinivasan, 2010).

Two main cell types support each blood vessel; the endothelial cells that line the lumen of the vessel and the mural cells, which line the basal surface of the endothelial cells. These cell types are integrally linked to maintain the structure and function of the blood vessels for each tissue bed. While many intricate histological, biochemical, and biological studies of the vasculature have been completed to generate a large body of knowledge, this thesis will focused on two areas, namely microvascular endothelial cells and smooth muscle cells. This review will highlight the individual properties of each cell type as it relates to its structure and function, and their role homeostasis and disease.

1.1.1 Endothelial cells
Endothelial cells provide the inner cell lining of all vessels. Historically, these cells were thought to be inert, however closer study has found that endothelial cells are metabolically active. They produce a number of signals that are required for their own survival and function (known as autocrine signals) as well as factors that are required by surrounding tissue (known as paracrine factors). Endothelial cells are quite diverse and they differ between vascular beds throughout the growth and development of the organism. Endothelial cells can also have varied structure and function even within the same vascular bed, and each
type of endothelial cell can be differentially regulated in health and disease (Aird, 2007a, 2007b).

1.1.1.1 Defining endothelial cells

While endothelial cells are easily defined by anatomical location, defining endothelial cells by genetic markers is not straightforward. Classic endothelial cell markers, including platelet/endothelial cell adhesion molecule (PECAM or CD31), thrombomodulin, vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1), vascular endothelial cadherin (VE-Cadherin), endoglin-1, and Tie-2 are all expressed by other cell types, and not expressed by endothelial cells in all vascular beds. Of these markers, only VE-Cadherin is expressed in all vascular beds, but at varying levels (Minami and Aird, 2005). Therefore multiple markers must be used for rigorous identification of endothelial cells. In addition, many studies have identified specific lectins and genetic markers for different types of endothelium (Ruoslahti and Rajotte, 2000; Chi et al., 2003; Oliver and Srinivasan, 2010). Arteries are defined by a set of specific markers including ephrinB2 and delta-like ligand 4, while veins express ephrinB4 and COUP-TFII. Similarly, expression of Prox-1 and lymphatic vessel endothelial receptor 1 (LYVE-1) define the lymphatic endothelium (Gerety et al., 1999; Krebs et al., 2000; Gale et al., 2001; Podgrabsinska et al., 2002; You et al., 2005; Oliver and Srinivasan, 2010). Pulmonary alveolar capillaries also have a unique signature that includes expression of angiotensin-1 converting enzyme, aminopeptidase P, and molecules that bind Griffonia simplicifolia lectin, but unlike other capillary beds, do not express von Willebrand factor (vWF) (Pusztaszeri et al., 2006; Aird, 2007a). Thus, the diversity of endothelial cells lends to having markers for defined types or subsets of endothelial cells, rather than a master endothelial cell marker that can be used to identify all endothelia.

1.1.1.2 Endothelial-specific transgenic mice

Transgenic mice have become an important tool to facilitate our understanding of the cellular processes required for vascular development, homeostasis and repair. Unfortunately, early studies that used constitutive knockout mice to abrogate the expression of endothelial cell related genes often result in embryonic lethality, making it impossible to study the function
of these genes in vascular homeostasis and repair. The development of conditional transgenic mice has facilitated the temporal and spatial deletion of genes, as well as the visualization of gene expression using reporter strains. Unfortunately, the lack of one distinct endothelial cell marker has complicated the use of promoter driven transgenic mice to understand endothelial cell function. Close examination of “endothelial specific” conditional transgenic mice typically exhibit promoter activity that is only present in discrete cell subtypes, leading to incomplete expression or deletion in all vascular beds (Minami and Aird, 2005). Often, genes associated with endothelial cell function are not exclusive to endothelial cells, which leads to deletion in other cell types and complicates the analysis of the phenotype.

Currently there are a number of conditional endothelial specific transgenics that use the Cre recombinase system, the most common use the Cdh5 (VE-Cadherin) or Tek (Endothelial-Specific Receptor Tyrosine Kinase, also known as Tie2) promoters to drive expression. The Tek-Cre was the first endothelial specific transgenic Cre developed and has been widely used within the field to facilitate the study of endothelial cell function because it deletes early in development in a wide variety of tissues including the heart, lung, kidney, intestine and brain, but is also detectable in most hematopoietic cells (Constien et al., 2001; Kisanuki et al., 2001, 2010). However, the Tek-Cre is also expressed in the female germline, and without careful breeding will result in a constitutive-null mouse (de Lange et al., 2008). The Cdh5-Cre transgenic mouse has recently gained acceptance in the literature as an endothelial specific Cre with similar expression patterns to the Tek-Cre, but only deletes in 50% of hematopoietic cells and is not expressed in the germline (Alva et al., 2006). Both promoters have now been used to generate transgenics that facilitate temporal control, which is critical to circumvent the numerous genes that are essential during development (Forde et al., 2002; Monvoisin et al., 2006). In summary, care is required in the selection of these endothelial specific transgenic mice, and for each study, extreme diligence is required in the analysis of promoter activity to be certain that it is active in the tissue of interest.
1.1.1.3  Endothelial cell basement membrane components and interactions

In all vessels, interaction of endothelial cells with the basement membrane is essential for maintaining vessel structure and function. Fibronectin is the first matrix to be laid down during vasculogenesis, and this is remodeled to a basement membrane composed of multiple extracellular matrix (ECM) proteins, including laminins and collagen type IV, which are linked by nidogens, and heparin sulfate proteoglycans. Attachment of endothelial cells to the basement membrane is required for vessel integrity, barrier function and signal transduction of mechanical signals from endothelial cells to the supporting mural cells that make up the vessel wall (Hallmann et al., 2005). Furthermore, individual components of the ECM interact with endothelial cells to regulate proliferation, migration and differentiation. This is especially important in the initiation of angiogenesis where collagen type I and fibronectin are both produced as early matrix components to initiate growth of endothelial cells. Similarly, maturation of the vessel requires remodeling of the collagen type I and fibronectin matrix to the normal laminin and collagen type IV basement membrane components (Schultz and Wysocki, 2009).

Laminin is the most active component of the endothelial cell basement membrane proteins (Hallmann et al., 2005). There are 15 different isoforms of laminin composed of an $\alpha$, $\beta$ and $\gamma$ chain and expression is regulated by endothelial cell type and vascular bed depending on the needs of the tissue. There are two laminins that are produced by endothelial cells, laminin 8 and laminin 10. Laminin 8 is expressed by all endothelial cells regardless of their stage of development and is composed of $\alpha 4$, $\beta 1$ and $\gamma 1$ chains. Laminin 10 is composed of the $\alpha 5$, $\beta 1$ and $\gamma 1$ laminin chains, which is expressed in more mature vessels. The laminin $\alpha 4$ chain (laminin 8) can be upregulated by growth factors and cytokines induced by inflammation, while the $\alpha 5$ chain (laminin 10) requires potent inflammatory signals such as tumor necrosis factor (TNF-$\alpha$) to induce its increased expression. Deletion of the laminin $\alpha 4$ gene in mice results in hemorrhaging and poor vessel structure caused by defective formation of the basement membrane. Unfortunately, understanding the role of laminin $\alpha 5$ is hampered
by the fact that laminin α5 deficiency in mice results in embryonic lethality before vessel formation is initiated (Miner et al., 1998; Thyboll et al., 2002).

Endothelial cells interact with the matrix primarily through integrin receptors. Integrins are transmembrane adhesion molecules that facilitate cell-cell and cell-matrix interactions. They are composed of heterodimers of alpha and beta subunits that control ligand specificity (Campbell and Humphries, 2011; Fu et al., 2011; Kim et al., 2011). Most of our understanding of integrin/matrix interactions are based on classic integrins matrix pairings that give binding specificity for each matrix listed in Table 1.1 (Rüegg and Mariotti, 2003; Silva et al., 2008).

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Major ECM protein ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1</td>
<td>CO*, LM</td>
</tr>
<tr>
<td>α2β1</td>
<td>CO*, LM, VN, TN-C, TSP</td>
</tr>
<tr>
<td>α3β1</td>
<td>CO, LM*, FN, TSP, EL</td>
</tr>
<tr>
<td>α4β1</td>
<td>FN*, TSP</td>
</tr>
<tr>
<td>α5β1</td>
<td>FN*, TSP</td>
</tr>
<tr>
<td>α6β1</td>
<td>LM*</td>
</tr>
<tr>
<td>α6β4</td>
<td>LM*</td>
</tr>
<tr>
<td>α8β1</td>
<td>FN, VN, TN-C</td>
</tr>
<tr>
<td>αvβ1</td>
<td>FN, VN</td>
</tr>
<tr>
<td>αvβ3</td>
<td>FN, VN, FB, TSP, TN-C, vWF, OP, MMP-2</td>
</tr>
<tr>
<td>αvβ5</td>
<td>FN, VN</td>
</tr>
</tbody>
</table>

Table 1.1: Integrins expressed on endothelial cells and their extracellular ligands
Abbreviations: CO, collagen; EL, elastin; FB, fibrinogen; FN, fibronectin; LM, laminin; MMP, matrix metalloproteinase; OPN, osteopontin; TN-C, tenascin-C; TSP, thrombospondin; VN, vitronectin; vWF; von Willebrand factor. * Identifies classic integrin/matrix binding partners. Adapted from Rüegg and Mariotti, Cell. Mol. Life Sci. 60 (2003) 1135–1157.

Integrin signaling is important in both vascular development and angiogenesis. Mice lacking either the α or β chain of many integrins results in altered angiogenesis and defective vascular development. α5β1 deficient mice have distended blood vessels in the yolk sac and embryo, as well as mesodermal defects that result in early embryonic lethality (Francis et al., 2002). Mice deficient for αv or β8 have placental defects that result in 65-80% of the
embryos to die mid-gestation, while those that survive exhibit brain vascular defects that results in hemorrhaging and die perinatally (Bader et al., 1998; Zhu et al., 2002). α4β1 deficient mice also have placental defects resulting in perinatal death, but the vascular beds within the embryo are normal (Yang et al., 1995). This suggests that to maintain vessel integrity, endothelial cells require integrins to maintain continuous contact to the matrix. Endothelial cells also utilize integrins as survival factors to maintain adhesion to the ECM. Indeed, endothelial cells cultured on substratum that prevents adhesion or spreading are sufficient to induce apoptosis (Re et al., 1994). Taken together, these studies show that the interaction of endothelial cells with the ECM through integrins is important for endothelial cell survival and function.

1.1.1.4 Endothelial cell function

Endothelial cells are typically quiescent with a lifespan of approximately 1 year in humans; however, they can be activated to proliferate to form new vessels in response to damage or disease (Aird, 2007b). Endothelial cells play an active role in a number of processes including blood homeostasis, vessel constriction and tone, inflammation, and selective permeability (Aird, 2007b). Endothelial cells regulate blood homeostasis through their immense repository of pro and anti-coagulants that allow them to maintain control of thrombosis and fibrinolysis as well as mediating platelet adhesion and aggregation, especially during vascular damage. Vessel constriction and tone (known as vasomotion), while often thought to be the main function of smooth muscle cells, is regulated by the release of dilation and constriction factors by endothelial cells. Since vasoconstriction can be measured using non-invasive techniques, the inability to regulate vasomotion is now being used in the clinic as an early indicator of endothelial dysfunction (Verma and Anderson, 2002). Endothelial cells also play an active role in mediating inflammation through many mechanisms including the production of cytokines and regulation of leukocyte recruitment. While all these functions are required for optimum health, the studies outlined in this thesis will focus mainly on the ability of endothelial cells to regulate endothelial barrier function by altering vascular permeability.
1.1.1.4.1 Vascular permeability
The semi-permeable nature of the endothelium is the primary function of the microcirculation. This permits the selective exchange of molecules from the blood to the tissue. Small molecules required for basic survival of cells such as gasses, ions and small solutes including glucose, and urea are able to pass freely between cells through passive diffusion. However, larger proteins such as plasma proteins including albumin are restricted in their passage. The filtration of plasma proteins and water is defined by the imbalance of hydrostatic conductivity, diffusion and osmotic pressure differences best described by Starling’s equation (Johnson, 2003; Nagy et al., 2008). This selective permeability regulates the osmotic gradient necessary to maintain fluid homeostasis. However, changes in the number of capillaries perfused, capillary pore size, and the resistivity to the protein in question, along with changes to blood pressure within the arterioles and veins, fluid levels within the interstitium, and the concentration of proteins within the plasma or tissue are all sufficient to alter vascular permeability within a given tissue (Johnson, 2003).

1.1.1.4.2 Structural heterogeneity of endothelial cells required for barrier control
Endothelial cells, from each vascular bed and even within the same tissue, maintain barrier function differently depending on the type of the vessels, the size of the vessels and the tissue environment. Veins and post-capillary venules inherently have increased permeability over other types of vessels, and are the first to alter permeability in response to inflammation (Aird, 2007a). Arterial endothelial cells, by contrast, are aligned by flow and contain numerous junctions in order to maintain high-pressure flow with limited basal permeability (Tarbell, 2010). Arterial endothelial cells can also increase their permeability through the increase of intracellular calcium stores and release of nitric oxide in response to shear stress caused by changes in blood flow (Gebb and Stevens, 2004). Interestingly, the study of vascular permeability has been facilitated by the fact that the molecules and structures that regulate vascular permeability in each vascular bed are maintained after culture in vitro.

Endothelial cells in capillaries can be defined as discontinuous, continuous, or fenestrated depending on the needs of the tissue (Aird, 2007b). Discontinuous endothelium, found only
within the liver sinusoids, has large fenestrae without membranes and large gaps between cells, while fenestrated endothelium is typically found in organs that require increased transport or filtration such as in the kidneys, intestinal mucosa as well as exocrine and endocrine organs. Fenestrae are 70µm transcellular pores that maintain a membrane over the pore and facilitate the transport of macromolecules. The permeability to solutes is proportional to the number of fenestrae present on the cell surface. Continuous endothelium is present in the lungs, heart and skin, and possess numerous caveolae to transport macromolecules. In comparison to other endothelial cells, continuous endothelial cells have increased barrier properties as measured by transendothelial electrical resistance, and display a 100-fold increased resistance to water permeability compared to large vessels (Blum et al., 1997; Gebb and Stevens, 2004). In summary, these structural features are required for the control of barrier function to the specific needs of each vascular bed.

1.1.1.4.3 Regulation of basal permeability

Within tissues, the capillaries are responsible for controlling basal permeability. Continuous endothelium, especially in the lung, maintains barrier function through 3 mechanisms (Michel and Curry, 1999; Mehta and Malik, 2006). These include 1) the glycocalyx on the surface of the endothelium, 2) the transcellular pathway that consists of the vacuoles and vesicle transport mechanisms and 3) the paracellular pathway composed of interendothelial junctions.

The glycocalyx

The glycocalyx has been described as a negatively charged fiber matrix on the surface of endothelium comprised of membrane-bound glycoproteins, proteoglycans and glypicans that are modified with multiple side chains of chondroitin sulfate, glycosaminoglycan, heparin sulfate, and hyaluronic acid (Fig 1.1) (Michel and Curry, 1999; Pries et al., 2000). In addition, there is a dynamic outer layer of proteins that are deposited from the plasma that together extend up to 200nm into the vessels lumen. The glycocalyx provides the primary interface between the blood and endothelial cells reducing the resistance of blood flow in
small vessels. It also limits the interactions of inflammatory cells with the receptors on the endothelial surface.

Figure 1.1: A schematic representation of the glycocalyx on the surface of endothelial cells. Transmembrane syndecans are shown to cluster in the outer edge of caveolae, and can interact with cytoskeletal elements (A) and intracellular signals when oligomerized (B). Syndecans also contain chondroitin sulfate modifications in the membrane proximal regions of the core protein (green dotted lines). A glycoprotein with its short oligosaccharide branched chains and their associated Sialic Acid ‘caps’ are displayed in the middle part of the figure (green). Caveolin-1 associates with lipids in the membrane (darker circles, left), and forms cave-like structures or caveolae (right). Molecules involved with endothelial nitric oxide synthase signaling localize in caveolae (C). Glypicans, along with their heparin sulfate chains (blue dotted lines) localize in these regions. Transmembrane CD44, a link-domain adhesion molecule, localizes to caveolae and can have chondroitin sulfate, heparin sulfate and oligosaccharides attached to it. Hyaluronic acid or hyaluronan is a very long glycosaminoglycan (orange dotted line), which weaves into the glycocalyx and binds with CD44. Plasma proteins (grey), along with cations and cationic amino acids (red circles) are known to associate with glycosaminoglycans. Reprinted from Tarbell J M, Shear stress and the endothelial transport barrier. Cardiovascular Research 2010; 87(2): 320-330 by permission of the European Society of Cardiology.

The glycocalyx regulates barrier function by controlling the transport of macromolecules to the endothelial surface. In microvessels, the glycocalyx is the primary barrier to plasma proteins, particularly albumin. Most circulating albumin carries a negative charge that is repelled by the negative charge of the glycocalyx. Disruption of the glycocalyx is sufficient to increase albumin permeability 2-3 fold. This can occur by treatments that cleave the negative side chains on proteins and lipids (including neuraminidase, pronase, heparinase or proteolysis of hyaluronan), or by neutralizing the negative charge by protamine or cationic
ferratin (Pries et al., 2000; Mehta and Malik, 2006; Becker et al., 2010). The glycocalyx can also respond to shear stress and alter barrier function. The hyaluronic acid and heparin sulfate chains, in particular, are sensitive to high shear stress and trigger the release of nitric oxide to increase permeability. How this is regulated mechanistically is still unknown (Tarbell, 2010).

**The transcellular pathway**

The endothelial cell layer controls fluid homeostasis by mediating the passage of albumin from the plasma into the interstitial tissue space through the transcellular pathways (Fig 1.2). Albumin also acts as a shuttle for hormones, enzymes and other hydrophobic molecules from the plasma into the tissue. Albumin transport, especially in microvessels, occurs through caveolae trafficking from the surface to the interstitial space. Gp-60 functions as a docking molecule within the caveolae, and activation of gp-60 is sufficient to increase albumin permeability 2 fold with no changes in interstitial water content (Tiruppathi et al., 1996, 1997). There are also specific receptors for the transport of water, the aquaporins, which are responsible for up to 40% of water flux. Additionally, vesicular vacuolar organelles (VVOs) present in endothelial cells of venules and tumor vasculature can be activated by vascular endothelial growth factor (VEGF) to provide a channel for solutes and fluids from the surface to both the lateral and basal surface of endothelial cells. Therefore, the transcellular pathways are important mechanisms to control the transport of albumin, plasma proteins and water in maintaining basal permeability.
Figure 1.2: Schematic of transport pathways in continuous endothelium.

Under basal conditions, the transcellular pathway can mediate the transport of plasma proteins (>3 nm) such as albumin by caveolae via an absorptive (receptor-mediated) or fluid-phase pathway. Transcellular channels can also form transiently in endothelial cells by fusion of multiple caveolae and allow albumin transport. Aquaporins form channels across the lipid bilayer that are highly selective for water molecules and allow their movement across the luminal or abluminal endothelial membrane, thus creating a transendothelial pathway for water. Small molecules including urea and glucose (<3 nm $M_r$) are transported around individual endothelial cells via the paracellular, i.e., interendothelial junction (IEJ) pathway. Reprinted from Mehta D, Malik AB, Physiol Rev 2006;86:279-367.

The paracellular pathway

The paracellular pathway, which is controlled by interendothelial junctions, plays a minor role in maintaining basal permeability (Fig 1.3). There are 2 major types of endothelial cell junctions, adherens junctions and tight junctions. Adherence junctions in endothelial cells are the first junctions to be formed in development, and consist of VE-Cadherin and catenins. VE-Cadherin on adjacent cells is responsible for cell-cell contact, while the catenins facilitate connection between VE-cadherin and the actin cytoskeleton. VE-Cadherin is important in regulating vascular permeability (Dejana et al., 2009). Inhibition of VE-Cadherin using blocking antibodies causes defects in cell-cell and cell-matrix interactions resulting in the exposure of the extracellular matrix and increased vascular permeability in vivo (Dejana et al., 2009). Tight junctions in endothelial cells are composed of occludins and claudins, and are connected to the actin cytoskeleton by zona occludins. Tight junctions are present in higher frequency in arterial vessels over venules, and are sufficient to block water and
macromolecule transport. However, there are limited data to explain the direct role of tight junctions in regulating basal permeability. Taken together, this data highlights that intracellular pathways facilitate basal permeability by restricting fluid movement and providing an impenetrable barrier to albumin.

Figure 1.3: Schematic representation of tight junctions and adherens junctions in endothelial cells

In endothelial cells, adhesion at tight junctions is mediated by claudins, occludins, members of the JAM family, and ESAM. The cytoplasmic components of tight junctions are ZO proteins, cingulin, ZONAB, and others. At adherens junctions, adhesion is promoted by cadherins (VE-cadherin and N-cadherin), which directly bind to p120, β-catenin, and plakoglobin. Nectins and their intracellular partner afadin/AF-6 participate in the organization of both tight and adherens junctions. A large set of actin binding proteins have been found to be associated to adherens junctions such as α-catenin, vinculin, α-actinin, eplin, and others. In addition, phosphatases (DEP-1, VE-PTP, PTPμ, SHP2, etc.) and kinases (src, csk, and others) are directly or indirectly associated to adherens junction components. Growth factor receptors: VEGF receptor 2 (also called flk-1 or KDR) and TGFβ receptor complex could bind to VE-cadherin complex. This interaction modulates their signaling properties. Reprinted from Developmental Cell, Volume 16, Issue 2, Elisabetta Dejana, Elisabeth Tournier-Lasserve and Brant M. Weinstein, The Control of Vascular Integrity by Endothelial Cell Junctions: Molecular Basis and Pathological Implications, 209-221, © 2009 with permission from Elsevier

Nonetheless, the paracellular pathway is the key regulator of vascular permeability induced by extrinsic factors such as mechanical stress, inflammation, and angiogenesis. As a result, much of our knowledge of the paracellular pathway comes from studying the effects of mediators of vascular permeability. While, many of the various factors that initiate vascular
permeability work through similar pathways, I will focus on inflammation-induced permeability, as a model in our studies.

1.1.1.4.4 **Inflammation-induced permeability**

In contrast to basal permeability, inflammation induced permeability is caused primarily by changes in the interendothelial junctions leading to the transport of macromolecules and facilitating immune cell migration. Inflammation releases many of the classic mediators of permeability including VEGF, thrombin, histamine, bradykinin, reactive oxygen species, and TNF-α (Orfanos et al., 2004; Dejana et al., 2009; Bates, 2010). These factors are able to induce increased permeability by phosphorylating VE-cadherin, which results in its internalization and disassembly of the adherens junctions (Dejana et al., 2009; Bates, 2010). Through their specific receptors, these factors can also activate release of intracellular calcium stores or phosphorylate myosin light chain (MLC), affecting the actin cytoskeleton to induce junctional gaps (Mehta and Malik, 2006).

Inflammation or angiogenic factors also alter the interactions of endothelial junction proteins and integrin receptors with the actin cytoskeleton (Dudek and Garcia, 2001; Komarova and Malik, 2010). The Rho GTPase family, namely Rho, Rac1 and Cdc42, act as the connection between the agonist-induced signaling and the actin cytoskeleton, junction proteins and integrins to induce the ultrastructural changes that result in aberrant cell-cell and cell-matrix contacts. For example, in response to VEGF, VEGFR signals through Rac1 to generate fenestrations and increase numbers of actin bundles, leading to cell retraction and unstable adherens junctions, which results in the increased permeability. Rac1 also results in the phosphorylation of MLC and MLC kinase (MLCK), which in turn generates stress fibers and contractile complexes, resulting in increased cell contraction and increased permeability (Tapon and Hall, 1997). Interestingly, activation of Cdc42 does not occur during the induction of vascular permeability, but rather during the repair of barrier function *in vivo* (Maniatis et al., 2008b). In summary, extrinsic factors are able to activate pathways which cause decreased contractile forces between cells and decreased adhesive forces in cell-matrix contacts resulting in increased vascular permeability.
1.1.1.4.5 Acute lung injury

ALI is a clinical malady that is characterized by defective gas exchange and respiratory failure as a result of non-cardiogenic pulmonary edema, and can be caused by a number of insults to the lung (Orfanos et al., 2004; Maniatis et al., 2008a). These include chemical damage; infectious and inflammatory damage, as a result of conditions such as pneumonia or sepsis; or mechanical damage, as a result of ventilation. Much of our understanding of inflammatory permeability in the lung has been gleaned through clinical experience and experimental models of acute lung injury (ALI), such as the administration of endotoxin, in vivo.

Hallmarks of ALI include increased vascular permeability, along with the influx of neutrophils and elevated cytokine production (Maniatis et al., 2008a). Neutrophils are necessary for the production of many of the factors that induce vascular permeability. These include the production of reactive oxygen species and proteases, which in turn lead to exposure of the basement membrane and activate thrombin release and the clotting cascade. Furthermore, neutrophil binding to endothelial cells is sufficient to disrupt of adherens junctions, rearrangement of the actin cytoskeleton and increased formation of junctional gaps that permit plasma leakage and facilitate neutrophil migration (Orfanos et al., 2004).

The alveolar epithelial barrier is also an important component in ALI (reviewed in Matthay et al., 2002; Sartori and Matthay, 2002; Kim and Malik, 2003). Under normal conditions, alveolar epithelial cells provide the primary barrier to solutes in the lung and prevent fluid from the vascular and interstitial spaces from entering the airways. Tight junctions within the epithelium provide significant resistance to the transport of solutes, as the pores between epithelial cells that allow for the diffusion of water-soluble molecules are 10 times smaller than those in endothelial cells (Taylor and Gaar, 1970). In the lung, the fluid balance controlled by the epithelial barrier, is regulated by ion transport mechanisms rather than the balance of hydrostatic and osmotic pressures as suggested by Starling’s Law (Matthay et al., 2002). The transport of sodium from the apical to basal surface of epithelial cells stimulates the osmotic water transport necessary for the removal of fluid from the airspaces. In ALI, pulmonary edema is typically caused by defects in vascular permeability, and patient
outcome is reliant on the ability of the epithelial cells to transfer fluid out of the airspaces (Sartori and Matthay, 2002). In many models of ALI, such as endotoxemia, neutrophils and monocytes are recruited to the lung and are able to migrate across the epithelial barrier. Through this process, the epithelial barrier undergoes only mild damage with no changes to fluid or protein transport into the airways (Berthiaume et al., 2002). In addition, septic shock can induce the release of catecholamines and cytokines, which in turn increase sodium and fluid transport such that fluid clearance is initiated within 12 hours. However, if the endotoxemia becomes systemic, the paracellular pathways are altered and the epithelial cells are damaged to the point at which they can no longer maintain fluid transport, thereby increasing the incidence of morbidity (Sartori and Matthay, 2002). Therefore both the endothelial and epithelial cells play critical roles in maintaining barrier function within the lung.

1.1.1.4.6 The study of barrier function and vascular permeability

In the literature, there are two different perspectives to define and measure barrier function and vascular permeability. Physiologists tend to define vascular permeability based on fluid and solute transfer as defined by Starling’s law, and therefore measure the properties of diffusion and hydraulic conductivity across a single vessel to quantify changes in permeability (Nagy et al., 2008). This method can detect subtle changes in permeability because of the “closed” nature of the system. Vascular biologists, on the other hand, view vascular permeability as the transport of a single solute, usually albumin, into the interstitial tissue in response to an agonist or pathology. Correspondingly, their measurements typically occur over a whole tissue, where the variability of measuring over a variety of vessel types and alterations in blood flow caused by the stimuli are significantly increased (Nagy et al., 2008). Therefore, changes in permeability measured in this manner must be detectable over the inherent noise of the system.

In vivo, the most common measurement of vascular permeability uses a variation of the Miles assay (Miles and Miles, 1952). This assay relies on the affinity of Evans Blue dye for albumin, and measures dye accumulation in the tissue as a surrogate marker of albumin
extravasation. Overall, the assay is reproducible and inexpensive, especially when the appropriate controls are included. However there are a number of caveats to the assay. First of all, it is best used in tissues, such as the skin, where the vascular volumes are small relative to the amount of dye, but this caveat can be mitigated somewhat by effective perfusion of the vessels before dye extraction (Nagy et al., 2008). Furthermore, visualizing changes in permeability using Evans Blue alone, one cannot take into account differences in albumin bound to the surface of vessels or the vessel dilation that is often caused as a result of most antagonists (Bates, 2010). Therefore, new methods include the use of dual fluorescent tracers with different sizes to measure both the plasma volume and albumin leakage. Additionally, the use of novel imaging techniques such as magnetic resonance imaging, microPET and confocal imaging are currently being tested to allow experimental testing on the same animal under both control and test conditions as well as for longitudinal studies (Curry and Adamson, 2010).

Much of our understanding of permeability inducing agents has come from the use of in vitro assay systems. The most common assay is to measure the flux of dye across static cultures of confluent monolayers grown on a transwell chamber, however, like most in vitro systems; there are a number of limitations. Cultured endothelial cells, whether they are from large or small vessels, are inherently more leaky in culture, have limited number of vesicles, and may or may not maintain the glycocalyx (Nagy et al., 2008). Thus, they do not possess many of the key structures that regulate the transport of solutes in vivo. Additionally, cultured endothelial cells are slower to react to permeability agents. For example, VEGF-A is able to mediate increased permeability within minutes in vivo, however cultured endothelial cells require hours to demonstrate a similar increase. As a result, Nagy et al. suggested that cultured endothelial cells offer a more robust model of vascular permeability under pathological conditions (such as during tumor vessel growth or in vessels exposed to chronic inflammation) rather than as a model for basal permeability (Nagy et al., 2008). Finally, barrier function in vitro is typically regulated by contractile mechanisms rather than by interendothelial junctional complexes, or transcellular pathways as seen in vivo. Nevertheless, this artificial caveat of cell culture, has allowed us to better understand the importance of cell-matrix adhesion and cell-cell contractile forces in vascular permeability.
In summary, it is clear that the use of both \textit{in vivo} and \textit{in vitro} assay systems can facilitate the delineation of specific actions of factors that modulate vascular permeability, but must they must be analyzed in the correct context to be able to truly understand their function.

1.1.2 Smooth muscle cells

“Mural cells” is a broad term used to describe the pericytes and smooth muscle cells that interact with the endothelial cells, depending on blood vessel size. Pericytes form a discontinuous single layer around the endothelium and have direct contact to endothelial cells through gap junctions. Vascular smooth muscle cells (vSMC), in contrast, are found in association with any vessel larger than 20 microns and are separated from the endothelium by the basement membrane and interstitial cells that compose the intimal layer. Currently, pericytes and vSMC are considered to be of the same lineage, and there is a growing body of evidence that these perivascular cell are actually mesenchymal stem cells. This includes the fact that there are no definitive markers that can distinguish pericytes from vSMC or mesenchymal cells (Armulik et al., 2005, 2011; Gaengel et al., 2009). Regardless of nomenclature, while these cells have become of high interest due to their role in vascular development and angiogenesis, in pathological conditions such as cancer and fibrosis, and as a putative supply of progenitor cells, my focus in this thesis will be on vSMC.

1.1.2.1 Defining smooth muscle cells

Adult vSMC are remarkably quiescent and provide structural support for large vessels. They are also known for their functional role in controlling blood pressure and blood flow throughout the vascular system by regulating vessel contraction and tone. Like endothelial cells, vSMC are incredibly diverse. During development, vSMC originate from 8 different embryonic tissues to form the vascular network (Majesky, 2007). Mapping techniques have shown that segments from the same vessel develop from distinct sources of progenitors with sharp boundaries that do not mix. For example, mapping the full length of the aorta has demonstrated that progenitors from the neural crest form the aortic arch and ascending aorta, progenitors derived from the somites contribute to the thoracic descending aorta, and progenitors from the splenic mesoderm are responsible for the formation of the abdominal
aorta. In addition, progenitors from the secondary heart field, pro-epicardium and mesothelium as well as various stem cells and mesangioblasts all contribute to form the smooth muscle cells within the vascular network (Majesky, 2007).

Surprisingly, even with these diverse origins, all vSMC express a similar repertoire of proteins necessary for contraction, including contractile proteins, ion channels, and signaling molecules (Owens, 1995). Differentiated, “contractile”, vSMC can be identified based on their expression of a panel of contractile proteins including smooth muscle α-actin (αSMA), smooth muscle myosin heavy chain (SM-MHC), h-caldesmon, h1-calponin, smooth muscle 22a (SM22α), desmin, and smoothelin, among others. SM-MHC is the most restricted marker of vSMC, as non-vSMC such as myofibroblasts will not express SM-MHC, even during pathological conditions such as wound healing (Owens et al., 2004). However, many of these proteins are not uniquely expressed in vSMC. For example, while αSMA is an excellent marker for vSMC differentiation, it can be upregulated under specific conditions by a variety of cells; including endothelial cells, fibroblasts, and skeletal and cardiac muscle. Therefore, rather than lineage markers that define the cell type, these proteins should be used as an index of the differentiation state of vSMC (Owens et al., 2004).

1.1.2.2 Smooth muscle cell plasticity

The diversity of vSMC is an evolutionary conserved mechanism to carry out a variety of functions including vascular development, maturation and repair. As a result, vSMC defy all conventional thoughts regarding cell differentiation, and instead display a spectrum of phenotypes throughout their lifetime that are generally described as “synthetic” or “contractile” (Owens, 1995; Halayko and Amrani, 2003; Owens et al., 2004). In development, vSMC are considered to be synthetic, as they have increased levels of proliferation, migration, and production of ECM proteins to support vascular development. As the vasculature matures, vSMC differentiate into the highly contractile, quiescent, fully differentiated/mature cell type for which vSMC are known for (Fig 1.4). Unfortunately this diversity can be corrupted during disease or in response to injury or disease, such that contractile vSMC can undergo phenotypic switching towards a “synthetic” phenotype in
response to local environmental cues. However, this switching can be manifested as any combination of components that are considered part of the synthetic phenotype depending on the signals provided by the environment. In addition, cells harvested from the aortae of multiple species can manifest as two vSMC morphologies; spindle-shaped cells that express high levels of differentiated vSMC markers, and epithelioid cells that have increased migration and proliferation and are more susceptible to pathological changes (Hao et al., 2003).

Figure 1.4: An integrated paradigm for functional and phenotypic plasticity of smooth muscle cells.

This schematic representation shows the association between phenotypic and mechanical plasticity in smooth muscle. Phenotypic plasticity results from reversible modulation and maturation of smooth muscle cells between a functionally ‘synthetic’ and ‘contractile’ state. This diversity is chiefly controlled through regulated expression of specific genes in response to a number of factors that can include pro-inflammatory stimuli, changes in ECM, and cell–cell interactions. Mechanical plasticity occurs in contractile myocytes as the result of subcellular reorganization of the contractile thick and thin filaments in response to acute or chronic muscle length change during contraction or due to stretch. Reprinted from Respiratory Physiology & Neurobiology, Volume 137, Issue 2-3, Andrew J. Halayko & Yassine Amrani, Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma, 209-222, © 2003 with permission from Elsevier

Defining vSMC phenotype by gene expression is also incredibly difficult. In development, vSMC express immature genes such as Smemb, but also express moderate levels of most vSMC contractile proteins (Owens et al., 2004). As the vasculature matures, the immature genes are downregulated and contractile proteins are upregulated to support vasomotion. However, contractile vSMC that undergo phenotypic switching, especially in atherosclerotic lesions, will typically downregulate the expression of most contractile genes (Hao et al.,
2003; Owens et al., 2004; Fisher, 2010). Therefore, although synthetic vSMC typically express lower levels of contractile proteins than contractile vSMC, these data emphasize the diversity in gene expression that accompanies the spectrum of vSMC phenotypes.

The diversity of vSMC phenotypes is regulated by a combination of genetic modifiers and environmental cues. Genetically, expression of vSMC contractile genes are regulated at the transcriptional level by the co-factors, myocardin and serum response factor (SRF), both of which bind to the CC(AT-rich)$_6$ GG (CArG) elements present in almost all vSMC contractile genes (Owens et al., 2004; Rzucidlo et al., 2007; Majesky et al., 2011). Expression of vSMC genes also requires the appropriate chromatin changes to generate the permissive state required for transcription. In addition, microRNAs (miR) control vSMC phenotypes. MicroRNAs are able to bind to complementary sequences within messenger RNA that leads to either their degradation or a block in their translation, which results in post-transcriptional regulation of gene expression. Expression of miR-143 and miR-145 are required for vSMC differentiation, and mice deficient for both exhibit thin artery walls and reduced blood pressure, suggesting changes in vSMC maturation or differentiation (Cordes et al., 2009; Elia et al., 2009).

Differentiation of vSMC can also be regulated by a number of environmental factors including local levels of transforming growth factor (TGF-$\beta$), mechanical forces, contractile agonists, contact with mature vascular basement membrane ECM, and contact with endothelial cells. TGF-$\beta$ regulates a number of processes in vSMC, including the control of growth, differentiation and matrix synthesis. Treatment of cultured vSMC with TGF-$\beta$ enhances SRF binding to the CArG domain in addition to an unknown factor that binds to the TGF-B control element (TCE), and leads to contractile protein transcription and vSMC differentiation (Hautmann et al., 1997). The basement membrane ECM also plays an important role in vSMC differentiation. Culture of vSMC on laminin or collagen type IV is sufficient to induce differentiation (Thyberg and Hultgårdh-Nilsson, 1994). Similarly, vSMC contact with endothelial cells will induce differentiation of vSMC, as will conditioned media from endothelial cell/vSMC co-cultures. The soluble factor(s) responsible for this differentiation has not yet been identified (Powell et al., 1996; Fillinger et al., 1997).
Growth factors, cytokines, and inflammatory mediators can stimulate the phenotypic switch of contractile vSMC to a synthetic phenotype, which in turn leads to proliferation and migration of cells that are normally quiescent. Platelet derived growth factor (PDGF) was the first growth factor defined to mediate phenotypic switching. Culturing post-confluent vSMC with PDGF-ββ is sufficient to suppress vSMC differentiation and downregulate a number of contractile proteins including αSMA and SM-MHC (Holycross et al., 1992) through transcriptional repression. PDGF-ββ phosphorylates Elk-1, which competitively binds to SRF, thereby inhibits myocardin binding and the transcription of vSMC contractile genes (Kawai-Kowase and Owens, 2007). During injury or disease, PDGF-ββ produced by endothelial cells, is sufficient to induce proliferation and migration of vSMC. In development, PDGF-β interacts with its receptor PDGFR-β on vSMC to recruit vSMC to developing vessels, and loss of either PDGF-B or PDGFR-β results in defective vessel formation (Majesky et al., 1990; Armulik et al., 2005). Inflammatory mediators, such as LPS also induce phenotypic switching by the transcriptional downregulation of αSMA in a TCE-dependent manner (Sandbo et al., 2007). Similarly, during atherosclerosis, inflammatory mediators and growth factors released by macrophages are sufficient to initiate phenotypic switching, resulting in modified vSMC migration into the subintimal space and synthesis of ECM proteins such as collagen, which contribute to the fibrous cap of the atherosclerotic plaque (Owens et al., 2004; Rzucidlo et al., 2007).

In vitro culture of vSMC has offered the opportunity to study vSMC phenotypic switching. Under normal culture conditions, vSMC possess a synthetic phenotype, including the propensity for proliferation and migration, and express a moderate level of contractile proteins, similar to immature vSMC. vSMC can be induced to adopt a contractile phenotype, simply by culturing these cells to confluence followed by serum starvation for several days. This leads to significantly increased expression of contractile proteins, and decreased proliferation and migration. Similarly, factors can be added to the differentiated cells to understand the mechanisms and cell behaviours associated with phenotypic switching in vitro.
1.2 Pulmonary development and disease
The lung provides the essential function of gas exchange between the environment and the body. During inhalation, the airways transport the incoming environmental air to the alveoli, where the air-blood barrier facilitates the exchange of oxygen into the blood stream and release carbon dioxide from the blood. Carbon dioxide is then exhaled back into the atmosphere with the remainder of the air. As an environmental interface, the lung is also under constant assault from inhaled particles and gasses, microorganisms, and other environmental stimuli. This requires constant surveillance by the immune system to detect exogenous insult, and appropriate clearance of damaged or dead cells. In this section I will highlight the research that has catalogued the development of the lung and its subsequent maintenance, and how the vasculature plays a critical role in mediating these processes.

1.2.1 Lung development
Lung development is a tightly regulated process requiring the coordination of airway morphogenesis and alveolarization along with vasculogenesis and angiogenesis (Thurlbeck, 1975; Burri, 1984, 2006; Warburton et al., 2000). In the mouse, the first stage of lung development, known as the pseudoglandular stage (E12-E16.5), leads to the formation of the conducting airways and the primitive primary septa by branching morphogenesis. The formation of the airways and septa continues through the canalicular stage (E16.5-E17.5), and coincides with the appearance of the air-blood barrier. During the saccular stage (E17.5-P4), the alveolar “lacy” appearance begins to emerge through the differentiation of type I and II epithelial cells to form thick walled saccules, as well as the loss of the mesenchymal interstitial tissue. The stage from postnatal day 4 through postnatal day 36, termed “alveolarization” coincides with the growth of new septa that expand into the distal spaces to form the smaller alveoli required for function in the adult. This process produces 90% of the alveolar surface area observed in adulthood (Mund et al., 2008). Alveolarization continues to occur in juveniles in the distal parenchyma, albeit at a much slower pace, along side the linear growth necessary to sustain the gas exchange functions of the growing animal.

The development of the pulmonary vasculature goes hand in hand with the epithelial development of the lung. However, less is known about the mechanisms governing
pulmonary vascular development (Galambos and deMello, 2007; Thébaud and Abman, 2007). One of the prevailing theories is that the pulmonary arteries are produced by angiogenic sprouting of the dorsal aorta into the lung bud, while the capillaries and vessels formed in the distal regions of the lung bud are generated by vasculogenesis. These primitive vessels are then remodeled and fuse with the pulmonary vessels to complete the circulation (Galambos and deMello, 2007). Others have suggested that vasculogenesis is the primary mechanism of vascular development in the lung, and this would agree with the general observations that continuous circulation of the lung occurs as soon as embryonic circulation is initiated (Schachtner et al., 2000). However, as a rule the pulmonary arteries develop alongside the bronchial tree, while the veins develop along the septa. Little is known about the mechanisms surrounding the formation of the air-blood barrier, but it is likely that this occurs through interactions between the endothelium and the epithelium during the saccular stage as each future airway is surrounded by a capillary layer (Burri, 1999; Schittny and Burri, 2004). Currently, there is limited research into the factors responsible for lymphatic development within the lung, other than it is completed before birth (Galambos and deMello, 2007).

Once the basic vascular tree has developed into a continuous circulation, the capillary network in the distal airspaces expands during the saccular stage to form a capillary layer around each airway. This becomes a double capillary layer as the airway tissue that will become the alveoli move toward each other (Schittny and Burri, 2004). As the new septa are formed during alveolarization, one of the capillary layers folds up along the edges of the septa to maintain the double capillary layer within the thick septum. During this stage, the capillary volume must expand 30-fold in order to develop sufficient surface area to maintain the air-blood barrier within the alveoli.

Therefore, concomitant with the alveolarization and expansion of the septa, from postnatal day 14 through day 21, the pulmonary vasculature undergoes expansion through sprouting and intussusceptive angiogenesis. Sprouting angiogenesis is important for the production of new vessels, where by angiogenic factors such as VEGF produced by the septa initiates the degradation of the vascular basement membrane, proliferation of existing endothelial cells,
and migration of these newly formed sprout cells to form new vessels. The rapid expansion of the lung also requires intussusceptive angiogenesis to mediate the expansion of the capillary network without additional proliferation. Intussusceptive angiogenesis forms transcapillary pillars within the vessel lumen to facilitate the addition of new meshes from existing vessels (Fig 1.5) (Burri and Djonov, 2002). While the addition of the transcapillary pillars often splits an existing vessel in two, the pillars can be formed by multiple mechanisms to facilitate vessel fusion, branching, and remodeling.

Figure 1.5: Model of how intussusceptive microvascular growth occurs

Various modes of pillar formation as observed in the lung an in the CAM. Panels 1 and 2 represent longitudinal sections of transcapillary pillars, 3–5 are cross-sections through the pillars. Arrows show sites of thinning and perforation of endothelial leaflets; second picture for each case illustrates location of interendothelial junctions.

Reprinted from Molecular Aspects of Medicine, Vol 6-supplement, Peter H. Burri and Valentine Djonov, Intussusceptive angiogenesis – the alternative to capillary sprouting, 1-27, ©2002, with permission from Elsevier

The ability of intussusceptive angiogenesis to participate in branching and remodeling also facilitates the final stage of lung development, which requires the vascular network to mature from a double capillary layer to a single capillary layer at the centre of a septum (Schittny and Burri, 2004; Mund et al., 2008). Once the vascular network has matured, the capillary network continues to expand with normal lung growth, although at a much higher volume ratio than that of the airspaces or tissue (Schittny and Burri, 2004).
1.2.2 Lung pathologies

Abnormal vessel growth can contribute to pediatric lung diseases (Galambos and deMello, 2007; Thébaud and Abman, 2007). Bronchopulmonary dysplasia (BPD) is a multi-factorial disease in pre-term infants that results in simplified alveolar structure and decreased alveolar numbers and decreased vascular involvement. This can be caused by genetic factors but can also be induced by hyperoxia caused by supplemental oxygen provided to preterm infants during ventilation. Infants with BPD present with decreased gene expression of endothelial markers including VEGF, CD31 and Tie-2. Failure to form the air-blood barrier is a fatal disorder defined as alveolar capillary dysplasia (ACD). ACD presents with thin walled vessels through the septa, thickened pulmonary arteries and veins that are located through the centre of the lung rather than at the periphery. A similar phenotype to ACD, which includes disorganized vessel structure and reduced gas-exchange surface area, is seen in mice deficient for eNOS and in mice that are deficient for VEGF164 and VEGF188 (Galambos et al., 2002; Han et al., 2004).

Vascular dysfunction has also been characterized in most chronic obstruction pulmonary diseases (COPD) including emphysema (Galambos and deMello, 2007; Voelkel et al., 2011). COPD describes a spectrum of lung diseases that are defined by chronic airflow limitations that is often progressive, not reversible, and associated with abnormal inflammatory responses to noxious particles and gases (Balkissoon et al., 2011). This includes emphysema and chronic asthma as well as late phases of ALI/adult respiratory distress syndrome (ALI/ARDS). Emphysema is characterized by abnormal airspace enlargement and destruction of the alveolar walls distal to the terminal bronchioles (Snider et al., 1985). In contrast to other COPD, emphysema does not typically present with high levels of inflammation. Two primary mechanisms have been identified that mediate the alveolar destruction observed in cigarette-smoke induced emphysema; namely, aberrant protease release caused by inflammation and alveolar cell apoptosis caused by defective/diminished VEGF expression and endothelial dysfunction. Interestingly, Voelkel et al. have proposed that emphysema is actually explained better by a defect in lung maintenance, rather than the chronic injury-repair disease (Voelkel and Taraseviciene-Stewart, 2005). They have also proposed that many of the cardiac complications associated with COPD including pulmonary
hypertension, is more likely caused by “sick lung circulation” than by heart overload (Voelkel et al., 2011). These studies demonstrate the clinical importance of the vasculature in both pediatric and adult lung diseases and highlight the need for further research into lung vascular development and vascular related mechanisms that facilitate lung maintenance and repair.

1.2.3 Factors that influence lung development

Lung development and alveolarization is controlled by a number of factors including matrix proteins, transcription factors and growth factors. These factors act through mesenchymal-epithelial inductive signaling to influence lung epithelial lineage differentiation and the development of the air-blood barrier (Warburton et al., 2000; Mariani et al., 2002; Maeda et al., 2007; Galambos and deMello, 2008). Fibroblast growth factors (FGFs) and their receptors, in particular, are known to play an important role in lung development. FGF10 is expressed by the mesenchyme and recruits epithelial progenitor cells to the lung bud. As a result, lack of FGF10, or its receptor FGFR2-IIIb in mice results in complete failure of the lung to develop (van Tuyl et al., 2004). FGF7 induces the epithelial cell proliferation necessary for lung bud expansion meanwhile FGFR3&4 are required for secondary septation (Weinstein et al., 1998; and reviewed in Van Tuyl et al., 2004).

A key player in lung alveolarization is PDGFA. PDGFA is expressed by the epithelium and signals through its receptor, PDGFRα, which is expressed on the adjacent mesenchyme. PDGFA is required for normal lung formation and the lungs of mice deficient for PDGFA lack septa and alveoli and possess only pre-alveolar sacs characteristic of the cannicular stage lung development (Lindahl et al., 1997). They are also devoid of alveolar myofibroblasts and elastic fibers. This data suggests that elastin deposition by myofibroblasts is required for alveolarization (Boström et al., 1996). Elastin, which provides the elastic property necessary for proper breathing, also contributes to lung development before alveolarization through its expression in the embryonic mesenchyme and the lung parenchyma at birth (Wendel et al., 2000). Consequently, like PDGFA-deficient mice, mice deficient for elastin display a terminal branching defect, fewer air sacs, and decreased septa. They also exhibit large cavities within the tissue caused by tearing of the tissue during normal inflation.
Matrix metalloproteinases (MMPs) are a large and diverse family of broadly expressed proteases that are also crucial for lung development. MMPs are zinc-dependent proteases that facilitate cleavage of cell surface receptors, degrade ECM proteins, and change the activation state of chemokines and cytokines. MMP2 is highly expressed in the developing lung and is downregulated in adult lung, while MMP16 is upregulated during alveolarization (Ryu et al., 2005; Hadchouel et al., 2008). Changes in oxygen concentration during post-natal development are sufficient to alter MMP2 and MMP9 regulation resulting in defective lung structure. MMP14 and MMP20 are both constitutively expressed in the developing lung while loss of MMP14 results in defective alveolarization and impaired contact between endothelial and alveolar epithelial cells (Atkinson et al., 2005; Irie et al., 2005; Ding et al., 2011). Therefore, lung development requires a number of factors that regulate epithelial cell growth, through factors expressed both by the surrounding mesenchyme as well as the epithelium.

1.2.4 Maintenance of lung structure requires communication between the airways and vasculature

The study of pulmonary vascular development along with the use of vascular knockouts and anti-angiogenic factors has highlighted the importance of the vasculature in development and maintenance of the lung (Thébaud and Abman, 2007). In the developing lung, VEGF produced by the distal tips of the lung buds, interacts with its receptor VEGFR2 on endothelial cells and this interaction is required for the formation of the primitive vascular network (Gebb and Shannon, 2000). The VEGF gene can be alternatively spliced to generate a number of isoforms depending on the species and in the mouse there are 3 major isoforms, VEGF120, VEGF164, and VEGF188 (Ferrara, 1999). Closer examination of VEGF isoforms suggests that VEGF164 and VEGF188, which bind heparin sulfate, rather than VEGF120, are necessary for the formation of the airways (Galambos et al., 2002). Constitutive deletion of either VEGF or VEGFR2 results in embryonic lethality due to an inability to develop normal vessels (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). Targeted deletion of VEGFR2 in adult lung, however, causes alveolar destruction leading to increased airspaces and increased lung compliance, (a phenotype reminiscent of emphysema), within 5
weeks of treatment (Tang et al., 2004). Similarly, treatment with SU5416, a VEGF receptor antagonist, is sufficient to reduce alveolarization in rats and treatment of adult mice with SU5416 is sufficient to induce an apoptosis driven emphysema (Jakkula et al., 2000; Kasahara et al., 2000; Tuder et al., 2003). Thus, there is experimental evidence to suggest that appropriate vascularization and integrity are required for normal epithelial function in the lung and that lesions in vascular function can present themselves epithelial defects.

A number of other endothelial cell related proteins have also been found to be important in development and maintenance of the lung. PECAM is a classic marker of endothelial cells and is required to maintain vascular integrity (Maas et al., 2005). Mice deficient for PECAM or treated with an anti-PECAM antibody exhibit defective alveolarization, without apparent changes in endothelial cell numbers in the lung (DeLisser et al., 2006). EphrinB2, which demarcates endothelial cells in arterial vessels and is a key regulator of vessel remodeling during development, is also essential for lung alveolarization. Mice carrying homozygous mutations in the PDZ domain docking site of EphrinB2 are unable to form alveolar structures and develop a progressive airspace enlargement and little to no discernable secondary septa (Wilkinson et al., 2008). These mice also have disorganized elastic structure, and decreased smooth muscle actin and fibronectin. Endothelial nitric oxide synthase (eNOS), the primary mediator of nitric oxide within endothelial cells, is expressed in both epithelial and endothelial cells within the developing lung (North et al., 1994). eNOS deficient mice display defects in pulmonary vascular development that defects in lung morphogenesis similar to those observed in bronchopulmonary dysplasia (Han et al., 2004). Furthermore, eNOS is important for mitigating the effects of hypoxic stress because under conditions of hypoxia, eNOS−/− mice have defective alveolarization as well as decreased vascular density and VEGFR2 expression (Balasubramaniam et al., 2003). Deletion of a number of other genes such as sonic hedgehog, Wnt7b, Foxf1α and Foxa2 among others, results in global defects in vascular morphogenesis, that are also involved with lung vascular development (Bridges and Weaver, 2006).
1.2.5 Lung regeneration

Aging leads to an intrinsic inability to repair or regenerate tissues after injury and typically results in pathological changes to the tissue. On the other hand, disease is typically mediated by extrinsic factors that often result in accelerated aging or aberrant repair mechanisms that culminate in remodeling of the tissue (Beers and Morrisey, 2011). It is the role of a number of progenitor cells to maintain normal function (Fig 1.6). In the lung, regeneration of alveolar tissue requires type II alveolar cells, which are able to self-renew and are able to differentiate into type I alveolar cells (Schittny and Burri, 2004). There have been reports that adherent cells from the bone marrow are also able to colonize the parenchyma and express type I epithelial cell markers after bleomycin-induced injury in mice (Kotton et al., 2001; Schittny and Burri, 2004). Meanwhile, bronchioalveolar stem cells that express Clara cell secretory protein and surfactant protein C as well as a variant Clara cell have been found within the bronchi and are able to regenerate both alveolar and bronchiolar epithelial cells (Beers and Morrisey, 2011; Kadzik and Morrisey, 2012).

![Figure 1.6: Adult lung airway and alveolar progenitor cells](image)

In the adult lung, the upper airways are populated with Clara cells (CC10+), ciliated cells (Foxj1+), basal cells (p63+), and neuroendocrine cells. The mature alveolus is the site of gas exchange in the lung, lined with alveolar epithelial type 1 and type 2 (AEC1 and AEC2 cells, respectively). AEC1 cells have flattened morphology, express aquaporin5 and T1α, and form close associations with the underlying vasculature. AEC2 cells have a more cuboidal shape and express surfactant protein C (Sftpc). Reprinted from Cell Stem Cell, 10(4), Rachel S. Kadzik & Edward E. Morrisey, Directing Lung Endoderm Differentiation in Pluripotent Stem Cells, 355-361, ©2012 with permission from Elsevier
Currently most of our understanding of the mechanisms of lung regeneration comes from pneumonectomies performed in human patients and animal models. This procedure removes a lung, or portion of a lung and results in global growth and hypertrophy of the remaining lobes. Depending on the age of the organism, the growth results in complete replacement of the lost tissue (Landesberg and Crystal, 2004). In the murine model, the compensatory growth is facilitated by the rapid expansion of existing alveolar tissue as well as the addition of new alveoli within 14 days of surgery (Fig 1.7) (Landesberg and Crystal, 2004; Beers and Morrisey, 2011). Mechanical stretch is one of the primary mechanisms that initiate the compensatory lung growth along with sheer stress, followed by changes in hormones and molecular factors. The space left by the missing lobe is sufficient to induce mechanical stretch of the remaining lobes and induce genes associated with growth. Interestingly, when the open space is filled with a plug, not all of the compensatory growth is prevented, suggesting mechanisms other than the sensing of vacant space, are important for the compensatory growth (Hoffman et al., 2010). The pneumonectomy also increases the sheer stress within the pulmonary vasculature by forcing all of the blood through the vessels of the remaining lobes. The increased blood flow causes sheer stress and cytokine production to trigger eNOS and iNOS that peak at day 7 with decreasing levels thereafter. eNOS is required for compensatory lung growth after pneumonectomy as mice lacking eNOS expression are unable to increase lung weight, lung volume or show any appreciative increase in cell proliferation, and this phenotype could be mimicked using an inhibitor of nitric oxide, L-NG-nitroarginine methyl ester (L-NAME) (Leuwerke et al., 2002). Thus, there is data to suggest that lung vasculature is a key player, and possibly an orchestrating player, in regulating lung regeneration.
Figure 1.7: Proposed model for regenerative alveolarization mediated by proliferation of lung epithelial progenitors.

Pneumonectomy (PNX)-induced alveolar regeneration is primarily mediated by amplification of bronchioalveolar stem cells (BASC), alveolar epithelial cells (AEC), and pulmonary capillary endothelial cells (PCECs). Reprinted from Cell, 147(3), Bi-Sen Ding, Daniel J. Nolan, Peipei Guo, Alexander O. Babazadeh, Zhongwei Cao, Zev Rosenwaks, Ronald G. Crystal, Michael Simons, Thomas N. Sato, Stefan Worgall, Koji Shido, Sina Y. Rabbany, Shahin Rafii, Endothelial-Derived Angiocrine Signals Induce and Sustain Regenerative Lung Alveolarization, 539-553, © 2011, with permission from Elsevier

There are a number of other molecular factors, many of which are involved with lung development, that are also required for lung regeneration (Landesberg and Crystal, 2004). For example, both elastin and collagen are necessary for normal alveolarization and during the cell proliferation stage (day 3-8 post-pneumonectomy), elastin and collagen type I mRNA expression is upregulated starting at day 3, continuing through day 7, and plateau at high levels through the completion of the expansion (Koh et al., 1996). A number of growth factors have also been shown to amplify the growth response post-pneumonectomy. Similar to its role described in developmental alveolarization, VEGF is increased in serum of animals undergoing pneumonectomy starting at day 3 and peaking at day 7 (Leuwerke et al., 2002). Administration of VEGF speeds up completion of lung growth from 10 days to 4 days post-pneumonectomy, but inhibiting VEGF through small molecule inhibitors does not affect lung growth. Nonetheless, conditional deletion of VEGFR2 ablates compensatory growth by inhibiting both endothelial and epithelial cell proliferation (Ding et al., 2011).

Similarly, hepatic growth factor (HGF) is a growth factor produced by macrophages and endothelial cells that can act as a mitogenic stimulus on airway epithelial cells. HGF is
regulated in a similar manner to eNOS and injections of HGF augment proliferation, while administration of an anti-HGF antibody is sufficient to decrease lung weight and DNA content when compared to control mice undergoing pneumonectomy (Sakamaki et al., 2002). Epidermal growth factor (EGF) has also been implicated in maintaining lung regeneration through expression of its receptor EGFR on airway epithelial cells. Studies in rats by Kaza et al. showed that administration of recombinant EGF accelerates and increased lung growth response, resulting in pronounced increases in lung growth and weight over controls (Kaza et al., 2000). In a recent study, Ding et al. showed that stimulation of alveolar progenitor cell proliferation required cleavage of EGFR ligands, heparin bound EGF (HB-EGF) and laminin α5 γ2, by MMP14 expressed on endothelial cells for compensatory growth post-pneumonectomy (Ding et al., 2011). Taken together, the data overwhelmingly link lung regeneration to the stimulation of vascular and epithelial cell growth through coordinated approach. They also suggest that under many, if not all, conditions, the vasculature is the upstream driving force behind lung development and regeneration.

1.3 CD34 family

1.3.1 Protein structure, genomic organization and binding partners

CD34, podocalyxin and endoglycan are all cell-surface sialomucin proteins and have been classified as a subfamily of the much larger family of cell surface mucins base on their shared structural similarity and genomic organization (as reviewed by (Furness and McNagny, 2006; Nielsen and McNagny, 2008). Their inclusion in the family of secreted and type I transmembrane mucins is based on their PTS domain (i.e. rich in proline, serine and threonine residues) which permits their extensively post-translational modification by the addition of O-linked glycosylations. O-linked oligosaccharides are added at S/T residues, and in many instances these also undergo sulfation. Sialomucins, in particular, are also heavily modified by the addition of a terminal sialic acid to terminal O-linked glycans.

The conserved genomic organization of genes encoding the members of the CD34-family is highly suggestive of a common ancestral gene and sufficient to warrant classification of the encoded proteins as a related family. Each protein is coded by eight exons, with the protein domains of each encoded by equivalent exons. CD34 and podocalyxin also have alternative
splice sites within the seventh intron that generate longer transcripts, which encode shorter proteins due to a premature stop codon. The result is proteins that contain a truncated cytoplasmic tail. To date, no splice variant has been identified for endoglycan. CD34-type genes are conserved in vertebrate evolution but no homologous proteins have been identified invertebrates or other lower eukaryotes.

CD34, podocalyxin, and endoglycan proteins share many structural components (Fig 1.8). All family members share a mucin domain, a disulfide-bonded globular domain, a juxtamembrane stalk domain, a transmembrane region, and a charged intracellular domain of about 75 amino acids. As is typical of most sialomucins, the extracellular domains of CD34 and Podocalyxin are poorly conserved across species however, the extracellular domain sequence of endoglycan is >67% identical between mice and humans. In contrast, sequence analysis across species confirms that the cytoplasmic domain of each protein is highly conserved, particularly the consensus docking sites for PDZ-domains at the C-terminus. (Furness and McNagny, 2006; Nielsen and McNagny, 2009).

CD34 protein contains ~380 amino acids, depending on the species, 35 kilodaltons with an apparent molecular weight of ~90 kilodaltons. The cytoplasmic domain concludes with an aspartate-threonine-gutamine-leucine sequence (DTEL) that is a typical PDZ-domain docking signal. Nevertheless, to date, no binding partners for this motif have been found. The only known intracellular binding partner for CD34 is CrkL (Felschow, 2001). Crk proteins contain SH2 and SH3 domains and act as scaffolds for signal transduction complexes, often allowing proteins without kinase activity to transmit signals indirectly to intracellular signalling cascades (Nielsen and McNagny, 2008).

Podocalyxin-like 1 protein (also known as PCLP1, gp135, MEP21, GCTM2, TRA-1-60, TRA-1-81 and thrombomucin; gene name PODXL) encoded a protein core of approximately 56 kilodaltons with an apparent molecular weight of 120-200 kilodaltons. The cytoplasmic domain of podocalyxin contains a membrane proximal ERM (ezrin, radixin, moesin) binding site allowing direct interaction with ezrin, as well as a PDZ-binding domain in the four C-terminal amino acids of the intracellular domain, aspartate-threonine-histidine-leucine
(DTHL). The best-characterized binding partners for this motif are NHERF-1 & 2 (solute carrier family 9 (Na+/H+ exchanger), member 3 regulator -1 and -2). These adapter proteins, (NHERF1, 2 and ezrin), have diverse binding partners that give podocalyxin the potential for interacting with a number of signaling pathways as well as the actin cytoskeleton.

Endoglycan is the third, and most recently identified, member of the CD34 family (also known as podocalyxin-like protein 2, gene name Podxl2) and consists of core protein of approximately ~605 amino acids with an apparent molecular weight of >170 kilodaltons, making it the largest of the family members (Sassetti et al., 2000). Endoglycan bears a number of additional extracellular features including, a poorly glycosylated N-terminal domain that is modified with chondroitin sulfate, and a poly-glutamic acid sequence of ~30 amino acids (Sassetti et al., 2000). Furthermore, there is an unpaired juxtamembrane cystine that may permit homodimerization. Like podocalyxin, the cytoplasmic domain of endoglycan also has a terminal DTHL motif allowing it to bind to NHERF-1 and 2 (Tan et al., 2006).
Figure 1.8: Protein structure, genomic organization and splicing of the CD34 family

(A) Schematic of protein structures. CD34, podocalyxin and endoglycan each have an extensively O-glycosylated (horizontal lines) and sialylated (horizontal lines with arrowheads) serine-, threonine- and proline-rich extracellular mucin domain (green), sites of N-glycosylation (lines with circles), a cysteine-containing globular domain (dark blue) and a juxtamembrane stalk region (yellow). Their single-pass transmembrane domains (light blue) are followed by short cytoplasmic tails (red) containing putative phosphorylation sites and C-terminal PDZ-domain docking sites (DTEL or DTHL). The extracellular unpaired cysteine residue of endoglycan can facilitate homodimerization; endoglycan also contains an unusual polyglutamic-acid-rich extracellular domain (pink box). (B) Genomic organizations. Each protein is encoded by eight exons, with individual exons encoding the corresponding domain in each protein. (C) Alternative splicing. CD34 and podocalyxin display identical patterns of alternative splicing, and can exist as truncated versions that lack most of the cytoplasmic tail. Reproduced with permission Nielsen and McNagny, Journal of Cell Science, 2008;121(Pt 22):3683-3692.
1.3.2 Cellular distribution of CD34 family

1.3.2.1 Vascular expression

All three members of the CD34 family have been identified in vascular tissue, although on different cell types (Table 1.2). Over the decades, most reports of CD34, podocalyxin, and endoglycan on vascular-related cells (including endothelial, smooth muscle cells and circulating endothelial progenitor cells (EPCs)) have focused on their utility as markers, but recent work by our lab and others have begun to delineate functional relationship between these molecules and vascular development and disease. Both CD34 and podocalyxin are expressed on blood endothelial cells, however, unlike other classic markers of endothelial cells such as Tie-2 or Flk-1, both CD34 and podocalyxin do not appear to be essential for endothelial cell growth and development (Cheng et al., 1996; Suzuki et al., 1996; Doyonnas et al., 2001).

1.3.2.1.1 Podocalyxin

Expression of podocalyxin by the vasculature has been observed in multiple species in all vessels of tissues except the sinusoidal or lymphatic endothelium (Horvat et al., 1986; Chi et al., 2003; Testa et al., 2009). Podocalyxin is expressed early in vascular development. Hara et al. identified podocalyxin as a specific marker of aorta-gonad-mesonephros (AGM) hemangioblast cells (Hara et al., 1999). Immunogold staining demonstrated that podocalyxin localizes to the luminal surface, with a patchy distribution, in both non-fenestrated and fenestrated capillaries as well as large arterial vessels including the coronary artery and abdominal aorta (Horvat et al., 1986). In continuous endothelium such as the capillaries of the lung, podocalyxin is primarily expressed on the plasma membrane, but is also associated with caveolae but not vesicles (Horvat et al., 1986; Oh et al., 2007). Podocalyxin is highly expressed by brain endothelial cells, with a 3.4 fold increase over lung microvasculature, and 7.5-9.7 fold increase over liver, kidney and heart microvasculature (Agarwal et al., 2010). Recent studies have begun to use podocalyxin as a marker of tumor vasculature and in human cases of hepatocellular carcinoma, over 90% of tumors were found to have greater than 50% podocalyxin-positive vessels, making it a novel marker for malignant liver tumors (Heukamp et al., 2006; Kazakov et al., 2012).
1.3.2.1.2 CD34

In mammalian tissue, CD34 is a pan-endothelial cell marker as it is expressed by blood, tumor and lymphatic endothelial cells, but not sinusoidal endothelial cells. During development, CD34 expression can be found in intraembryonic vessels soon after their formation, including the capillary plexus, as well as presumptive hemangioblastic progenitors, but not in vessels that form through coalescence, (Young et al., 1995; Wood et al., 1997). CD34 is considered an excellent marker of capillary endothelial cells in almost all vascular beds throughout development except in the fetal liver. It is also expressed by larger vessels, although to a lesser extent (Fina et al., 1990; Young et al., 1995; Chi et al., 2003). Levels of CD34 are typically downregulated by adult endothelial in many tissues including the lung and kidney (Schlingemann et al., 1990; Delia et al., 1993; Young et al., 1995; Sauter et al., 1998; Siemerink et al., 2012).

Despite the variations in levels noted above, all normal adult vascular tissues express CD34 (Baumhueter et al., 1994). Although early reports suggested that CD34 was not highly expressed on vessels in the central nervous system (Fina et al., 1990), it is now used as a classic marker for endothelial cells in pathological studies of the brain and spinal cord, including multiple sclerosis and Alzheimer’s disease (Kalaria and Kroon, 1992; Bennett et al., 2010). CD34 was characterized as one of the original markers of adult circulating endothelial progenitor cells (EPCs) (Asahara et al., 1997), while in lymphoid tissues, CD34 is expressed by high endothelial venules (HEV) under both naïve and inflammatory conditions (Baumhueter et al., 1994). Within the kidney and lymphatic vasculature, CD34 expression is highest on the luminal face and at the junctions, but it can also be detected on the abluminal domains (Sauter et al., 1998; Acevedo et al., 2008). In the lung, CD34 is highest on capillaries, followed by arteries and veins (Müller et al., 2002). CD34 is variably detected in the heart within the endocardial endothelium, and can detect more intramyocardial capillaries than CD31 (Pusztaszeri et al., 2006).
CD34 has emerged as a classic marker of tumor vasculature, particularly to identify microvessel density (MVD), a negative prognostic indicator in most solid tumor lesions. CD34 is expressed on >90% of tumor vasculature. It is upregulated two-fold on hemangiomas and lymphomas and has been used as a diagnostic for MVD and angiogenesis in cervical and prostate tumors, among others (Sauter et al., 1998; Vieira et al., 2004; Sasano and Suzuki, 2005). In breast cancer studies, CD34 is the most sensitive and robust antibody to detect MVD, over both CD31 and vWF (Martin et al., 1997), however in gastric cancers, the predictive value of CD34 expression to overall survival was good, but did not correlate as well as CD105 to other pathological parameters (Ding et al., 2006). The use of CD34 and the lymphatic endothelium marker D2-40 in evaluation of melanoma histological samples increased the ability to diagnose lymphovascular invasion, and therefore disease severity (Rose et al., 2011). Hepatocellular carcinomas can also be differentiated from non-neoplastic tissue, (particularly cirrhotic tissue) using anti-CD34 antibodies (Ruck et al., 1995). Finally, with the recent progression to computer aided analysis of staining, the use of CD34 has facilitated more accurate diagnostics because it has proven superior in detecting vessels found in intratumor areas of necrosis as well as developed vessels, over other endothelial cell markers. Therefore, CD34 has become an important diagnostic marker in the detection of MVD.

More recently, CD34 has emerged as a marker of endothelial tip cells. Filapodia of sprouting vessels within the embryo express high levels of CD34, and similar cells have been found in cultured human umbilical vein endothelial cells (HUVECs), suggesting a possible function in angiogenesis (See Section 1.3.3) (Schlingemann et al., 1990; Young et al., 1995; Sauter et al., 1998; Siemerink et al., 2012).

1.3.2.1.3 Endoglycan

The initial characterization of endoglycan distribution by Sassetti et al. showed mRNA expression in human pancreas, kidney and brain, with low expression also found in the liver, foreskin vasculature and HUVECs. While they argue that the broad tissue distribution in human samples is a result of endothelial expression, this is contradicted by the fact that they
are unable to find expression of endoglycan in the lung or placenta; two incredibly vascularized tissues (Sassetti et al., 2000). Endoglycan is also expressed on 25% of HEV, a highly specialized endothelium, within human tonsils; but interestingly, endoglycan is not apparent on mouse HEV (Kerr et al., 2008). Endoglycan has also been detected throughout the zebrafish vasculature as a marker of the apical membrane of endothelial cells (Herwig et al., 2011).

<table>
<thead>
<tr>
<th></th>
<th>CD34</th>
<th>Podocalyxin</th>
<th>Endoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrovessels</td>
<td>+</td>
<td>++</td>
<td>-/+</td>
</tr>
<tr>
<td>Microvessels</td>
<td>+</td>
<td>++</td>
<td>-/+</td>
</tr>
<tr>
<td>Tumor vessels</td>
<td>++</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Lymphatics</td>
<td>+</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>Developing aortic lumen</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemangioblasts</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Human EPCs</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Wound angiogenesis</td>
<td>++</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>-/+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1.2: CD34, podocalyxin and endoglycan expression in vascular related cells
CD34, podocalyxin and endoglycan expression in vascular related cells (++ = highly expressed, + = expressed, - = not expressed, -/+ = low or variable expression. ? = not tested)

1.3.2.2 Hematopoietic and additional sites of expression
Podocalyxin, CD34 and endoglycan have both common and unique distribution patterns within hematopoietic and non-hematopoietic tissues (Table 1.3). Outside of the vasculature, hematopoietic progenitors are the primary cell type to exhibit common expression of podocalyxin, CD34 and endoglycan. Neural tissues express both podocalyxin and endoglycan, while mature hematopoietic cells and tissue specific cells typically have unique distribution patterns. These include unique expression of podocalyxin on podocytes, mesothelial cells and human cancers, while CD34 is only expressed on hair follicle stem cells and muscle satellite cells.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>CD34</th>
<th>Podocalyxin</th>
<th>Endoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematopoietic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem Cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Multipotent precursors</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>-</td>
<td>+ (anemic)</td>
<td>-</td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
<td>-</td>
<td>(thymocytes)</td>
</tr>
<tr>
<td>B cells</td>
<td>-</td>
<td>-</td>
<td>+ (TLR activated)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mast cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Non-Hematopoietic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood vessels</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurons</td>
<td>+/-</td>
<td>+ (small subset)</td>
<td>+ (30%)</td>
</tr>
<tr>
<td>Mesothelia</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Podocytes</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Muscle satellite cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hair follicle stem cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibrocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1.3: Expression patterns of podocalyxin, CD34 and endoglycan**

CD34, podocalyxin and endoglycan have common and unique distribution patterns within both hematopoietic and non-hematopoietic tissues. (+ = expressed, - = not expressed, +/- = low or variable expression) Table contents updated and adapted with permission from Nielsen & McNagny (2008).
1.3.2.2.1 Podocalyxin

Podocalyxin was initially characterized as a marker for podocytes, a specialized epithelial cell within the kidney and its expression is required for normal podocyte morphogenesis (Kerjaschki et al., 1984; Doyonnas et al., 2001). Careful analysis of rat kidneys suggest podocalyxin is expressed along the apical surface of the podocytes, just above the slit diaphragms, rather than near the glomerular basement membrane (Kerjaschki et al., 1984; Schnabel et al., 1989). Podocalyxin is also expressed on kidney tubules in some species, as well as other epithelial ductal cells such as mammary and ovary ducts (Cheng et al., 2005; Lin et al., 2006; Graves, 2008; Cipollone et al., 2012). Within the developing embryo, mesothelial cells lining many organs including the gut and liver and peritoneum express podocalyxin, however it is typically downregulated after birth (McNagny et al., 1997; Doyonnas et al., 2001; Gómez-Gil et al., 2009; Onitsuka et al., 2010).

Careful analysis by in situ hybridization has shown that podocalyxin is also expressed by small subsets of neuronal cells, starting from E14 through adulthood (Vitureira et al., 2005). Podocalyxin is highly expressed in the cerebral cortex and in the cerebellum, and by highly proliferative areas such as the subventricular zone and external granular layer (Vitureira et al., 2005, 2010). Moderate expression can be found in forebrain structures such as the hippocampus, neocortex and olfactory bulb. Closer examination of the cellular expression of podocalyxin revealed expression on postmitotic neuron cell bodies and dendrites, axons including the growth cones, ependymal cells, and Purkinje cells during development (Vitureira et al., 2005, 2010; Lin et al., 2006).

Retrospective analyses of human cancers have found podocalyxin expression in a number of cancers including testicular cancer, astrocytomas, glioblastomas, leukemia and epithelial carcinomas of the kidney, prostate, breast, thyroid, and ovaries. Podocalyxin expression typically identifies aggressive tumors, with high potential for metastasis and correlates with poor patient outcome (McNagny et al., 2012).

During development, podocalyxin is expressed in all active sites of hematopoiesis, with the highest expression found at the initiation of each stage of hematopoiesis and is
downregulated with time (Doyonnas et al., 2005). Consequently, podocalyxin is expressed in erythroid and multi-lineage progenitors at the beginning of hematopoiesis in the yolk sac and blood (E10-12), followed by the fetal liver (E15), the spleen and bone marrow (E18-birth). Shortly after birth, there is a wave of podocalyxin expression, presumably as the bone marrow undergoes necessary expansion to seed all of the niches, followed by a gradual decline to levels found during adulthood (Doyonnas et al., 2005). Similar expression patterns can also be found in the developing chicken, where podocalyxin, known as thrombomucin, is also expressed in erythroid and hematopoietic multipotent progenitors (McNagny et al., 1997).

In the adult, podocalyxin expression is restricted to megakaryocytes and platelets, stress-induced erythroid cells, and hematopoietic progenitor cells. In the rat, podocalyxin is expressed by megakaryocytes, and in the intracellular store membranes of resting platelets. It is subsequently re-localized to the surface after thrombin activation (Miettinen et al., 1999). In the mouse, podocalyxin can be detected in megakaryocytes, while in the chicken, podocalyxin can be detected in thrombocytes (platelets in the chicken), however its localization has not been confirmed (McNagny et al., 1997; Pericacho et al., 2011). Adult erythroid cells can express podocalyxin in response to stress-induced anemia. Specifically, podocalyxin is expressed on early progenitors (BFU-E) through reticulocytes, through a erythropoietin-STAT5 dependent manner in response to hemolytic anemia (Doyonnas et al., 2005; Sathyanarayana et al., 2007; Maltby et al., 2009). Finally, in normal adult hematopoiesis, podocalyxin is expressed in a very rare subset of hematopoietic progenitor cells in adult bone marrow. These cells are able to give rise to both myeloid and lymphoid cells in serial transplantation experiments, suggesting they are long term repopulating hematopoietic stem cells (Doyonnas et al., 2005).

1.3.2.2.2 CD34

CD34 is best known as a marker of hematopoietic stem cells where it has been used clinically for over 30 years (Ogawa, 2002). In committed hematopoietic cells, CD34 is also expressed on both human and mouse committed mast cell progenitors, and remains present in mature
mast cells in the mouse (Drew et al., 2002, 2005), while low levels of CD34 are also expressed on committed eosinophil progenitors as well as eosinophils from the bone marrow or in resident tissues (Blanchet et al., 2007; Maltby et al., 2010; Rådinger et al., 2011). Similarly, dendritic cell precursors, resident alveolar dendritic cells, along with activated lung dendritic cells all have low levels of CD34 (Blanchet et al., 2011). In non-hematopoietic tissues, CD34 is also present on fibrocytes (Bucala et al., 1994), muscle satellite cells (Beauchamp et al., 2000; Alfaro et al., 2011), and on hair follicle bulge cells (Trempus et al., 2007).

### 1.3.2.2.3 Endoglycan

Endoglycan is expressed on human hematopoietic and lymphatic tissues in the fetal liver, spleen, lymphnode, peripheral blood, and thymus as well as CD34+ bone marrow cells (Sassetti et al., 2000). Endoglycan is also detectible on primary B and T cells, as well as a number of human B cell monocyte cell lines, with highest expression on germinal center and memory B cells (Kerr et al., 2008). Endoglycan expression on B cells can be augmented 10 fold with phorbol myristate acetate (PMA) stimulation likely through transcriptional mechanisms as a result of the time required for this upregulation (Kerr et al., 2008).

In the mouse, endoglycan is expressed on many of the same hematopoietic cells as in the human but also on resting macrophages and thymocytes (Sassetti et al., 2000; Lam, 2005). Like human B cells, endoglycan is also expressed highly on mouse germinal B cells, and stimulation of resting B cells with lipopolysaccharides (LPS) induces surface expression of endoglycan in a toll-like receptor-4 dependent manner (Lam, 2005). Further examination of the developing mouse by \textit{in situ} hybridization has shown expression in smooth muscle cells and the central nervous system (van der Zwaag et al., 2005). This includes the cerebellar primordium, the hindbrain and medulla oblongata, the spinal cord and the ganglia of cranial nerves. Other tissues of the developing embryo (E14.5) expressing endoglycan include the cortical region of the kidney, the olfactory epithelium and smooth muscle cells in several organs including stomach, intestine, tongue and bronchi (van der Zwaag et al., 2005).
1.3.3 Emerging role of the CD34 family in the vasculature

In the context of the vasculature, both podocalyxin and the related CD34, play an important role in the development and function of the vasculature. In a recent landmark paper dissecting the mechanism of aortic development, Strilic et al. determined that podocalyxin and CD34 provide an anti-adhesive role at the luminal face of endothelial cords, and that loss of podocalyxin was sufficient to delay aortic vascular lumen formation (Strilić et al., 2009). Endoglycan has been shown to function in a similar manner in zebrafish. During anastomosis, endoglycan is present in the membrane, shortly after initial contact, during the formation of junctional rings, and defines the apical surface even before lumen formation. In addition, once the vessels mature, endoglycan can be found in all zebrafish vasculature (Herwig et al., 2011). Further work by Strilic et al. has shown that lumen formation for any vessel is dependent on the negative charge provided by sialomucins such as CD34 and podocalyxin. Removal of the sialic acids either with neuraminidase or neutralization with cationic protamine sulfate is sufficient to impair lumen formation in vitro and in vivo, as well as inhibit the growth of new angiogenic sprouts in vitro (Strilić et al., 2010). Partial rescue of lumen formation could be obtained with the addition of dextran sulfate, which binds to the ECs, increasing the negative charge, and thereby generating the necessary force to separate the cells.

CD34, podocalyxin and endoglycan have been shown to play a role in leukocyte adhesion to the vasculature. In HEVs, CD34, podocalyxin and endoglycan act as pro-adhesive molecules through ability to bind to L-selectin, facilitating lymphocyte tethering (Baumheter et al., 1993; Sassetti et al., 1998; Kerr et al., 2008). Recognition of CD34-family sialomucins by selectins require an unusual carbohydrate post-translational modification (sulfated sialyl Lewis-X (sLe^x)), which is present on only a fraction of CD34-family sialomucins expressed by HEV of lymph nodes (Baumhueter et al., 1994; Mir et al., 2009; Leppänen et al., 2010). Since these modifications are not typically found on CD34-type proteins expressed by the vast majority of all endothelia, this function is arguably not of global importance. Secondly, CD34 has been proposed to block leukocytes adhesion to the endothelium. Using HUVECs, Delia et al. showed that under inflammatory stimulation, including interleukin-1, TNF-α and LPS; CD34 was downregulated (Delia et al., 1993). Therefore, it was proposed that in naïve
endothelium, CD34 play an anti-adhesive role to inhibit leukocyte binding and subsequent transmigration into tissues. However, during inflammation CD34 is downregulated to promote the necessary transmigration of cells from the blood through the endothelium into the damaged tissue. Therefore, depending on the type of endothelium, and its activation state CD34, podocalyxin and endoglycan can play multiple roles in the regulation of adhesion of leukocytes.

Figure 1.9: Proposed functions for CD34-type proteins based on published literature

A) **Pro-adhesive.** High endothelial venule-specific glycosylation of CD34 and podocalyxin converts them into ligands for L-selectin expressed by activated leukocytes. “Rolling” on these molecules is the first step in lymphocyte migration into lymph nodes.

(B) **Anti-adhesive.** The vast majority of vessels express CD34 and podocalyxin in a form that cannot bind L-selectin. In addition, podocalyxin overexpression experiments suggest that it blocks adhesion, probably through charge repulsion and steric inhibition of receptors. *Reproduced with permission Nielsen and McNagny, Journal of Cell Science, 2008;121(Pt 22):3683-3692.*

There is an emerging role for CD34 in angiogenesis. Within vessels, the development of tip cells from quiescent endothelial cells is essential to initiate sprouting angiogenesis and guide the migration of nascent vessels towards angiogenic factors (reviewed in Eilken & Adams...
These cells are characterized by their extensive filapodia at the distal end of the cell. CD34 is highly expressed on these filapodia in both the developing embryo as well as active sites of angiogenesis in tumor and wound healing (Ito et al., 1995; Young et al., 1995). In culture, CD34 has been found on a subset (~10%) of cultured HUVECs, and its expression correlates with a number of tip cell markers including VEGFR2, VEGFR3, ephrinB2, plexin D1, as well as neuropillin 1 and 2. CD34 is also dynamically regulated by angiogenic stimuli such as VEGF (Schlingemann et al., 1990; Siemerink et al., 2012). CD34+ cells also contribute to alveolar capillary angiogenesis. The lung pneumonectomy model can be used as a unique model of angiogenesis as it allows for the study of lung alveolar capillary angiogenesis, without tissue injury or endothelial denudation. Using this model, Chamoto et al. identified that at the peak of angiogenesis 7 days after pneumonectomy, CD34+ cells were 12% of all the newly formed CD31+ endothelial cells, and CD34+ cells had proliferation rates four times that of CD34- cells (Chamoto et al., 2011). Interestingly, when the lung pneumonectomy model was performed in mice transplanted with GFP+ bone-marrow, 73.4% of CD34+ endothelial cells (CD31+/CD45-) were derived from GFP+ cells. This suggests CD34 is as a marker for both resident and migratory cells that can contribute to lung angiogenesis.

Furthermore, using controlled subcutaneous angiogenic implant, we demonstrated that neo-angiogenesis was defective in Cd34−/− mice (Heine, 2010). After the first two weeks, significantly more endothelial cells, pericytes and macrophages had migrated from Cd34+/− mice into the implant when compared to implants placed in B6 mice. In spite of the increased cellularity, the migrated cells from Cd34+/− mice displayed less directionality, and resulted in more cells at the edge. Detailed analysis of the implants in CD34 deficient mice also showed that newly formed vessels had altered structure. In comparison to the smooth, well-branched vessels found in control implants, the de-novo vessels from Cd34−/− mice were more clumped, tightly packed and ragged in nature. Vessels within the Cd34−/− implants also had a decreased incorporation of pericytes and blood pools outside of vessel lumens, suggesting a defect in vascular integrity (Heine, 2010). Angiogenesis is also crucial for tumor metastasis. Using a B16 melanoma model, Maltby et al. was able to show loss of CD34 on the vasculature was sufficient to decrease the size of tumors at an early time point.
(Maltby et al., 2011). This reduction in tumor size is associated with altered vessel structure and vascular integrity rather than overall vessel density. Similar to the angiogenic implants, vessels in tumors from $Cd34^{-/-}$ mice displayed shorter, less branched microvessels at the center of the tumor, with more rotund vessels at the periphery, and these vessels also exhibit increased vascular permeability compared to tumors grown in control mice (Maltby et al., 2011). Thus, CD34 plays a role in angiogenesis; likely through alterations in endothelial tip cells, and endothelial-pericyte interactions however, further work is needed to define the mechanisms involved.

Normal function of the vasculature requires exquisite control of the semi-permeable nature of the endothelium. Recently, we have identified a role for CD34 in regulating inflammation induced vascular permeability. In the KBxN-serum induced auto-immune based model of arthritis, $Cd34^{-/-}$ mice show exacerbated symptoms of arthritis caused by increased vascular permeability at the onset of disease (Blanchet et al., 2010). Utilizing bone-marrow chimeras, we determined that loss of CD34 specifically on the endothelium rather than hematopoietic cells resulted in an exacerbated disease. This was confirmed in a parallel study using intravital microscopy to monitor the migration of FITC-labeled bovine serum albumin (BSA) in the cremaster by TNF$\alpha$, which is expressed at the onset of disease. $Cd34^{-/-}$ mice treated with TNF$\alpha$ exhibit increased vascular permeability over control mice, as measured by dye leakage, while naïve permeability in $Cd34^{-/-}$ mice was comparable to control B6 mice (Blanchet et al., 2010). Therefore, CD34 regulates vascular permeability in response to inflammatory stimuli.

1.3.4 CD34 function in hematopoietic cells

With the use of CD34 as a marker of hematopoietic stem cells, many proposed that development of a CD34 knockout mouse would have potentially lethal implications. To the surprise of everyone in the field, $Cd34^{-/-}$ mice from two independent groups were viable and only had subtle defects in eosinophil recruitment in an asthma model, or a delay progenitor differentiation $in vitro$ (Cheng et al., 1996; Suzuki et al., 1996). No defects were found in hematopoietic cell development or the ability to recover from sub-lethal doses of radiation (Cheng et al., 1996).
However, much of our understanding about the function of CD34 in hematopoietic cells has come from challenging $Cd34^{-/-}$ mice in vivo. In these experiments, $Cd34^{-/-}$ mice exhibit a pronounced defect in hematopoietic cell homing and migration. In a transplantation model using lethal-irradiated hosts, the ability for CD34 deficient stem cells to reconstitute the host bone marrow is impaired (Drew et al., 2005). This is even more pronounced in a sub-lethal irradiated transplantation model when the cells were required to migrate through normal endothelium, suggesting that CD34 is required for efficient migration of stem cells to the bone marrow niche (Nielsen and Mcnagny, 2009). CD34 is also important for mast cell migration into tissues. Loss of CD34, along with the related molecule CD43, resulted in decrease mast cell migration into sites of colorectal polyp tumors, leading to changes in integrity and decreased polyposis (Gounaris et al., 2007). Additionally, CD34 is required for effective migration of peritoneal mast cells necessary for the reconstitute the mast cell population after water ablation (Drew et al., 2005). Furthermore, CD34-deficient mice are protected from asthma, hypersensitivity pneumonitis, and DSS-induced colitis because CD34 is necessary for efficient homing of mast cells, eosinophils and dendritic cells to sites of inflammation (Blanchet et al., 2007, 2011; Maltby et al., 2010). In asthma, CD34 expression by eosinophils and mast cells is required for migration from the blood into the lung to facilitate disease progression, while in DSS-induced colitis CD34 is necessary for the migration of eosinophils into the colon to initiate disease (Blanchet et al., 2007; Maltby et al., 2010). Similarly, in hypersensitivity pneumonitis CD34 is required for the migration of activated dendritic cells from the lung to the lymphnodes, in order to initiate the transition to a chronic T cell response necessary for disease progression (Blanchet et al., 2011). Therefore, CD34 is necessary for efficient homing and migration of hematopoietic cells, dendritic cells, mast cell and eosinophils.

CD34 also influences hematopoietic cell-cell contact by regulating homotypic adhesion. Careful observations during in vitro culture of bone-marrow derived mast cells (BMMCs) identified that CD34 deficient mast cells formed aggregates when compared to B6 control mast cells and this phenotype could be exacerbated by the loss of CD43, a related sialomucin (Drew et al., 2005). The increased cell aggregation is divalent cation-dependent suggesting
that it may be mediated by integrins or cadherins, and is reversed when CD34 is ectopically re-expressed in \textit{Cd34-/-} mast cells. Interestingly, the naturally-occurring, splice variant of CD34 lacking the bulk of the cytoplasmic tail was more effective in reversing the aggregation phenotype than full-length CD34, suggesting a potential role for CD34’s intracellular domain in regulating anti-adhesive function. Therefore, in hematopoietic cells, CD34 is important in the regulation of hematopoietic cell-cell adhesion and cell migration.

1.3.5 \textbf{Podocalyxin function outside the vasculature}

Podocalyxin is named for its expression on the podocytes, the foot processes of the glomeruli epithelial cells, which together with the endothelium make up the filtration unit within the kidney (Nielsen and McNagny, 2009). Currently, the only essential function identified for podocalyxin is the formation of the glomerular filtration apparatus of the kidney. The negative charge provided by podocalyxin is required for maintaining podocyte structural integrity. Disruption of the sialic acids in the kidney is sufficient to alter foot process architecture, decreasing or reorganizing slit diaphragms, reducing the number of slits (Kurihara et al., 1992; Guo et al., 2009). Germline deletion of podocalyxin resulted in perinatal lethality within 24hr of birth and anuria (Doyonnas et al., 2001). Careful examination of the kidney architecture by transmission electron microscopy showed a decrease in major processes and complete lack of foot processes and slit diaphragms, and the complete coverage of the capillary loops by podocyte bodies. Along with structural changes, podocalyxin deficient podocytes were unable to convert adherence junctions to tight junctions between podocytes. Taken together this demonstrates that podocalyxin, which provides the bulk of the sialic acids within the kidney, is required for maintaining normal podocyte structure and function.

Analysis of podocalyxin deficient mice have also demonstrated that podocalyxin is important in adhesion at tissue boundaries, in neural development and in platelet function. Podocalyxin is necessary as a general anti-adhesive factor at tissue boundaries, reducing the adhesion of the mesothelial lining of the gut surface to the umbilicus during embryogenesis (Doyonnas et al., 2001). Podocalyxin expression is also required in the developing brain. Loss of podocalyxin results in increased axonal elongation, growth, branching and synaptogenesis,
all of which are not dependent on the negative charge provided by the sialic acids (Vitureira et al., 2010). Furthermore, conditional deletion of podocalyxin using a pan-neuronal Cre (Cre3), results in enlarged ventricles and increased capillary density within the intraventricular spaces, but otherwise the mice develop normally (Nowakowski et al., 2010). Furthermore, mice deficient for podocalyxin displayed increased bleeding time after tail injury, which suggests that podocalyxin could regulate either blood pressure or clotting (Doyonnas et al., 2001; Pericacho et al., 2011). Recently, the role of podocalyxin in clotting has been investigated with the use of transgenic mice that delete podocalyxin specifically within megakaryocytes (Podxl^{lox/lox}:: Pf4- Cre mice) (Pericacho et al., 2011). While naive Podxl^{lox/lox}:: Pf4-Cre mice had normal numbers of megakaryocytes and platelets, after thrombosis, these mice exhibited lower numbers of platelets were detected when compared to control mice. Additionally, under flow conditions in vitro, platelets lacking podocalyxin exhibited decreased aggregation in response to fibrinogen contact. This suggests that podocalyxin may contribute the thrombogenic response (Pericacho et al., 2011).

Podocalyxin has also been shown to play an important role in adhesion and migration of cells. Typically, podocalyxin acts as an anti-adhesion molecule and is able to block adhesion through steric hindrance and negative charge provided by the mucin domain. This can be seen in suspension cell cultures, whereby overexpression of podocalyxin is sufficient to block aggregation of cells. This phenotype is a charge dependent phenotype as cleaving the sialic acid residues with neuraminidase treatment is sufficient to abrogate the anti-adhesive phenotype (Takeda et al., 2000). Overexpression of podocalyxin in polarized epithelial cells such as MDCK, a canine renal tubule cell line, or MCF7 breast cancer cells results in the formation of apical microvilli, apical bulging of monolayers, delamination and shedding of cells from monolayers in vitro (Somasiri et al., 2004; Nielsen et al., 2007). When these polarized epithelial cells were cultured in 2-D cultures, overexpression of podocalyxin was sufficient to disrupt cell junctions as measured by reduced transepithelial resistance, but did not alter junctional expression (Takeda et al., 2000; Somasiri et al., 2004). However in 3D spheroid epithelial cell cultures, forced expression of podocalyxin enhances the exclusion of β1-integrins from the free, apical surface and recruits F-actin from the basolateral ECM-adhesion complexes to an expanded apical membrane domain (Graves,
Consequently, changes in β1 integrin localization results in a concordant decrease in fibronectin-dependent static adhesion. Surprisingly, unlike 2D cultures, podocalyxin expression in cells grown in the 3D cultures has no effect on cell-cell junctions expression and localization, or cell-matrix interactions (Graves, 2008; Cipollone et al., 2012).

Forced expression of podocalyxin in MCF-7 or PC3 prostate cancer cell lines can also enhance cell motility and invasive potential in vitro (Sizemore et al., 2007). The enhanced motility of podocalyxin overexpressing cells depends on the interactions of podocalyxin with its intercellular ligands, NHERF1 and ezrin, to upregulate extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT. Cells that over-express podocalyxin also upregulate levels of MMP1 and MMP9 to facilitate digestion of the matrix and enhance migration (Sizemore et al., 2007). Furthermore, silencing of podocalyxin in A549 carcinoma cells alters cytoskeleton ruffling and cell spreading that results in decreased TGF-β induced migration (Meng et al., 2011). Clinically, podocalyxin expression on tumor cells demarcates those with highest metastatic potential, and is a predictor of poor outcome (Somasiri et al., 2004; Sizemore et al., 2007; McNagny et al., 2012). Podocalyxin also regulates migration in hematopoietic cells. Homing and engraftment of bone marrow hematopoietic stem cells is mediated primarily by CXCL12/CXCR4 axis. Primary hematopoietic cells derived from Podxl⁻ fetal liver (E15.5) display impaired migration to CXCL12 in an in vitro assay (Nielsen and McNagny, 2008; McNagny et al., 2012). Additionally, stimulation of mouse fetal liver with CXCL12 induces enhanced surface exposure of CXCR4 that is distributed with podocalyxin to a common polarized membrane domain, which suggests that podocalyxin and CXCR4 can physically associate at the cell surface. As a result, we hypothesize that podocalyxin has an active role in hematopoietic cell migration and tissue homing by stabilizing or enhancing chemokine receptor signaling. In summary, while podocalyxin is only essential for maintaining podocyte structural integrity, it also play an important role in mediating cytoskeletal changes required for adhesion and migration.

1.3.6 Published function of endoglycan

In B-cells, endoglycan facilitates L- and P-selectin-mediated rolling through sialylation and fucosylation in transfected cells or B-cell lines in vitro (Kerr et al., 2008). Using peptides
modeling the 37 N-terminal amino acid residues of human endoglycan, Leppanen et al showed endoglycan was able to bind L- but not P-selectin with a similar affinity to P-selectin glycoprotein ligand-1 (PSGL-1). This required the core-2 threonine linked O-glycan modified with sialyl Lewis x in conjunction with the tyrosine (Y) sulfate at position Y124 and Y118 for L-selectin recognition and binding respectively (Leppänen et al., 2010). It is likely that similar to CD34 and podocalyxin, these specific modifications are found only on a selectin in HEV, and is not a global function of endoglycan on all cells.

1.4 Aims and rationale of the study
My aim for this thesis was to better characterize the expression patterns of CD34, podocalyxin and endoglycan in vascular structures, and to use genetic deletion models to delineate the biological roles of 1) CD34 and podocalyxin in vascular development, integrity and repair necessary for normal lung development and maintenance, as well as 2) the role of endoglycan in vascular smooth muscle biology. My findings have greatly improved our understanding of CD34, podocalyxin and endoglycan function in the vasculature and that overlapping expression patterns of these molecules is not always indicative of overlapping function.

Early studies examining the role of podocalyxin in the vasculature were hampered by the fact that conventional Podxl−/− mice die perinatally. Therefore, we set out to examine the role of podocalyxin in adult vasculature by generating a conditional knockout in the vasculature. Since the vasculature is necessary for normal development and maintenance of the lung and Podxl−/− mice exhibit edema, suggesting defects in endothelial barrier function, we hypothesized that podocalyxin is required for normal lung vessel development and permeability in vivo.

Additionally, isolating endothelial cells from the lung is the most common method for developing mouse primary endothelial cells (mEC), thereby providing us the opportunity to understand the role of podocalyxin in vitro. Previous work by we and others have identified a role for podocalyxin in defining the apical domain and that altered levels of podocalyxin
can modify epithelial cell adhesion and monolayer integrity. Therefore we hypothesized that **podocalyxin is necessary for normal endothelial cell adhesion and spreading.**

CD34, podocalyxin and endoglycan share overlapping expression in a number of cell types including hematopoietic progenitors and endothelial cells. Since *Podxl<sup>−/−</sup>* mice have increased CD34 mRNA expression in the lung and no overt vascular phenotype, we hypothesized that **loss of both podocalyxin and CD34 on endothelial cells results in a more severe phenotype than loss of each gene individually.**

Finally, within the literature, our understanding of endoglycan has focused on its role in hematopoietic cells, namely B cells and T cells, where endoglycan expression is upregulated with activation. In the vasculature, there have been reports that both smooth muscle cells and endothelial cells express endoglycan, although there is significant debate about the latter. Therefore our objective was to confirm the distribution of endoglycan expression in vascular related cells and its regulation with activation. Based on previous work in our lab, we hypothesized that **endoglycan is a marker of smooth muscle cells and not endothelial cells and is upregulated with activation.**
Chapter 2: Vascular inactivation of the *Podxl* gene leads to increased vascular permeability, altered lung structure and defective endothelial cell adhesion and spreading

2.1 Introduction

Lung development is a tightly controlled process requiring coordinated vascular growth by vasculogenesis and angiogenesis and airway development through morphogenesis and alveolarization (Burri 1984; Burri 2006). In the mouse, the conducting airways and the primitive primary septa are formed through branching morphogenesis during the first 3 stages of lung development that occur during embryogenesis. From postnatal day 4 through postnatal day 36, the fourth stage, termed “alveolarization” results in the growth of new septae that expand into the distal spaces to form the smaller alveoli required in adults. This process produces 90% of the alveolar surface at adulthood (Mund et al., 2008). Alveolarization is influenced by a number of factors including matrix proteins, transcription factors and growth factors, which affect alveolar growth through their interactions with the interstitial fibroblasts and epithelial cells that form the septa (Warburton et al., 2000; Mariani et al., 2002; Maeda et al., 2007; Galambos and deMello, 2008). Defects in alveolarization are found in mice deficient a number of genes including elastin, MMP14, FGFR3 and 4, and retinoic receptors (M. Weinstein et al. 1998; Wendel et al. 2000; Atkinson et al. 2005; Mitani et al. 2009; Nicola et al. 2009; and reviewed in Bridges & Weaver 2006).

Concomitant with alveolarization, the pulmonary vasculature undergoes expansion through sprouting and intussusceptive angiogenesis starting at postnatal day 14. Sprouting angiogenesis causes new vessel growth by initiating endothelial cell proliferation while intussusceptive angiogenesis allows for the expansion and remodeling of the capillary network without additional proliferation through the addition of transcapillary pillars within the vessel lumen (Burri and Djonov, 2002). Subsequently, the fifth and final stage of lung development requires that the vascular network matures from a double capillary layer to a single capillary layer at the centre of a septum for effective gas exchange (Mund et al., 2008). Afterwards, expansion continues with the growth of the animal until adulthood.
Abnormal vessel growth and vascular dysfunction contribute to a number of pediatric and adult lung pathologies (Galambos and deMello, 2007; Voelkel et al., 2011). Consequently, vascular knockouts and anti-angiogenic factors have been used to clarify some of the factors produced by the vasculature necessary for normal development and maintenance of the lung. Disruption of VEGF or its receptors is sufficient to cause endothelial cell dysfunction and apoptosis, which leads to defects in alveolarization and lung maintenance. During lung development, treatment with SU5416, a VEGF receptor antagonist, is sufficient to reduce alveolarization in rats and treatment of adult mice with SU5416 is sufficient to induce an apoptosis driven emphysema (Jakkula et al., 2000; Kasahara et al., 2000; Tuder et al., 2003). Furthermore, targeted deletion of VEGFR2 in the lung caused alveolar destruction leading to increased airspaces as well as increased lung compliance within 5 weeks of treatment. Disruption of endothelial cell growth or signaling can also affect alveolarization as increased airspaces can be found in mice deficient for CD31, EphB2, eNOS (Han et al., 2004; Tang et al., 2004; DeLisser et al., 2006; Wilkinson et al., 2008; Vadivel et al., 2010).

Podocalyxin-like 1 protein (gene name PODXL) is a sialomucin that shares genomic and structural similarities to CD34 and endoglycan (reviewed in Nielsen & K. M. McNagny 2008). Initial characterization of podocalyxin identified it as the primary sialylated protein in the glycocalyx of kidney glomeruli epithelial cells (Kerjaschki et al., 1984). Podocalyxin has also been found on vascular endothelia; hematopoietic stem cells; hemangioblasts; stress erythrocytes; megakaryocytes and platelets (in some species); ductal cells of the kidney, breast and ovaries; the mesothelial cells lining many organs, metastatic cancers and neurons (Kerjaschki et al., 1984; Horvat et al., 1986; Kershaw et al., 1997; McNagny et al., 1997, 2012; Hara et al., 1999; Miettinen et al., 1999; Doyonnas et al., 2001, 2005; Kerosuo et al., 2004; Vitureira et al., 2010).

Podocalyxin can function as either a positive or negative regulator of adhesion depending on its post-translational modifications and subcellular localization. In HEV, podocalyxin is modified with an unusual glycosylation (sialyl-Lewis X modification) that allows podocalyxin to act as ligand for L-selectin (a mammalian leukocyte transmembrane C-type lectin) and facilitate lymphocyte tethering to the endothelium (Sassetti et al., 1998). Since
this modification to podocalyxin only occurs in HEV, and not in the vast majority of other cells that express podocalyxin, it is likely that this function represents an exception rather than the rule. Indeed, under the majority of circumstances there is strong evidence that podocalyxin functions as an anti-adhesin.

Podocalyxin, like other mucins, is highly negatively charged as a result of numerous sialylated O-glycosylations, which are known to block adhesion through steric hindrance and negative charge (Nielsen and McNagny, 2009). In suspension cell cultures, overexpression of podocalyxin is sufficient to block aggregation of cells. This phenotype is charge dependent as cleaving the sialic acid residues with neuraminidase treatment is sufficient to abrogate the anti-adhesive phenotype (Takeda et al., 2000). Loss of podocalyxin \textit{in vivo} also leads to increased adhesiveness of mesothelial linings, which, in turn, leads to high frequency of newborn mice with herniation of the gut (omphalocele) into the umbilical space (Doyonnas et al., 2001). Thus, the data would argue that under most circumstances, the negatively charged glyco-domain of podocalyxin serves to prevent non-specific adhesion, possibly by shielding integrins and other adhesion molecules (Nielsen and McNagny, 2008).

Podocalyxin can also affect adhesion by altering the size of apical and basolateral membrane domains. Overexpression of podocalyxin in mammary epithelial cells is sufficient to expand the apical domain at the expense of the basal surface (Nielsen et al., 2007). This restricts $\beta 1$ integrin to such a small domain that it can no longer provide sufficient contact to maintain adhesion to the substrate (Economou et al., 2004; Somasiri et al., 2004; Nielsen et al., 2007; Graves, 2008). Consequently, there is a decrease in fibronectin-dependent static adhesion through changes in $\beta 1$ integrin localization (Graves, 2008; Cipollone et al., 2012). While changes in podocalyxin expression do not alter junctional protein expression, junction localization is clearly altered (Takeda et al., 2000). Podocalyxin also regulates changes to cell-cell junctions required for the formation of the glomerular foot processes during development (Doyonnas et al., 2001). As a result, $Podxl^{-/-}$ mice are anuric and die within 24 hours of birth. $Podxl^{-/-}$ mice also have an increase red cell flow rate, presumably as a result of fluid build-up caused by the kidney defect. In addition, approximately 25% of pups
present with edema at embryonic day 15 and 18, which is likely caused by leaky vessels or secondary to the kidney defect.

In the context of the vasculature, both podocalyxin and the related CD34, play an important role in the development and function of the vasculature. Podocalyxin is an excellent marker of blood endothelial cells in all tissues, but it is absent from lymphatic and sinusoidal endothelial cells (Horvat et al., 1986; Schnitzer et al., 1990; Testa et al., 2009). Podocalyxin is highly expressed in brain and lung microvasculature, and to a lesser extent in liver, kidney and heart microvasculature (Agarwal et al., 2010). In a recent landmark paper examining vascular lumen development, Strilic et al. determined that podocalyxin and CD34 provide an anti-adhesive role at the luminal face of endothelial cords and that the loss of podocalyxin was sufficient to delay aortic vascular lumen formation (Strilić et al., 2009). Interestingly, Podxl null mice have otherwise normal vascular beds within most tissues just before birth (Doyonnas et al., 2001). Intriguingly, these mice upregulate CD34 expression in the vascular beds, presumably as a compensatory mechanism, which may explain why there is a limited vascular phenotype (Doyonnas et al., 2001).

CD34 is known to be pan-endothelial cell marker, and is highly expressed on endothelial tip cells (Schlingemann et al., 1990; Young et al., 1995; Sauter et al., 1998; Siemerink et al., 2012). The use of disease models has allowed us to better understand the function of CD34 in endothelial cells. First of all, in an autoimmune serum transfer model, we identified that CD34 deficient mice were more susceptible to arthritis and that loss of CD34 expression on endothelial cells and not expression on mast cells, eosinophils or other hematopoietic cells resulted in an exacerbated disease (Blanchet et al., 2010). Closer examination identified that the exacerbated disease phenotype was caused by an increase in vascular permeability during both the early stages of disease and in response to TNFα. Secondly, using a tumor-angiogenesis model Maltby et al. was able to show that the loss of CD34 resulted in altered vessel structure and vascular integrity but normal vessel density within the tumor (Maltby et al., 2011). The vessels within the tumors from Cd34−/− mice displayed shorter, less branched microvessels at the center of the tumor, with more rotund vessels at the periphery. Further analysis also showed that Cd34−/− tumor tissues exhibit increased vascular permeability in
compared to those grown in control mice (Maltby et al., 2011). Taken together, this data identifies a pivotal role for CD34 in regulation of induced permeability and vessel patency during angiogenesis. Unfortunately, ubiquitous deletion of the Podxl gene leads to perinatal lethality and thereby precludes a similar analysis of the importance of this molecule in adult vasculature.

To circumvent this problem and examine the function of podocalyxin in the adult vasculature, we disrupted the podocalyxin gene in a vascular specific manner with transgenic mice containing a Podxl-floxed allele (Podxl\textsuperscript{flox}) crossed to transgenic mice containing the Cdh5-Cre allele. Through a detailed analysis of the resulting mice, I found that loss of endothelial podocalyxin resulted in a striking increase in basal pulmonary vascular permeability, enlarged airspaces and changes to the matrix composition. While there were no changes to in expression of classic endothelial cell markers, podocalyxin deficient endothelial cells were unable to spread on laminin, exhibited enhanced adhesion to fibronectin, and upregulated transcription of integrins responsible for binding to these matrix proteins. Surprisingly, we found that deletion of both podocalyxin and CD34 from the vasculature (Podxl\textsuperscript{ΔECCd34/-} mice) resulted in no additional phenotypes beyond those observed in the Podxl\textsuperscript{ΔEC} mice suggesting that CD34 does not, in and of itself, play a compensatory role in vasculature function. My data reveals a key role for Podxl in adult vasculature and provides a genetic tool for analyzing the importance of vascular integrity in lung remodeling.

2.2 Materials & methods

2.2.1 Animals

All animals were maintained in a pathogen-free facility, and all experiments were performed in accordance with the University of British Columbia Animal Care Committee regulations. Cd34/- mice were a gift from Dr. Tak Mak and were routinely backcrossed to control C57Bl/6J (B6) mice. Cdh5-Cre mice were purchased from JAX (B6.Cg-Tg(Cdh5-cre)7Mlia/J, Stock number 006137). Rosa26-YFP mice were a gift from Dr. Thomas Graf.
2.2.2 Generation of conditional podocalyxin knockout

A mutant podocalyxin (Podxl) genomic targeting construct was generated from a 129SvJ mouse genomic library clone by introducing two loxP recombination target sequences upstream of exons 3 and 8. A neomycin resistance cassette (Neo\(^R\)) was also inserted between exons 2 and 3 and flanked with flp-recombinase target sequences (frt) to permit targeted deletion of the drug resistance cassette subsequent to the identification of drug resistant ES cell clones (Fig 2.1). When exposed to Cre-recombinase, this substrate DNA should undergo deletion of the intervening DNA between loxP sites, removing exons 3-7.

The resulting vector was electroporated into R1 embryonic stem (ES) cells and selected with G418. Proper targeting of the Podxl locus in the resulting clones was confirmed in six ES cell clones by PCR and Southern blot. Four clones were chosen for further analysis. These targeted ES cells were injected into albino C57Bl/6J-Tyr-C2J blastocysts and implanted into pseudo pregnant female mice to generate chimeras as per standard protocols. Seven chimeric mice were born from 3 ES cell clones and then backcrossed with B6 mice to identify mice bearing a germline mutation of the Podxl allele. Three chimeras gave germline transmission, and PCR screening identified 2 founders from a single ES clone. Finally, the residual neomycin cassette was removed from the targeted-Podxl locus by breeding these mice to mice ubiquitously expressing Flp-recombinase (Rodriguez et al., 2000). From these 2 founders, 50% of the offspring were able to delete the Neo\(^R\) cassette by PCR screening. Two male mice were selected as founders from these offspring to generate the loxP-flanked (“floxed”) Podxl allele (Podxl\(^{flo}x\)).

The diagnostic PCR strategy for identifying the conditional podocalyxin allele in mice is shown in Figure 1.1 and relies on the PCR primers listed in Table 1.3. Briefly, successful integration of the Neo\(^R\) gene between frt sites in intron 2 was confirmed using primers that bound upstream of the first loxP site (PCK-UFP), within Neo\(^R\) cassette (RPFCK-neg), and downstream of the final frt sites (RFPCK-pos) resulting in a 437bp product to indicate germline transmission, and no product was generated for the wild type allele. Identification of mice that underwent Flp-dependent deletion of the Neo\(^R\) cassette was confirmed using primers upstream of the first loxP site (Podxl-ExF1) and downstream of the final frt site.
(RFPCK-pos), which results in a diagnostic 632bp product in deleted mice, or a 519bp band was in mice retaining the NeoR cassette. $\text{Podxl}^{\text{Annlo}}$ mice with successful deletion of exons 3-7 through either ubiquitous Cre (ear clip, Fig 1.1C) or $\text{Cdhl5-Cre}$ (isolated lung endothelial cells, Fig 2.2A) mediated recombination was detected using primers upstream of the first loxP site (Podxl-Ex F1), upstream of the final loxP site (Podxl-Cstr F2) and downstream of the final loxP site (Podxl-U R1) results in a 285bp product, otherwise a 171bp product was generated.

2.2.3 Cells

The mouse brain endothelial cell line, bEND.3 (Montesano et al., 1990) was purchased from ATCC. Cells were routinely maintained in complete DMEM media (DMEM supplemented with 10% FBS, 1x penicillin/streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM L-glutamine). Cells were passaged using 0.25% Trypsin-EDTA or TrypLE Express (Invitrogen) and maintained between passages 2-30.

Mouse primary lung endothelial cells (mEC) were isolated as previously described with modifications (Mahabeleshwar et al., 2006). In brief, mice of either gender were sacrificed and the lungs were dissected, taking care to remove the bronchi and connective tissue. Lungs were minced and digested at 37°C on a rotator with 3mg/mL Collagenase/Dispase (Roche Diagnostics) in complete DMEM for 2hr. Tissue suspension was trituated using a 20-gauge needle on a 3mL syringe until a single cell suspension was formed. Following red cell lysis, cells were passed through a 70µm tissue strainer (BD Falcon), rinsed with FACS buffer (PBS containing 2 mM EDTA and 10% FBS) and centrifuged. Cells were stained with anti-CD31-PeCy7 (eBiosciences) and CD45-PerCP (BD Pharmingen) for 30min, washed and strained before sorting. Endothelial cells, identified by CD31+/CD45- staining were isolated from the mixture of cell populations using a BD FACSVantage or FACSaria with the help of the UBC FLOW cytometry facility. Cells were plated at a density of 2x10^5 cells per well in a 24 well plate coated with 2% (w/v) gelatin in EC Media (DMEM with 1x penicillin/streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM L-glutamine, 20% FBS, 100ng/mL endothelial cell growth supplement (ECGS) (BD Biosciences), 25mM HEPES, 90µg/mL heparin sulfate). Cells were verified to be
endothelial cells by CD31, ICAM-2, Tie-2 and endoglin positive staining and were maintained until passage 8.

2.2.4 Genomic PCR and real-time qPCR

For the purposes of genotyping mice, genomic DNA was isolated from ear clips or isolated cells by proteinase K digestion. Primers used to identify the various genotypes are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podxl-Ex F1</td>
<td>CTCATACTACCCAGTCAGTGG</td>
</tr>
<tr>
<td>Podxl-Cstr F2</td>
<td>TTACTCTAGGTAGCCAGTT</td>
</tr>
<tr>
<td>Podxl-U R1</td>
<td>TCTCCACGCTAGAGACAGAAG</td>
</tr>
<tr>
<td>PCK-UFP</td>
<td>TGGCCTCAAACTCAGAGATCTATC</td>
</tr>
<tr>
<td>RFPCK-pos</td>
<td>ACCGGTGAAAGGTTCTACT</td>
</tr>
<tr>
<td>RFPCK-neg</td>
<td>CTTCTATGAAAGGTTGGCTT</td>
</tr>
</tbody>
</table>

RNA isolation for cultured cells was performed using RNeasy mini kits (Qiagen), and isolation for tissue was performed using Trizol (Invitrogen). RNA quantification was performed using a ND1000 spectrophotometer (Nanodrop). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real time qPCR (qRT-PCR) gene expression analysis was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences), on a 7900HT Real Time PCR system (Applied Biosystems). Sequence information for the primers is listed in Table 2.2. Data was acquired using SDS 2.0. The relative mRNA expression was normalized to simultaneous amplification of Gapdh gene and the average of each of the Podxl\textsuperscript{flox} tissues set to 1.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>CATTCTTCGCTGCCATTCTG</td>
<td>GCACATTGCCATGTTGAATC</td>
</tr>
<tr>
<td>Ang-2</td>
<td>TAGCACAAGGGTACGGACGACGAAAT</td>
<td>TTTTGTGGTGGTACTGCTGCAATC</td>
</tr>
<tr>
<td>CD34</td>
<td>GACTTGAGAAGCTGGGATCC</td>
<td>TCCAACTGGAAGGTTCAATC</td>
</tr>
<tr>
<td>Cdh5</td>
<td>TCTCTGTACCTCACTATACACA</td>
<td>GTAAGTGAACACTGCTGGTGAAT</td>
</tr>
<tr>
<td>Col1a1</td>
<td>CCAAGGTTACACGACGGA</td>
<td>CTCGTTTTTCTCTTCTCCG</td>
</tr>
<tr>
<td>Col1a2</td>
<td>TGTTGCCCATCTGTAAGAA</td>
<td>CAGGGAATCCAAGTGGCC</td>
</tr>
<tr>
<td>eNOS</td>
<td>TTTTGTAGTTCCAGCACCCACCAGCGCCC</td>
<td>TTTGGCGGTTAGGACTTGGTCAAC</td>
</tr>
<tr>
<td>FGF-7</td>
<td>AAAGAGGCAAAAGTGAAGGGAC</td>
<td>CTTTGAGTTGCAATCCTAATTCGATTC</td>
</tr>
<tr>
<td>FGF10</td>
<td>TTTAGCTGTCCGTACAGTGTCCTG</td>
<td>AAATTCCTTATCTCTCCTTCAGC</td>
</tr>
<tr>
<td>FGFR1</td>
<td>AACCTCTAACCAGAGCAAC</td>
<td>GAGACTCAGCACTCCAGAGAGAG</td>
</tr>
<tr>
<td>FGFR2</td>
<td>ATCATCGCCTGCTCCATC</td>
<td>GCTGTGGTACTGCTGGTC</td>
</tr>
<tr>
<td>FGFR3</td>
<td>TCTCTGTGCTGTGTAAC</td>
<td>TCTCTGTCCATCTTTACG</td>
</tr>
<tr>
<td>FGFR4</td>
<td>ATGACCTGTTCGTAACATACTACAC</td>
<td>TGTCAGTGGTGGTTCG</td>
</tr>
<tr>
<td>Flk1</td>
<td>TTTAGGTGCCTCCCCATACCCTG</td>
<td>CTTTCCTGCGGTTAGTGTGAG</td>
</tr>
<tr>
<td>Fn1</td>
<td>AATCCAGTCCACAGCCATTC</td>
<td>TGTGGCCACCATGAGTCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGATGCCGCTTGGAGAAACC</td>
<td>TGAAGGTCAGCGGAGTGGTC</td>
</tr>
<tr>
<td>iNOS</td>
<td>CGAAACGCTTCACTTGCCAA</td>
<td>TGAAGACTATATGTTGTCG</td>
</tr>
<tr>
<td>Itga1</td>
<td>GGAGAAGAAAGAAGACAGAAG</td>
<td>CACGTTGAGTTCTTTATACG</td>
</tr>
<tr>
<td>Itga2</td>
<td>GGTATCCCTGCCAATTGAGTG</td>
<td>GGTTGAGTGCTGCCAGG</td>
</tr>
<tr>
<td>Itga3</td>
<td>GAGGATATGTGTTGGCTGAGTG</td>
<td>ATCTCGTGGTGTGATGTCG</td>
</tr>
<tr>
<td>Itga4</td>
<td>TCTATCGTGGACAGTTCCTCCCA</td>
<td>AGTCAGTAGAGTTGCTG</td>
</tr>
<tr>
<td>Itga5</td>
<td>AGCTGGATGTTATGGGAG</td>
<td>CAGACTGGCGGAGGAGTT</td>
</tr>
<tr>
<td>Itga6</td>
<td>TCTCTTCAACAGAATTGACCTCCG</td>
<td>CTGAAGCTCAGTGGCAACCC</td>
</tr>
<tr>
<td>Itgb1</td>
<td>GGATATTGTTGAGATGTGTCG</td>
<td>TGGTGAAGATGAGTTGGGAG</td>
</tr>
<tr>
<td>Itgb4</td>
<td>GCTTTGTGGTCCAGGTGTTT</td>
<td>TGTGGCAGGCTGACTT</td>
</tr>
<tr>
<td>Lama4</td>
<td>AACAAACCTTAGAAGGTGGTTATAGGA</td>
<td>AATAAAGCTTGCCCGTGTTGAATATAG</td>
</tr>
<tr>
<td>Lama5</td>
<td>ACCCAAGGACCACACTGTAG</td>
<td>TCTGTGGTGGTACTGCTC</td>
</tr>
<tr>
<td>Lamb1</td>
<td>GGCAAACTGCCAACAGTCTCG</td>
<td>CTGGTATGCTTCAGG</td>
</tr>
<tr>
<td>Lamc1</td>
<td>TGCCGGAGTTGTTAATGCC</td>
<td>CTGGTTGTTAGTCCGTCAG</td>
</tr>
<tr>
<td>Mmp14</td>
<td>GCTTGGAGCTTCACTTACGA</td>
<td>TCTTGAGGCTGACCCGTCA</td>
</tr>
<tr>
<td>NHERF-1</td>
<td>CCACTTACCTCCAGCAGTG</td>
<td>TCTACGTCCTCCCTGCT</td>
</tr>
<tr>
<td>NHERF-2</td>
<td>CACATTCCACAGAGTACG</td>
<td>TGCCATCTCTTGTGGTCT</td>
</tr>
<tr>
<td>PECAM</td>
<td>AGGGGACCAGCTGCACATAGG</td>
<td>AGGGCGCTCTCTCTGACCACCTT</td>
</tr>
<tr>
<td>Podxl</td>
<td>TACACCAAACATTTGGGGCA</td>
<td>CACGCGAGCGCCACATTGAGA</td>
</tr>
<tr>
<td>SDF-1</td>
<td>ACCCTGGCATCCATAGTCGCCC</td>
<td>GAGATGCTAGCTTCTCCCGGG</td>
</tr>
<tr>
<td>SftpC</td>
<td>CCACTGGCATCGCTGTGATG</td>
<td>GTAGGTTCCGAGCCTGCCAAG</td>
</tr>
<tr>
<td>Eln</td>
<td>TGTTATTTGGTGCACCCGG</td>
<td>CTGGTTGTTAGCTG</td>
</tr>
<tr>
<td>VEGF164</td>
<td>CATAGAGAGAATGAGCTTCTACACG</td>
<td>TGCTTTCTCCGCTTCTGGAACCAAGG</td>
</tr>
<tr>
<td>ZO-1</td>
<td>GACTCCAGACAACATCCCGAA</td>
<td>AACGCTGGAAAATACCTCTG</td>
</tr>
</tbody>
</table>

Table 2.2: Real time qPCR primers
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>CATTCTTCGCTGCCCATTCTG</td>
<td>GCACATTGCCCATGTTGAATC</td>
</tr>
<tr>
<td>ZO-2</td>
<td>CAGCCACAAATCAACGTGAATTC</td>
<td>CTGTCCTTCAAGCTGCCAAC</td>
</tr>
</tbody>
</table>
2.2.5 Antibodies and reagents

All media components unless otherwise stated were obtained from Gibco (Life Technologies). LPS, and cell culture grade BSA was obtained from Sigma. Primary and secondary antibodies used in this study are listed in Tables 2.3 and 2.4, respectively.

Table 2.3: Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Concentration</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-mouse CD31 PECy7</td>
<td>rlgG2a</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 25-0311</td>
</tr>
<tr>
<td>α-mouse Podocalyxin</td>
<td>gIgG</td>
<td>2µg/mL</td>
<td>IHC WB</td>
<td>R&amp;D Systems AF1556</td>
</tr>
<tr>
<td>α-mouse Podocalyxin APC</td>
<td>rlgG2b</td>
<td>2µg/mL</td>
<td>FC</td>
<td>R&amp;D Systems FAB1556A</td>
</tr>
<tr>
<td>α-mCD45 PerCP</td>
<td>rlgG2b</td>
<td>1µg/mL</td>
<td>FC</td>
<td>BD Pharmingen 550994</td>
</tr>
<tr>
<td>α-mEndoglin PE</td>
<td>rlgG2a</td>
<td>0.5µg/mL</td>
<td>FC</td>
<td>eBiosciences 12-1051</td>
</tr>
<tr>
<td>α-mTie2 PE</td>
<td>rlgG2a</td>
<td>4µg/mL</td>
<td>FC</td>
<td>eBiosciences 12-5987</td>
</tr>
<tr>
<td>α-mICAM-2 eFluor 450</td>
<td>rlgG2a</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 48-1021</td>
</tr>
<tr>
<td>α-alpha6 integrin</td>
<td>rlgG2a</td>
<td>2µg/mL</td>
<td>FC</td>
<td>BD Pharmingen 555734</td>
</tr>
<tr>
<td>α-beta1 integrin</td>
<td>hamIgG2</td>
<td>2µg/mL</td>
<td>FC</td>
<td>BD Pharmingen 553837</td>
</tr>
<tr>
<td>α-von Willebrand factor (vWF)</td>
<td>rabIgG</td>
<td>8µg/mL</td>
<td>IHC</td>
<td>Sigma F3520</td>
</tr>
<tr>
<td>α-MMP14</td>
<td>rabIgG</td>
<td>1:1000</td>
<td>WB IHC</td>
<td>Abcam Ab51074</td>
</tr>
<tr>
<td>α-Ki67 (SP6)</td>
<td>rabIgG</td>
<td>1:200</td>
<td>IHC</td>
<td>Thermo Scientific RM-9106-S</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Isotype</td>
<td>4µg/mL</td>
<td>FC</td>
<td>R&amp;D Systems AB-108-C</td>
</tr>
<tr>
<td>Rat IgG2a PECy7</td>
<td>Isotype</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 25-4321</td>
</tr>
<tr>
<td>Rat IgG2a</td>
<td>Isotype</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 14-4321</td>
</tr>
<tr>
<td>Rat IgG2a PE</td>
<td>Isotype</td>
<td>2µg/mL</td>
<td>FC</td>
<td>eBiosciences 12-4321</td>
</tr>
<tr>
<td>Antibody</td>
<td>Type</td>
<td>Concentration</td>
<td>Application</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------</td>
<td>---------------</td>
<td>-------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>α-mouse CD31 PECy7</td>
<td>rIgG2a</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25-0311</td>
</tr>
<tr>
<td>Rat IgG2a eFluor 450</td>
<td>Isotype</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48-4321</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Isotype</td>
<td>2µg/mL</td>
<td>IHC</td>
<td>Vector</td>
</tr>
<tr>
<td>Rat IgG2b APC</td>
<td>Isotype</td>
<td>2µg/mL</td>
<td>FC</td>
<td>eBiosciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17-4031</td>
</tr>
</tbody>
</table>

- g – goat species, r – rat species, m – mouse species, h – human species, rab – rabbit species
- FC – flow cytometry, IHC – immunohistochemistry, IF – immunofluorescence, WB – western blot

### Table 2.4: Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-goat AF555</td>
<td>4µg/mL</td>
<td>IF</td>
<td>Invitrogen A-21432</td>
</tr>
<tr>
<td>Donkey anti-rabbit AF555</td>
<td>4µg/mL</td>
<td>IF</td>
<td>Invitrogen A-31572</td>
</tr>
<tr>
<td>Rabbit anti-goat IgG biotin</td>
<td>2µg/mL</td>
<td>IHC</td>
<td>Vector BA-5000</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG biotin</td>
<td>~5µg/mL</td>
<td>IHC</td>
<td>Vector PK-4000</td>
</tr>
<tr>
<td>Donkey anti-goat IR800</td>
<td>66ng/mL</td>
<td>WB</td>
<td>Odyssey 926-32214</td>
</tr>
<tr>
<td>Goat anti-rabbit IR700</td>
<td>66ng/mL</td>
<td>WB</td>
<td>Odyssey 926-32221</td>
</tr>
</tbody>
</table>

- FC – flow cytometry, IHC – immunohistochemistry, IF – immunofluorescence, WB – western blot

### 2.2.6 Flow cytometry analysis

Adherent cells were detached with TEN buffer and then washed with FACS buffer, incubated with primary antibodies (Table 2.3) for 20 min at 4 °C, washed, incubated with secondary antibodies if necessary (Table 2.4) for 20 min at 4 °C and washed again before analysis. Data was collected with a FACSCalibur or BD LSRII using CellQuest or FACSDiva software, respectively. FlowJo software (Tree Star Inc.) was used for analysis of FACS data.
2.2.7 Immunohistochemistry

For immunohistochemical analysis of tissue, tissues were removed from both male and female mice, post-fixed for 24hr in 4% paraformaldehyde and transferred to 70% ethanol at 4°C for storage until paraffin-embedded. Sections (thickness=4µm) were deparaffinized with xylene (2x 5min), rehydrated in decreasing concentrations of ethanol (100% 2x 3min, 95% 3min, 90% 3min, 70% 3min), and rinsed in distilled water (2x 5min) in preparation for staining.

2.2.7.1 Podocalyxin staining

To assess podocalyxin expression in tissue, sections underwent antigen-retrieval in sodium citrate buffer (pH 6.0) in a steamer for 30min, cooled for 20min at room temperature and rinsed in PBS (3x 5min). Sections were then placed in a humidified chamber, pre-incubated with EnVision Flex antibody diluent (Dako) before the primary antibody or isotype control was added overnight at 4°C. Tissues were rinsed (3min, 2x 15min), and stained with biotinylated secondary antibodies for 30min. To block endogenous peroxidase, tissues were immersed in 0.3% hydrogen peroxide in methanol for 30min. Kidney sections underwent an additional 15min wash in fresh peroxide to block the exceptionally high amounts of peroxidase present in the tissue. The VECTASTAIN ABC kit (Vector Labs) was used to visualize the antibody binding, developed with DAB (3,3’-diaminobenzidine) (Vector Labs) and counterstained with Methyl Green or Hematoxylin (Vector Labs) as per manufacturer’s instructions. Sections were then dehydrated in increasing concentrations of ethanol followed by xylene and mounted with Permoun™ mounting media.

2.2.7.2 Von Willebrand factor and Ki-67 staining

Staining for von Willebrand factor in lung tissues required a pepsin enzymatic antigen retrieval using Digest-All 3 (Invitrogen) for 15min and then rinsed in PBS (3x 5min). Staining for Ki-67 required heated sodium citrate buffer antigen retrieval as described in 2.2.7.1. Sections were then placed in a humidified chamber, pre-incubated with Blocking buffer (5% donkey serum, 1% BSA, 0.2% TritonX-100, 0.05% Tween 20 in PBS) before the primary antibody or isotype control in Staining buffer (1% BSA, 0.2% TritonX-100, 0.05%
Tween 20 in PBS) was added overnight at 4°C. Tissues were washed in PBS (3min, 2x15min) then stained with fluorescently labeled secondary antibodies for 1hr, rinsed in PBS (3x 15min), counterstained with DAPI (4’,6-diamidino-2-phenylindole) and mounted with Fluoromount G (Southern Biotech).

### 2.2.8 Morphometric analysis

The lungs were inflated with 1% low melting agarose (Invitrogen) to 25cm water and post-fixed for 24hr in formalin before processing. Mean linear intercept (MLI) was determined from 4µm paraffin-embedded lung histological sections stained with hematoxylin and eosin. Images were taken of 8 randomly chosen sites and the MLI was calculated with a grid of 130 lines and 250 points and a line length of 118.93 µm utilizing the ImagePro system (MediaCybernetics, Bethesda, MD) (Thurlbeck, 1994).

To quantify vessel density, images were taken of 5 randomly chosen sites, which did not include conducting airways from 4µm histologic sections of formalin-fixed, paraffin-embedded lung tissue stained with vWF. Vessel density was calculated with a grid of 192 points to determine the ratio of the number of vWF immunoreactive points to the number of total lung parenchyma points (Balasubramaniam et al., 2003).

To quantify the percentage of Ki67 positive cells, images were taken of 5 randomly chosen sites, which did not include conducting airways, from 4µm histologic sections of formalin-fixed, paraffin-embedded tissue stained with Ki67 and DAPI. The percentage of Ki67 cells was determined to be the number of Ki67 immunoreactive nuclei to the total number of nuclei per image.

### 2.2.9 Determination of vascular permeability and lung edema

Vascular permeability was quantified by Miles assay as previously described (Moitra et al., 2007). Female mice were anesthetized with isoflurine before intra-tracheal instillation of 2mg/kg LPS (Strain 0127:B8, Sigma) 24hr before sacrifice. Naïve or LPS treated mice were injected i.v. with 20mg/kg Evans Blue 1hr before sacrifice. Mice were perfused through the right ventricle with 10mL PBS using a syringe with a 22 gauge needle until the lungs were
white. Lung tissue was excised, and placed in 1 ml of formamide for 96 h to achieve Evans blue extraction. OD of the supernatants was read at 620 and 740 nm on a spectrophotometer with standard curve in the same solution. Tissue specific correction factor was applied to account for heme and the tissues were weighed, and the µg/g tissue was calculated (Moitra et al., 2007). Lung edema was measured as a ratio of wet lung weight to dry lung weight. Lungs were excised and weighed immediately after, and subsequently dried at 60°C for 36 hr.

2.2.10 Lung volumes and right ventricular hypertrophy
Lung volumes were measured by water displacement (Scherle, 1970). Right ventricle hypertrophy was measured as a ratio (RV:LV+S) of the independent weight of the right ventricle (RV) and the left ventricle with septum (LV+S) (Vadivel et al., 2010).

2.2.11 Lung function measurements
Mice were anesthetized with an intra-peritoneal injection of ketamine and xylazine (450µl of 10 mg/mL ketamine/1 mg/mL xylazine solution). Mice were tracheostomized and intubated with an 18-G catheter and all parameters were measured with a Flexivent apparatus (SCIREQ, Montreal, QC). Mice were given 0.8 mg/kg pancuronium bromide to prevent spontaneous breathing. Respiratory frequency was set at 160 breaths/min with a tidal volume of 0.2 mL, and a positive end-expiratory pressure of 2 to 4 mL H₂O was applied. A “snapshot perturbation” maneuver was imposed to measure resistance (R), compliance (C), and elastance (E) of the whole respiratory system (airways, lung, and chest wall). Forced oscillation perturbation (“primewave-8”) was consequently applied, and resulted in Rn, inertia of the air, tissue damping (resistance) (G), H, and tissue hysteresivity (tissue damping [G]/H). Maximal pressure-volume loops (PV-loops) were subsequently determined to measure vital (total) lung capacity, IC from zero pressure (B), form of deflating PV loop (K), static C (Cst), static elastance (Est), and hysteresis (area between inflating and deflating part of the loop). For each parameter, an average of three measurements was calculated and depicted per mouse (Vanoirbeek et al., 2010).
2.2.12 Multiphoton and second harmonic generation imaging

The utilized microscope system has been described in detail elsewhere (Abraham and Hogg, 2010). The laser used for second harmonic generation (SHG) as well as the multi-photon excitation fluorescent microscopy (MPEF) was a mode-locked femto-second Ti:Sapphire Tsunami (Spectra-Physics, Mountain View, CA) synchronously pumped by a Millenia Xs J (Spectra-Physics) diode-pumped solid-state laser capable of delivering up to 10W pumping power at 532 nm. The laser was directed to a Leica AOBS scan head coupled with Leica upright microscope system (Heidelberg, Germany) at an average power that was below the damage threshold of the samples. Upon entering the Leica microscope system, the laser beam was directed to the scanning mirrors, then through a 690 nm long pass dichroic mirror (690 DCXRU, Chroma Technology) and subsequently focused on the specimens through a 20x/0.95 NA water dipping objective lens (Olympus) and the backscattered emission from the sample was collected through the same objective lens. Leica Confocal Software TCS SP2 was used for the image acquisition. Non-de-scanned detectors in the reflection geometry located close to the objective were used for capturing the 3D images. In the non-de-scanned PMT detectors (R6357, Hamamatsu, Shizuoka, Japan), a 700 nm short pass filter (E700SP, Chroma Technology, USA) was used to prevent the scattered IR laser radiation from reaching the detector and a 455 long pass dichroic beam splitter (455 DCXRU, Chroma Technology, USA) was used to separate SHG signal from the MPEF signal.

For 3D image data set acquisition, the multiphoton excitation beam was first focused at the maximum signal intensity focal position within the tissue sample and the appropriate PMT levels (both the gain and offset levels) were then selected to obtain the pixel intensities within range of 0–255 (8-bit images) using a color gradient function. Later on, the beginning and end of the 3D stack (i.e., the top and the bottom optical sections) were set based on the signal level degradation. Series of 2D images for a selected 3D stack volume were then acquired at slow scan speed i.e., 10 s per 512 x 512 pixels. The 3D stack images with optical section thickness (z-axis) of approximately 1.12 µm were captured from tissue volumes. For each tissue volume reported here, z-section images were compiled and finally the 3D image restoration was performed using Volocity software (Improvisions, UK). A noise-removal filter, whose kernel size of 3x3, was applied to these 3D image volumes.
2.2.13 **Cell viability assay**
Cells were cultured for 48 h in 96-well plates, and the number of viable cells was determined using the ProMega CellTiter 96 AQueous non-radioactive cell assay (MTS) according to the manufacturer’s instructions. With the understanding that this assay measures the metabolic activity of cells, “cell viability” is defined as a combined measure of cells undergoing both apoptosis and proliferation.

2.2.14 **Static adhesion assay**
Cell adhesion to various matrix proteins was performed as previously described with modifications (Grutzmacher et al., 2010). Briefly, 10ug/mL fibronectin (Millipore), collagen type I (Serva Electrophoresis), and laminin (BD BioSciences) and 0.2% gelatin (Sigma) prepared in PBS were coated on 96-well plates (75 μl/well) overnight at 4°C. A set of control wells were coated with 1% BSA. Plates were blocked with 200 μl of 1% BSA prepared in PBS for at least 1 h at room temperature. Cells were prepared by removal from the dish with TrypLE Express buffer (Gibco), washed with PBS, and resuspended at 1 × 10⁶ cells/ml in EC Media. After blocking, liquid was dumped and 100 μl of cell suspension was added to each well. The cells were allowed to adhere to the plate for 90min at 37°C. The non-adherent cells were removed by gently washing the plate four times. The number of adherent cells in each well was quantified by crystal violet staining as previously described (Humphries, 2000). All samples were done in triplicate.

2.2.15 **Spreading assay**
Primary mECs were removed with TrypLE Express, and 0.5x10⁵ cells were plated in uncoated 0.4μm 24-well cell culture inserts or inserts coated with fibronectin, laminin, collagen 1 (BD Biosciences) and grown for 48hr. Images were acquired using OpenLab 4.0 (PerkinElmer) and cell spreading was defined by % thresholded area of cells within the images using ImageJ software (NIH).
2.2.16 Statistical analysis
Data was expressed as means ± standard error of the mean. Statistical analysis was performed using Prism 5 (GraphPad Software). For comparisons between Podxl\textsuperscript{flx} and Podxl\textsuperscript{EC} the Student’s t test was used, and for comparisons between all three genotypes statistics were assessed by ANOVA with a Bonferroni post-test. p<0.05 was considered as a statistical significance.
2.3 Results

2.3.1 Podocalyxin is ablated in Podxl^{ΔEC} mice

Conventional Podxl^{-} mice die perinatally from a kidney defect, making it difficult to evaluate the post-natal role of podocalyxin in other tissues such as the endothelium (Doyonnas et al., 2001). In order to address the function of podocalyxin in vascular function, we generated a vascular specific Podxl knockout mouse by crossing mice carrying conditional floxed to an endothelial-cell specific driven Cre recombinase. The Cdh5-Cre mice have been reported to have Cre activity as early as E7.5 of embryogenesis, with the potential to delete genes expressed in the endothelium of all major and minor vessels including those in all tissue-bed vessels after E14.5 (Alva et al., 2006).

The conditional vector was designed to delete exons 3-7 by Cre-mediated recombination. Figure 2.1 shows the schematic representation of the targeting strategy. Consecutive crosses with mice systemically expressing Flp-recombinase to remove the neo-cassette (ActFlpe mice) and Cre recombinase (Cdh5-Cre) were performed to produce a vascular-null mutation on a mixed genetic background. Subsequently, we crossed our conditional knockout mice to the Rosa-rsYFP. This strain permits the monitoring of Cre-recombinase via a fluorescent reporter (Srinivas et al., 2001). Mice on the Rosa-rsYFP reporter were maintained as a heterozygous floxed (Podxl^{flox/wt}Rosa-rsYFP) in order to maintain the fluorescent reporter in control mice. In all studies, littermates were used as controls, and there were no differences found between Podxl^{ΔEC} mice maintained with or without the Rosa-rsYFP reporter.
Figure 2.1: Targeted, conditional deletion of the Podxl locus

(A) Schematic representation of wild type and transgenic allele. Exons are depicted in red with corresponding exon numbers and introns are in white. loxP and frt sites are depicted with blue and green boxes with arrowheads, respectively. The NeoR cassette is represented by a green box. PCR primers with product sizes are indentified by directional half-arrows. PCR primer sequences are listed in Table 2.1.

(B) Genotyping of conditional Podxl locus to. PCR genotyping using primers in Table 2.1 confirmed deletion of the NeoR cassette to yield “floxed” allele in 3 of 5 mice. Flp-mediated deletion produced a 632bp product (*); otherwise a 438bp product was produced. The 519bp band was detected in mice heterozygous for the floxed allele.

(C) Genotyping of conditional Podxl locus to generate PodxlΔflox mice. PCR genotyping using primers in Table 2.1 confirmed recombination of PodxlΔflox allele from mice crossed to ubiquitous Cre mice. Cre-mediated deletion produced a 285bp product (*); otherwise a 171bp product was produced. A 122bp band was detected in mice heterozygous for the floxed allele.
*Podxl*ΔEC* m*ice are viable, fertile and are undistinguishable from *Podxl*flox littermates. Genotyping of isolated lung endothelial cells by PCR revealed efficient deletion of *podxl* locus as identified by the highly expressed ΔFlox band in *Podxl*ΔEC mice (Fig 2.2A). To confirm podocalyxin expression was ablated in the major vascular beds of *Podxl*ΔEC mice, qRT-PCR was performed on isolated RNA from naïve adult lung, heart, kidney, liver, small intestines and aorta. Podocalyxin mRNA expression was decreased in the lung, aorta, small intestines, and the heart (94%, 89%, 83% and 68% respectively), indicating extremely efficient deletion. We could not detect a noticeable reduction in podocalyxin mRNA in the kidney, likely due to the fact that the bulk of podocalyxin expression in this organ is in the glomerular epithelial cells that obscure the levels of vascular expression (Fig 2.2B).

Histological examination of podocalyxin expression in vascularized tissues confirmed the qRT-PCR expression data (Fig 2.3). In the kidney, the podocytes of the glomeruli and the ductal cells were stained with podocalyxin, but not the endothelial cells found at the centre of the glomerulus (Fig 2.3A arrows) or in large vessels (Fig 2.3A arrowhead). Staining showed complete abrogation in the lung (Fig 2.3B), while small patches of podocalyxin positive endothelial cells remained within the aorta and small intestines (Fig 2.3C,D). A number of other tissues we examined demonstrated weak deletion. Deletion in the heart was less consistent; large vessels such as the pulmonary artery exhibited efficient deletion in all but isolated cells, but deletion was less robust within in the septum and heart muscle (Fig 2.3E) and deletion in the liver was restricted to blood endothelial cells and not sinusoidal endothelial cells (Fig 2.3F). To our surprise, we were able to detect podocalyxin expression within the brain microvasculature (Fig 2.3G arrows) and larger vessels. However, on closer examination of the initial publication by Alva *et al.* it appears that Cre activity was scant in the microvasculature and only detectable at reasonable levels in the large vessels of the brain (Alva *et al.*, 2006).
Figure 2.2: Analysis of podocalyxin expression in PodxlΔEC tissues

(A) Genotyping of isolated lung endothelial cells. Genomic PCR using primers listed in Table 2.1 identifying wild type (WT), transgenic (flox) or functionally deleted Podxl alleles (ΔFlox) via Cdh5-Cre in endothelial cells.

(B) Highly vascularized adult tissues assayed for expression of podocalyxin mRNA by qRT-PCR. cDNA was generated from mRNA isolated from Podxl<sup>flox</sup> (black bars) and Podxl<sup>ΔEC</sup> (white bars) tissues. Gene expression by qRT-PCR from 3 mice per genotype was normalized to Gapdh, and the average of each of the Podxl<sup>flox</sup> tissues set to 1. qRT-PCR primer sequences are listed in Table 2.2. * represents p<0.05 by Student’s t test, when compared to Podxl<sup>flox</sup> mice; error bars = SEM.
Figure 2.3: Histological analysis of podocalyxin expression in PodxlEC tissues

Cdhl-Cre drives deletion of podocalyxin in different organ vascular beds.

(A) Within the kidney, podocalyxin normally expressed by both the epithelia cells (podocytes) as well as the endothelial cells. Deletion is efficient in the PodxlEC mice within the endothelial cells (arrows) as well the larger vessels but not the podocytes (arrow heads).

(B) Expression of podocalyxin is completely abrogated in the lung of PodxlEC mice, while podocalyxin expression can be found in all endothelial cells including in large vessels and the alveolar tissue of Podxlfllox mice.

(C, D) The endothelial cells that line the lumen of the aorta normally express podocalyxin as does the endothelial cells within vessels of the small intestine (arrows), which is lost in all but a few isolated cells in the PodxlEC mice.

(E) In the heart podocalyxin can be found in all endothelial cells, however deletion in the PodxlEC mice in the vessels of the heart muscle is variable, while deletion in large vessels such as the pulmonary artery is complete in all but isolated cells similar to the aorta.

(F) In the liver, PodxlEC mice have expression abrogated in blood endothelial cells, but not in sinusoidal endothelial cells.

(G) In the brain, podocalyxin is normally expressed in the ventricles and endothelial cells, including the microvasculature (arrows). Sections of the brain from PodxlEC mice display similar staining to control mice indicating deletion has not occurred (arrows).
Podxl<sup>flox</sup>

Kidney

Lung

Aorta

Sm. Intestine

Heart

Liver

Brain

Podxl<sup>ΔEC</sup>
Due to the highly efficient deletion of podocalyxin on the lung vasculature, we focused our investigation of the role of podocalyxin in lung endothelium. This also allowed us to take advantage of the fact that isolating endothelial cells from the lung is the most common method for developing mouse primary endothelial cells (mEC), thereby providing us the opportunity to perform both in vitro and in vivo assays. Since Podxl<sup>-/-</sup> mice have increased CD34 mRNA expression in the lung and no overt vascular phenotype, it was proposed that CD34 might compensate for the loss of podocalyxin in endothelial cells (Doyonnas et al., 2001). Therefore, to test this hypothesis we generated double transgenic mice by crossing the constitutive Cd34<sup>-/-</sup> to the Podxl<sup>ΔEC</sup> mice to generate Podxl<sup>ΔEC Cd34<sup>-/-</sup></sup>, which were tested side by side with Podxl<sup>ΔEC</sup> in most in vivo assays.

**2.3.2 Isolation and characterization of Podxl<sup>ΔEC</sup> and Podxl<sup>ΔEC Cd34<sup>-/-</sup></sup> lung endothelial cells**

To determine if there were any gross changes in the endothelial cells within the lung, we analyzed adult lung tissue for genes and cell surface markers characteristic of the endothelium. While mRNA expression of Cdh34 and Cdh5 was upregulated in Podxl<sup>ΔEC</sup> mice and Vegf164, Ang1 and Ang2 were downregulated in Podxl<sup>ΔEC Cd34<sup>-/-</sup></sup> mice (Fig 2.4B), surface expression of endothelial cell surface markers such as CD31, CD34, endoglin, Tie2, and ICAM2 were identical to Podxl<sup>fl<sub>x</sub></sup> control mice (Fig 2.4A). The increased CD34 mRNA expression is likely due to intracellular stores of the protein that is not picked up by the FACS surface staining.
Figure 2.4: Gene expression and surface expression of endothelial cell markers in PodxlEC and PodxlECCd34−/− lungs

(A) Lung tissue was assessed for expression of endothelial cell markers by flow cytometry. Shown are representative flow cytometry histograms. The red profile shows background staining in the presence of isotype control antibody, Podxl^flox in blue, Podxl^ΔEC in green, and Podxl^ΔECCd34−/− in orange. Note the similar expression levels of the cellular markers that have not been genetically altered.

(B) Lung tissue assayed for expression of endothelial cell markers by qRT-PCR. cDNA was generated from mRNA isolated from Podxl^flox (black bars), Podxl^ΔEC (white bars), and Podxl^ΔECCd34−/− (striped bars) lung tissue. Gene expression by qRT-PCR from 4 mice per genotype was normalized to Gapdh, and the average of the Podxl^flox set to 1. qRT-PCR primer sequences are listed in Table 2.2. * represents p<0.05 by ANOVA with Bonferroni post-test when compared to Podxl^flox mice; error bars = SEM.
2.3.3  *Podxl^ΔEC* mice have increased basal and inflammation-induced lung vascular permeability in vivo

Regulation of vascular permeability is an important component of homeostasis. We utilized the Miles assay to measure basal vascular permeability in naïve mice and inflammation-induced permeability using LPS as a broad-spectrum inducer of inflammation. In contrast to naïve *Cd34^-/-* mice, naïve *Podxl^ΔEC* mice exhibit a two-fold increase in permeability in the lung. Similar results were found with the *Podxl^ΔEC Cd34^-/-* mice suggesting that podocalyxin and not CD34 is responsible for regulating basal permeability. When mice were treated intra-tracheally with LPS 24hr before sacrifice, *Podxl^ΔEC* mice showed an additional increase in permeability when compared to *Podxl^flax* mice that was mirrored by the *Podxl^ΔEC Cd34^-/-* mice (Fig 2.5A). Closer examination of basal permeability in other organs showed no changes when compared to *Podxl^flax* mice (Fig 2.5B), which suggests that podocalyxin specifically regulates basal permeability in the lung. However, further work is needed to characterize the expression levels of podocalyxin in all tissues examined to determine whether the efficiency of deletion might influence the permeability in these tissues. Since increased permeability if often associated with edema, we determined the ratio of water in the lungs of naïve mice. To our surprise, there were no overt changes in the water weight, suggesting no increased edema in the lungs of *Podxl^ΔEC* mice (Fig 2.5C). Additionally, we questioned whether the vascular density in these mice was comparable. Classically, vWF staining is used to identify vessels within the lung rather than the capillaries found within the alveolar structures (Müller et al., 2002; Pusztaszeri et al., 2006). Using vWF vessel staining, no differences in vascular density between groups were identified (Fig 2.5D). Finally, a number of genes implicated in regulating barrier function including eNOS and iNOS, and ZO-2 were found to have increased mRNA expression in naïve lungs of *Podxl^ΔEC* but not *Podxl^ΔEC Cd34^-/-* mice (Fig 2.5E).
Figure 2.5: Basal and inflammatory induced vascular permeability in $Podxl^{EC}$ and $Podxl^{EC}Cd34^{-/-}$ mice

(A, B) Vascular permeability assessed by Miles Assay. Mice were treated with PBS (naïve) or LPS (2mg/kg) intra-tracheally 24hr before sacrifice (A). Data from (B) are from naïve mice. Mice were injected I.V. with Evans Blue 1hr before sacrifice. Mice were subsequently perfused with PBS until the lungs were white, lung tissue was excised, and placed in formamide for 96 h to extract the dye from tissues. The tissues were weighed; dye concentration measured by OD at 620 and 740nm on a spectrophotometer with a standard curve in the same solution and the $\mu$g dye/g tissue was calculated. Data represent 5-7 mice per genotype. * represents p<0.05 by ANOVA with Bonferroni post test when compared to $Podxl^{flox}$ naïve mice, # represents p<0.05 by ANOVA when compared to $Podxl^{flox}$ LPS treated mice.

(C) Lung edema measured by lung water weight. Lungs from naïve mice were excised and weighed immediately to determine wet weight. Dry weight determined after lungs were dried for 36hr at 60°C. Lung edema presented as a ratio of wet/dry lung weight. Data represent 6 mice per genotype.

(D) Vessel density assessment by vWF expression. Vessel density was determined by the ratio of vWF vessel staining to total lung tissue. Data represent 4 images per mouse, 4 mice per group.

(E) Lung tissue assayed for expression of permeability related genes by qRT-PCR. cDNA was generated from mRNA isolated from $Podxl^{flox}$ (black bars), $Podxl^{EC}$ (white bars), and $Podxl^{EC}Cd34^{-/-}$ (striped bars) lung tissue. Gene expression by qRT-PCR from 4 mice per genotype was normalized to Gapdh, and the average of the $Podxl^{flox}$ set to 1. qRT-PCR primer sequences are listed in Table 2.2.

* represents p<0.05 by ANOVA with Bonferroni post-test when compared to $Podxl^{flox}$ mice; error bars = SEM.
2.3.4 Deletion of vascular Podxl contributes to enlarged airspaces

Lung alveolar function requires the development of a tight alveolar-capillary complex to facilitate pulmonary gas exchange. There has been substantial data recently showing that the loss of vascular related genes, as well anti-angiogenic treatments, are sufficient to cause defects in alveolarization (Han et al., 2004; Voelkel and Taraseviciene-Stewart, 2005; Bridges and Weaver, 2006; Galambos and deMello, 2007; Knudsen et al., 2010). Previous analysis of the constitutive Podxl<sup>+/+</sup> mice showed no defects in embryonic lung development (Doyonnas et al., 2001). However, much of lung development, including extensive alveolarization, occurs post-natally. Therefore we examined the histological sections of control, Podxl<sup>ΔEC</sup> and Podxl<sup>ΔEC Cd34<sup>−/−</sup></sup> to determine whether post-natal alveolarization was impacted. At post-natal day 14, we found enlarged airspaces in Podxl<sup>ΔEC Cd34<sup>−/−</sup></sup>, as quantified by mean linear intercept analysis. This was resolved by postnatal day 28, suggesting a transient delay, but then increased again into adulthood (10 weeks) (Fig 2.6). Interestingly, the Podxl<sup>ΔEC</sup> mice also showed increased airspaces but only in adulthood (10 weeks of age). It is known that with advanced age, there is a progressive increase in the airspaces and by 7 months we found that control mice had caught up to Podxl<sup>ΔEC</sup> mice (Sueblinvong et al., 2011). We conclude that loss of podocalyxin leads to a premature increase in mean linear intercept, which is indicative of increased airspaces.
Figure 2.6: Alveolarization in Podxl<sup>EC</sup> and Podxl<sup>EC Cd34<sup>-/-</sup></sup> mice.

H&E sections of lungs from Podxl<sup>floxflox</sup>, Podxl<sup>ΔEC</sup>, and Podxl<sup>ΔEC Cd34<sup>-/-</sup></sup> obtained at 14 days, 28 days, 10 weeks and 7 months. Loss of podocalyxin results in defects in alveolarization as early as 14 days in Podxl<sup>EC Cd34<sup>-/-</sup></sup> that is resolved by postnatal day 28. However, both Podxl<sup>ΔEC</sup> and Podxl<sup>ΔEC Cd34<sup>-/-</sup></sup> mice exhibit increased MLI at 10 weeks of age. Mean linear intercepts determined by computer-assisted image analysis. Data represent 6 mice per genotype, * represents p<0.05, ** represents p<0.01 by ANOVA with Bonferroni post-test when compared to Podxl<sup>flox</sup> mice; error bars = SEM.
2.3.5 *Podxl\textsuperscript{ΔEC}* mice have increased lung volume and resistance in both the central airways and lungs

Pathological changes to the tissue, such as increased airspaces, often result in defects in lung function. To evaluate lung structure defects, we inflated the lungs of mice to a fixed pressure of 25cm water and measured the resulting lung volume at different time points during development. Both *Podxl\textsuperscript{ΔEC}* and *Podxl\textsuperscript{ΔEC} Cd34\textsuperscript{-/-}* mice lung volumes were significantly larger at 28 days (45% and 29% larger, respectively) and 10 weeks (66% and 62% larger, respectively) than the *Podxl\textsuperscript{flox}* controls (Fig 2.7A). This suggests either lung hypertrophy or a defect in lung compliance, similar to that seen with emphysematous lungs and other transgenic mice with defects in alveolarization (Kasahara et al., 2000; Mitani et al., 2009). COPD patients display increased airspaces and lung compliance, and can develop complications including pulmonary hypertension (Voelkel et al., 2011). Although right ventricle hypertrophy often precedes pulmonary hypertension, we found no differences in right ventricle to left ventricle plus septum (RV/LV+S) mass ratios between groups (Fig 2.7B).
Figure 2.7: Physiological analysis of PodxlEC and PodxlEC Cd34−/− mice

(A) Lung volume of Podxlflx, PodxlEC, and PodxlEC Cd34−/− mice. Mean lung volumes at each stage of development as determined by volume displacement. Data represent 4-6 mice per genotype, ** represents p<0.01 by ANOVA with Bonferroni post-test when compared to Podxlflx mice at each time point; error bars=SEM.

(B) Representative images of inflated lungs (4 left lobes) at 28 days and 10 weeks

(C) Right ventricular hypertrophy. Data represent 12 mice per group. No differences were found in RV/LV+S weight measurements between Podxlflx, PodxlEC, and PodxlEC Cd34−/− mice.

To understand whether the enlarged airspaces affected lung function, we examined the PodxlΔEC mice using an invasive pulmonary function apparatus (Flexivent, SCIREQ). This allowed us to look at a number of functional parameters similar to those used in human studies (Vanorbeek et al., 2010). PodxlΔEC mice exhibited increased resistance in both the airways and lung parenchyma, but there were no changes to other parameters such as total lung capacity and elastance (Fig 2.8 & 2.9). The obvious discrepancy between this data and the increased lung volume described above can likely be attributed to the fact that the functional parameters measured by the flexivent is limited by the chest wall, while the above measurements are not.
Previous analysis of the \( Cd34^{-/-} \) using the Flexivent showed changes in response to methacholine challenge, but naïve mice did not display defects in any other lung function parameters including, lung resistance (Blanchet et al., 2007). Further studies will be required to characterize the lung function in \( Podxl^{ΔEC}Cd34^{-/-} \) mice and we hypothesize that there will not be any additional phenotype in these mice because there has been no apparent contribution of CD34 to the lung phenotype over and above that seen with the \( Podxl^{ΔEC} \) mice.

![Figure 2.8: Lung mechanical analysis of \( Podxl^{flk} \) mice](image)

Resistance measurements from primewave-8 (A) and snapshot (B) perturbation. On the FlexiVent (SCIREQ), the snapshot and primewave-8 perturbations were performed, in naïve tracheotomized mice. In the primewave-8 perturbation the resistance is measured within the large airways (Rn) and in the snapshot perturbation, the resistance is indicative of the whole thorax (lung and chest wall). Data represent ten mice per genotype, * represents p<0.05 by Student’s \( t \) test, when compared to \( Podxl^{flx} \) mice at each time point; error bars = SEM.
**Figure 2.9: Lung mechanics of Podxl<sup>EC</sup> lungs**

Mechanical measurements on the FlexiVent (SCIREQ) including snapshot and primewave-8 perturbation and PV-loop pressure regulated (PVr-P). On the snapshot (elastance [E], and C) and primewave-8 perturbations (tissue damping (resistance) [G], and tissue elasticity [H]) and from the PV-loops, perturbation parameters, such as total lung capacity (A), form of deflating PV loop (K), static compliance (Cst), static elastance (Est), and hysteresis (area in PV loop) were determined. All measurements were obtained from naive tracheotomized mice. In the snapshot perturbation, the lung is seen as a single compartment and the measurements are indicative of the whole thorax (lung and chest wall). In the primewave-8 perturbation the measurements are indicative of the lung tissue. Data represent 10 mice per genotype, no significant differences found in any measurements by Student’s t test; error bars = SEM.
2.3.6 Lung maintenance is impaired in \( Podxl^{ΔEC} \) mice

Since the increased airspaces are detected primarily in adult but not the developing lungs of juvenile \( Podxl^{ΔEC} \) mice, we speculated that there might be defects in lung maintenance. A recent landmark paper by Ding et al. revealed that pulmonary endothelial cells provide key angiocrine signals to initiate and maintain regenerative lung alveolarization (Ding et al., 2011). They found that MMP14 is induced in a pneumonectomy model during active alveolar regeneration and that MMP14 expressed by the pulmonary endothelial cells is required for expansion of the epithelial progenitor cells during alveologenesis. Therefore we investigated MMP14 expression levels in the lung tissues of \( Podxl^{fl}\), \( Podxl^{ΔEC} \) and \( Podxl^{ΔEC Cd34^{-/-}} \) mice in adult mice. Typically MMP14 is expressed early in the lung development, but transcription is shut off around postnatal day 10 and remains so throughout the life of the mouse (Mariani et al., 2002; Atkinson et al., 2005). Surprisingly, we found that MMP14 was re-expressed in adult \( Podxl^{ΔEC} \) lungs, but not in the lungs of control (\( Podxl^{fl} \)) or \( Podxl^{ΔEC Cd34^{-/-}} \) (Fig 2.10A). Furthermore, increased vWF expression can also be detected in the alveolar tissues of patients who have undergone a pneumonectomy and endothelial cells undergoing regeneration after injury will increase expression of vWF in culture (Reidy et al., 1989; Jin et al., 2001; Maeda et al., 2001). Upon closer examination of the vWF staining, we found that vWF was consistently increased in the alveolar tissue of \( Podxl^{ΔEC} \) mice but not \( Podxl^{ΔEC Cd34^{-/-}} \) mice when compared to control \( Podxl^{fl} \) mice (Fig 2.10B).

Since MMP14 was required to induce epithelial progenitor cell proliferation, we investigated whether we could detect increased proliferation within the alveolar tissue of \( Podxl^{ΔEC} \) mice. Peculiarly, using Ki-67 staining to quantify cells undergoing proliferation, we determined that \( Podxl^{ΔEC} \) mice have decreased proliferation in the alveolar spaces when compared to control \( Podxl^{fl} \) mice (Fig 2.10C). Further work is required to determine exactly which cell types are proliferating, however the cells appear to have large nuclei, suggestive of type 2 alveolar cells. These data would suggest that naïve \( Podxl^{ΔEC} \) are trying to initiate regeneration by re-activating MMP14 and vWF, but lack the ability to initiate proliferation of
the epithelial cells, possibly due to impaired release of angiocrine signals by the endothelium that result in a downstream failure to support epithelial survival.

Figure 2.10: MMP14 expression and lung proliferation by Ki67 staining
(A) Lung tissue was assessed for expression of MMP14 expression at 10 weeks. cDNA was generated from mRNA isolated from Podxl$^{\text{flox}}$ (black bars), Podxl$^{\Delta \text{EC}}$ (white bars), and Podxl$^{\Delta \text{EC} \text{Cd34}^-}$ (striped bars). Relative gene expression by qRT-PCR from 4 mice per genotype was normalized to Gapdh, and the average of the normalized expression is shown for each time point. qRT-PCR primer sequences are listed in Table 2.2. N.D = no transcript detected after 40 cycles.
(B) Alveolar vWF staining density. vWF staining density was determined by the ratio of vWF staining in alveolar tissue to total lung tissue. Data represent 4 images per mouse, 4 mice per group. N.S = not significant when compared to Podxl$^{\text{flox}}$ mice
(C) Proliferation by Ki67 staining. Lung sections were stained with Ki67 and DAPI expressed as a percentage of total alveolar cells in a 20x field. Data represent 3 mice per group.
* represents p<0.05 by ANOVA with Bonferroni post-test, when compared to Podxl$^{\text{flox}}$ mice; error bars = SEM.

2.3.7 Gene profiling of adult lungs show changes in matrix components, and structural genes as a result of deletion of podocalyxin in endothelial cells
To understand how the loss of podocalyxin affects other genes involved with lung maintenance, qRT-PCR was performed on lung samples at 10 weeks. Levels of Sftpc and Fgfr4 were upregulated in Podxl$^{\Delta \text{EC}}$ lungs, as were transcripts for Colla1, Colla2, and elastin (Fig 2.11). Surfactant protein C (Sftpc) is a classic marker for lung epithelial progenitor cells and fibroblast growth factor receptor 4 (Fgfr4) is known to be upregulated during lung alveolarization (Weinstein et al., 1998); both suggesting that there is active alveologenesis in adult Podxl$^{\Delta \text{EC}}$ lungs. Collagen 1 and elastin are key components of the lung extracellular
matrix, and are essential for maintaining the structure and function of the lung (McGowan, 1992; Cardoso et al., 1993; Chapman, 2004). Haploinsufficiency of the elastin gene is sufficient to modulate lung regeneration, and Col1a1, Col1a2, and Eln are all upregulated early in the regeneration cascade (Mariani et al., 2002; Hoffman et al., 2010; Wolff et al., 2010). This led us to question whether the organization of collagen and elastin were disrupted as a result.

Figure 2.11: Gene expression of matrix, adhesion and lung structure related genes in PodxlEC and PodxlEC Cd34−/− lungs

(A,B) Lung tissue was assessed for expression of matrix and adhesion related transcripts (A) and lung structure transcripts by qRT-PCR (B). cDNA was generated from mRNA isolated from Podxllox (black bars), PodxlEC (white bars), and PodxlEC Cd34−/− (striped bars) lung tissue. Relative gene expression by qRT-PCR from three mice per genotype was normalized to Gapdh, and the average of the Podxllox set to 1. qRT-PCR primer sequences are listed in Table 2.2. * represents p<0.05 by ANOVA with Bonferroni post-test when compared to Podxllox mice; error bars = SEM.
2.3.8 Structural matrix components are mislocalized in lungs lacking podocalyxin

To visualize the 3-dimensional (3D) structure of matrix components in adult lung alveolar walls, we utilized second harmonic generation (SHG) paired with multi-photon excitation fluorescent microscopy (MPEF). The non-centrosymmetric collagen molecules produce a specific second harmonic signal, while elastin molecules generate endogenous fluorescent signal allowing us to image both of these extra-cellular structural proteins without any exogenous labeling with spatial resolution and specificity (Abraham and Hogg, 2010). In contrast to healthy human alveolar tissues, where the arrangements of fibrillar collagens and elastin complement each other, healthy mouse alveolar tissues have relatively less amounts of fibrillar collagen but where present, fibrillar collagen is always co-localized with elastin (Fig 2.12A Orange Arrows). The round objects in the alveolar tissue likely represent macrophages and red cells that are resident within the lung, since both are known to emit within the elastin spectrum (Pena et al., 2007; Abraham et al., 2011). Our direct comparison of the 3D images, which show the distribution of collagen and elastin in Podxl\textsuperscript{floex} and Podxl\textsuperscript{\textDelta EC} mice, identified significant amounts of fibrillar collagen in Podxl\textsuperscript{\textDelta EC} alveolar regions that was not localized with any elastin (Fig 2.12A Yellow Arrows). This is further represented in the co-localization scatter plots (Fig 2.12B) that show increased fibular collagen intensity that is not associated with any elastin pixels (Fig 2.12C). Therefore, we conclude that Podxl\textsuperscript{\textDelta EC} mice produce increased amounts of fibrillar collagen in enlarged alveolar spaces, likely, in order to maintain lung structure.
Figure 2.12: Second harmonic generation and multi-photon imaging

(A) Representative optically magnified SHG image originating from the collagen matrix overlaid with the MPEF images from 2 mice. Scale bar: 120µm. These are 3D extended focus views representing ~150 µm thick tissue section. These optically magnified images clearly show the ultrastructural differences between collagen and elastin components. The collagen appeared to be in the form of spirally wound collagen (violet color) while the lung elastin (green color) consisted of fine fibers. Areas of co-localization appear white in colour (orange arrows) and are found throughout PodxlPlus lung samples, while the PodxlΔEC exhibit areas of collagen that do not co-localize with elastin found primarily in larger alveolar spaces (yellow arrows). n= 2 images per mouse, 3-5 mice per group.

(B) The respective scatter plots of the images presented in (A) where SHG pixel intensities (y-axis) plotted as a function of elastin pixel intensities (x-axis) show voxel to voxel colocalization relationship between fibrillar collagens and elastic fibers. Non-colocalized SHG signals in gated areas are considerably higher in PodxlΔEC lungs when compared to PodxlPlus lung samples.

(C) Quantification of non-colocalized collagen pixels in the scatter plots represented in (B). % collagen is calculated base on the number of pink pixels, which represent collagen, compared to the total number of pixels in each dot plot.
A  Podxl<sup>flox</sup>  Podxl<sup>ΔEC</sup>

B  Podxl<sup>flox</sup>  Podxl<sup>ΔEC</sup>

C  Non-colocalized collagen

% Collagen Pixels

- Podxl<sup>flox</sup>  Podxl<sup>ΔEC</sup>
2.3.9 Isolation and characterization of cultured Podxl^{ΔEC} lung endothelial cells

In order to further evaluate the functional defects in lung endothelial cells without the complications of multiple cell types contained within the alveoli, we isolated primary mouse pulmonary capillary endothelial cells from Podxl^{flx} and Podxl^{ΔEC} mice. Briefly, CD31^{+}/CD45^{-} cells were purified from lungs and analyzed within the first 8 passages after culture. Five primary lung mEC cultures from each genotype were developed. Similar to freshly isolated endothelial cells, cultured lung mEC from Podxl^{flx} and Podxl^{ΔEC} mice maintained identical expression of endothelial cell surface markers in culture (Fig 2.13A compared to Fig 2.4A). Furthermore, no significant differences were seen in mRNA levels of these EC genes (Fig 2.13B). We next assessed cell viability in lung mEC under normal growth conditions using an MTS assay. Since this assay is a true measure of cell metabolic activity, we defined “cell viability” here as a combined measure of cells undergoing both apoptosis and proliferation. With the exception of one Podxl^{ΔEC} line, all knockout primary cell cultures exhibited lower cell viability over 48hr (Fig 2.13C). This resulted in loss of endothelial cell marker expression or senescence at passage 6, while control cells could be consistently maintained up to passage 10.
Figure 2.13: \( \text{Podxl}^{\text{EC}} \) lung mEC have identical endothelial markers to \( \text{Podxl}^{\text{flox}} \) lung mEC

(A) Lung mEC assayed for expression of endothelial cell markers by flow cytometry. Shown are representative histograms. The red profile shows background staining in the presence of control isotypes, with \( \text{Podxl}^{\text{flox}} \) in blue and \( \text{Podxl}^{\text{EC}} \) in green. Note the similar expression of these cellular markers by all cells.

(B) Cultured lung mEC assayed for expression of endothelial cell markers by qRT-PCR. cDNA was generated from mRNA isolated from \( \text{Podxl}^{\text{flox}} \) (black bars) and \( \text{Podxl}^{\text{EC}} \) (white bars) cultured lung mECs. Relative gene expression by qRT-PCR from 3 primary cell cultures per genotype was normalized to GAPDH, and the average of the \( \text{Podxl}^{\text{flox}} \) mEC set to 1. qRT-PCR primer sequences are listed in Table 2.2. * indicates \( p < 0.05 \) by Student’s \( t \) test, error bars=SEM

(C) Cultured lung mEC assayed for viability. 1000 cells were plated in a 96 well plate, and viable cells were assessed by ProMega CellTiter 96 AQueous assay after 48hr of growth. Graph representative of 2 experiments, with 4 primary cell cultures per genotype. Error bars=SEM, * indicates \( p < 0.05 \) by Student’s \( t \) test.
2.3.10 Loss of podocalyxin affects static adhesion and spreading of endothelial cells, likely through modulation of integrin expression

To understand the role of podocalyxin in endothelial cell adhesion, lung mECs were allowed to adhere to tissue culture wells coated with fibronectin, laminin, collagen type I, collagen type IV and gelatin for 90 min. This time frame is sufficient to permit static adhesion, but does not allow cell spreading. *Podxl*ΔEC ECs exhibited increased adhesion to fibronectin, but not to laminin, collagen type I, or collagen type IV (Fig 2.14A).

We also evaluated the role of podocalyxin in endothelial spreading. Lung mEC were plated on pre-coated 0.4 μm pore inserts, which provide an environment that is more typical of an *in vivo* setting. Control and podocalyxin-deficient lung mECs exhibit a similar ability to spread on fibronectin-coated inserts over 48 hr (Fig 2.14B&C Fibronectin). In stark contrast, *Podxl*ΔEC lung mEC exhibited a severe impairment in their ability to spread on laminin-coated inserts in comparison to control lung mEC. *Podxl*ΔEC lung mEC made initial contact with the substrate but failed to flatten and elongate. Instead these cells formed clumps that remained attached to the insert surface that could be maintained through 96 hr (Fig 2.14B&C Laminin). Plating *Podxl*ΔEC lung mEC on collagen type I yielded an intermediate phenotype; the cells displayed a limited ability to spread on this matrix, but not as readily as control lung mEC. In summary, our data suggest a profound and selective alteration in the ability of *Podxl*ΔEC lung mEC to adhere to a subset of extracellular matrices.
Figure 2.14: Adhesion and spreading of lung mECs on matrix

(A) Static adhesion of lung mEC to fibronectin, laminin, gelatin, collagen I, and collagen IV. Podxlflox (black bars) and PodxlΔEC (white bars) cultured lung mEC were plated on matrix-coated plates for 90min, washed and adhesion quantified by crystal violet absorbance. The average absorbance for the uncoated wells was normalized to 1 with relative amounts shown.

(B, C) Spreading of lung mEC on matrix coated transwells. Podxlflox (black bars) and PodxlΔEC (white bars) cultured lung mEC were plated on coated 0.4μm transwells and cultured for 48hr. Percent monolayer coverage (spreading) was assessed by the threshold area of the cell monolayer via ImageJ on at least 3 independent cell lines per genotype. Error bars=SEM, * indicates p<0.05 by Student’s t test.
Cell adhesion to the extracellular matrix is largely determined by the expression of and activation status of integrin adhesion receptors. Integrins are composed of alpha and beta chains that determine the specificity of binding to each type of matrix. Therefore we evaluated the mRNA expression of the individual integrin alpha and beta subunits responsible for fibronectin, laminin and collagen type I binding. Levels of Itga5, Itga6, and Itgb1 were selectively upregulated in Podxl\textsuperscript{ΔEC} lung mEC when compared to Podxl\textsuperscript{floxed} cells (Fig 2.15A). The integrin chains α5 and α6 heterodimerize with the β1 chain to form receptors for fibronectin and laminin respectively. We also evaluated the mRNA expression level of extracellular matrix proteins of control and Podxl\textsuperscript{ΔEC} lung mEC. Within the lung, Laminin 8 and 10 are the predominant laminin isoforms present and are composed of chains α4β1γ1 or α5β1γ1 respectively (Hallmann et al., 2005). Laminin α4 and γ1 transcripts were both upregulated in Podxl\textsuperscript{ΔEC} mEC suggesting an increase in Laminin 8 synthesis. At the protein level, we verified the increased surface expression of β1 integrin by flow cytometry, however there were no changes in α6 integrin surface expression (Fig 2.15B&C).
Figure 2.15: Expression analysis of integrins involved in matrix binding

(A) Gene expression of integrins and laminin isoforms. cDNA was generated from mRNA isolated from \textit{Podxl}\textsuperscript{flox} (black bars) and \textit{Podxl}\textsuperscript{ΔEC} (white bars) cultured lung mEC. Gene expression by qRT-PCR from 3 primary cell cultures per genotype were normalized to \textit{Gapdh}, and the average of the \textit{Podxl}\textsuperscript{flox} ECs set to 1. qRT-PCR primer sequences are listed in Table 2.2.

(B, C) Lung vECs assayed for expression of laminin binding integrins by flow cytometry. Shown are representative histograms. The red profile shows background staining in the presence of control isotypes, \textit{Podxl}\textsuperscript{flox} in blue and \textit{Podxl}\textsuperscript{ΔEC} in green and quantification of the mean fluorescent intensity (MFI) (C). Protein expression by flow cytometry from 2 lines per genotype were analyzed and averaged between 2 experiments. Error bars=SEM, * indicates p<0.05, ** indicates p<0.01 by Student’s t test.
2.4 Discussion

Podocalyxin expression was identified in endothelial cells over 20 years ago (Horvat et al., 1986), but only recently have any insights into its function been revealed. In the developing aorta, expression of podocalyxin is required for lumen formation. The negative charge provided by the sialic acid modified glycans on the extracellular domain of podocalyxin defines the luminal face of the endothelial cells and is thought to facilitate the expansion of the aortic lumen. Thus, in the absence of podocalyxin there is a delay in aortic lumen formation (Strilić et al., 2009). Despite this delay, the Podxl⁻/⁻ mice show otherwise normal vasculature at birth, but further work in understanding the role of podocalyxin in the adult vasculature has been inhibited by the fact that Podxl⁻/⁻ mice die perinatally, likely as a result of a kidney defect (Doyonnas et al., 2001). Therefore, to understand the role of podocalyxin in adult vasculature, we generated an endothelial specific knockout, PodxlΔEC, utilizing the Cre/LoxP system (Nagy, 2000). While there are a number of documented endothelial-specific Cre-deleter mice, we selected the transgenic mouse containing the Cre recombinase driven by the VE-Cadherin promoter (gene name Cdh5). This mouse is more applicable for our purposes because it has less contribution to secondary cell types, such as hematopoietic cells, leading to a reduced chance of off-target deletion effects (Alva et al., 2006). Another transgenic mouse commonly used for endothelial specific deletion is the Tek-Cre (Kisanuki et al., 2001; Rawlins and Perl, 2012). However, these mice are not appropriate for our studies because they can induce germline deletion that is not under control of the Cre recombinase, which in our case would result in similar perinatal lethality to the Podxl⁻/⁻ mice (de Lange et al., 2008).

I determined that deletion of podocalyxin in PodxlΔEC mice was very efficient in the glomerular endothelial cells, the aorta, and in both macrovessels and alveolar microcapillaries of the lung. Deletion was variable in both the heart and brain, where podocalyxin expression was lost in large vessels but not capillaries within those tissues. Although in principle, this could reflect selection against mice that efficiently delete in these structures to a deleterious effect, several observations argue against this interpretation. First, we have observed a normal Mendelian frequency of mice with the PodxlΔEC genotype as part
of generous litters (up to 10 pups per litter), suggesting there is no loss of a subset of these mice during development. Secondly, upon careful re-evaluation of the published literature of the Cdh5-Cre deleter strain we note that Cre activity in this strain appears to be most efficient in the lung, “patchy” in the heart and particularly poor in the brain endothelium: a pattern very similar to what we observe for podocalyxin deletion. Therefore it is likely that the tissue specific deletion pattern reflects the Cre expression rather than the selection of animals that have poor efficiency in deleting podocalyxin in the brain and heart.

With this in mind, I focused my attention on evaluating podocalyxin function in the lung. This offered an organ system for evaluating podocalyxin function in the absence of other cell types that express the gene (mesothelial, hematopoietic progenitors, etc) and where we were certain of high efficiency of Podxl gene inactivation. Importantly, this study represents the first attempt to assess the functional importance of podocalyxin in adult endothelial cell biology in vivo and in vitro.

2.4.1 Podocalyxin is required to maintain vascular permeability

Ex vivo analysis of Podxl\textsuperscript{ΔEC} and Podxl\textsuperscript{ΔEC}Cd34\textsuperscript{-/-} mice showed similar relative numbers of lung endothelial cells with no changes in classical endothelial cell surface markers when compared to control mice. In addition, there are no differences in vessel density by vWF staining. However, my analysis of vascular permeability found podocalyxin to be required in the maintenance of barrier function. Podxl\textsuperscript{ΔEC} mice exhibit increased basal vascular permeability in the lung, without any edema. Interestingly, further analysis of other organs does not display any alteration in basal permeability. When Podxl\textsuperscript{ΔEC} mice undergo an inflammatory challenge such as intra-tracheal instillation of LPS, Podxl\textsuperscript{ΔEC} mice display a further increase in permeability. To our surprise, there was no additional increase in vascular permeability in either naïve or LPS treated Podxl\textsuperscript{ΔEC}Cd34\textsuperscript{-/-} mice over Podxl\textsuperscript{ΔEC} mice. Previously we showed that CD34 is involved in regulating vascular permeability in tumor vasculature and during inflammatory insults (Blanchet et al., 2010; Maltby et al., 2011). Coupled with this new data, this would suggest that podocalyxin plays the dominant role in regulating permeability that cannot be further exacerbated by the deletion of both genes.
In the lung, the capillaries maintain extremely tight control of vascular permeability through 3 mechanisms: the glycocalyx, the transcellular pathway and the paracellular pathway and podocalyxin could be envisioned to regulate lung vascular permeability by any one of these pathways. First of all, podocalyxin could regulate basal permeability by modulating the endothelial glycocalyx. This vascular luminal barrier consists of glycoproteins, glycosaminoglycans, enzymes and proteins, which carry a strong negative charge, and is necessary to maintain homeostasis of the vessel wall and inhibit its interaction of the blood cells and products (van den Berg et al., 2006; Curry and Adamson, 2010). Simply modulating the charge of the glycocalyx, either by the removal of sialic acids with neuraminidase or by neutralizing the negative charge with protamine sulfate, is sufficient to increase albumin permeability (Swanson and Kern, 1994; Singh et al., 2007; Curry and Adamson, 2010). Considering podocalyxin provides the majority of the negative charge found in the kidney epithelium (Kerjaschki et al., 1984), it is presumable that it could do so in endothelial cells as well. As such, endothelial specific loss of podocalyxin would decrease the negative charge of the glycocalyx, thereby increasing basal vascular permeability to albumin in the lung.

Secondly, the phenotype seen in the Podxl\textsuperscript{ΔEC} mice is reminiscent of the phenotype seen with activation of gp-60, namely a 2-fold increased basal permeability without any lung edema and increased eNOS mRNA expression. Gp-60 functions as a receptor for albumin and regulates caveolae-mediated transcytosis of albumin (Tiruppathi et al., 1997). Activation of gp-60, through Src, is sufficient to dissociate eNOS from caveolin-1, resulting in eNOS phosphorylation and increased NO production (Maniatis et al., 2006). Interestingly, it is possible that podocalyxin could associate in caveolae through interactions with NHERF-1/2. Therefore it is tempting to speculate that podocalyxin could regulate basal permeability by localizing in or near caveolae and restrict albumin association with gp-60 through its bulky negative charge and limit albumin transcytosis.

Additionally, podocalyxin could regulate vascular permeability through the paracellular pathway by altering the actin cytoskeleton or endothelial cell junction localization and
integrity. Podocalyxin is able to recruit scaffolding proteins such as, NHERF 1/2, cortactin, and ERM proteins (ezrin, moesin and radixin) to the apical membrane at the expense of the junction domains. Presumably, loss of podocalyxin from the apical domain of cells would no longer provide a “sink” for these cytoskeletal ligands. Except for NHERF1/2, these scaffolding proteins can regulate actin cytoskeleton re-organization downstream of factors that induce vascular permeability. For example, vascular hyperpermeability induced by TNF-α depends on the phosphorylation of ERM proteins to alter the actin cytoskeleton (Koss et al., 2006). Similarly, cortactin regulates the cortical actin/stress actin ratio necessary to maintain normal endothelial cell contacts through the Epac/Rac1 pathway. Consequently, loss of cortactin increases basal permeability by decreasing the levels of activated Rac1 necessary to maintain endothelial cell contacts (Schnoor et al., 2011). It is then possible that loss of podocalyxin would be sufficient to change the localization of its cytoplasmic ligands such as cortactin and the ERM proteins and thus alter how they interact with the actin cytoskeleton to maintain cell-cell and cell-matrix contacts necessary to maintain vascular permeability.

Loss of podocalyxin could also be sufficient to alter vascular permeability simply by changing the localization endothelial cell junctions and integrity. In epithelial cells, overexpression of podocalyxin in kidney or mammary epithelial cells is sufficient to expand the apical domain, shifting adherence and tight junctions toward the basal surface. This expansion of the apical domain is also responsible for altering the localization of E-Cadherin, resulting in decreased transepithelial electric resistance (Takeda et al., 2000; Schmieder et al., 2004). Podocalyxin also facilitates the rearrangement of junctions necessary for aortic tube formation (Strilić et al., 2009). Therefore, the loss of podocalyxin could be sufficient to alter vascular permeability by changing the location of endothelial cell junctions and adhesive receptors between cells. Further analysis of the junctional localization in vivo, as well as evaluating whether the loss of podocalyxin alters F-actin, cortactin or ERM protein expression and localization may clarify the mechanisms leading to the defects observed in the Podxl\textsuperscript{ΔEC} mice.
2.4.2 Lung regeneration is activated but cannot be maintained as a result of endothelial cell specific loss of podocalyxin

Maintaining healthy vasculature in the lung is necessary for optimal lung structure and function. Based on the fact that multiple endothelial related genes have been implicated in postnatal lung development, namely alveolarization, and its downstream effects on lung function, I hypothesized that podocalyxin is required for normal lung structure and function. Characterization of Podxl\(^{-}\) mice revealed normal lung architecture at birth, illustrating that podocalyxin does not influence branching morphogenesis (Doyonnas et al., 2001). To our surprise, despite careful analysis of the lung tissue during alveolarization, I found no changes in airspace size in Podxl\(^{AEC}\) mice, but instead found enlarged airspaces at postnatal day 14 in the Podxl\(^{AEC}Cd34^{-}\) mice that were resolved by postnatal day 28. Subsequently, both Podxl\(^{AEC}\) and Podxl\(^{AEC}Cd34^{-}\) mice display increased airspaces during adulthood, which is not progressive with advanced age. Podxl\(^{AEC}\) and Podxl\(^{AEC}Cd34^{-}\) mice also have pathological changes to the lung. Both of these mice have increase lung compliance at postnatal day 28 that continues into adulthood, and Podxl\(^{AEC}\) display increased airway and thoracic resistance. Therefore, rather than influence alveolarization, it is more likely that podocalyxin plays a role in lung maintenance and regeneration.

Recent work aimed at understanding the role of endothelial cells in lung regeneration has shown that MMP14 produced by endothelial cells was necessary for epithelial progenitor expansion and facilitates regeneration of the lung in a murine pneumonectomy model (Ding et al., 2011). In the mouse lung, MMP14 is expressed at birth with peak expression at postnatal day 10, but expression is silenced after alveolarization is complete (Mariani et al., 2002; Atkinson et al., 2005). Interestingly, although MMP14 is regulated normally during juvenile lung development in podocalyxin deficient mice, we find that it is re-expressed in the lungs of adult Podxl\(^{AEC}\), but not Podxl\(^{flo}\) or Podxl\(^{AEC}Cd34^{-}\) mice. The fact that acute lung injury, such as that observed upon bleomycin-induced epithelial cell destruction, is not sufficient to induce MMP14 expression in B6 mice would argue that activation of MMP14 in Podxl\(^{AEC}\) reflects an ongoing lung maintenance or regeneration, rather than a pathological
condition such as COPD. Furthermore, \(Podxl^{\Delta EC}\), but not \(Podxl^{\text{flo}}\) or \(Podxl^{\Delta EC}Cd34^{-/}\) mice express vWF in alveolar tissue, which is typically only expressed in alveolar capillaries during regeneration or after injury (Reidy et al., 1989; Jin et al., 2001; Maeda et al., 2001). Podocalyxin deficient lungs at 10 weeks also express increased mRNA of matrix proteins \(Col1a1, \text{Col1a2, and Eln}\) as well as genes expressed in epithelial cells such as \(Sftpc\) and \(Fgfr4\). Increased collagen mRNA expression may be expected in pathological changes in the lung such as COPD, but typically there is not a coordinated increase in elastin expression, again suggesting that the gene expression profile in the \(Podxl^{\Delta EC}\) mice reflects a maintenance or regeneration pathway. However, despite all of the observations that suggest the mice are activating the regeneration pathway, the enlarged airspace pathology suggests it cannot be maintained. MMP14 is responsible for cleavage of angiocrine signals such as HB-EGF produced by the endothelial cells, which provide proliferation signals to the epithelial cells during lung regeneration. As podocalyxin has been implicated in EGF signaling, it is possible that the loss of podocalyxin is sufficient to impair the release of these signals by the endothelium that result in a downstream failure to support epithelial survival, and cause defective lung maintenance.

2.4.3 Podocalyxin and CD34 have different functions in the endothelium
Previously, other than in hematopoietic progenitor cells, our research has focused on studying to role of CD34, podocalyxin or endoglycan in systems in which they are uniquely expressed to simplify analysis. This work represents the first evaluation of the loss of both CD34 and podocalyxin in the adult vasculature. At the outset, I hypothesized that when multiple members of the CD34 family were expressed in a cell type, compensation by the remaining family member(s) would mitigate the loss of another family member. Correspondingly, I hypothesized that mice in which multiple genes were deleted would have a more severe phenotype than the loss of either one gene. This hypothesis was driven in part by the observation that the neonatal lungs and kidneys of \(Podxl^{-/}\) mice exhibit increased expression of CD34 mRNA (Doyonnas et al., 2001). When comparing \(Podxl^{\Delta EC}\) and \(Podxl^{\Delta EC}Cd34^{-/}\) mice in lung vascular permeability, we found that the phenotype of \(Podxl^{\Delta EC}\) mice was dominant in both basal in inflammation-induced permeability. This suggests, that
rather than compensating for each other’s loss, in endothelial cells CD34 and podocalyxin appear to provide independent functions. Recent data by Siemerink et al. have confirmed that CD34 identifies tip cells in cultured endothelial cells, whereas podocalyxin seems to be on all endothelial subsets (Siemerink et al., 2012). Furthermore, we have shown that $Cd34^{-/-}$ mice develop faulty (leaky) vasculature during tumor driven angiogenesis, which would argue for a pro-angiogenic function for CD34 in endothelial cells (Maltby et al., 2011). Taken together, this data along with the data I have presented in this chapter suggests that indeed rather than selectively regulating angiogenesis, podocalyxin is involved in maintaining homeostasis and vascular integrity of vessels.

Furthermore, subsequent deletion of CD34 did not alter or exacerbate the lung structure and indeed, one could propose that the subsequent deletion of CD34 abrogates the lung maintenance phenotype. Closer examination of CD34 expression in the lung proves that CD34 is not only expressed in the endothelial cells, but in almost all progenitor and mature epithelial and mesenchymal cell types in the lung, except airway epithelial cells (Fig 2.16). Therefore, loss of CD34 in the alveolar type II cells, which are the lung progenitor activated in lung maintenance and regeneration to maintain the alveolar type I cells, is a plausible explanation for the abrogated phenotype exhibited in the $Podxl^{Alb}Cd34^{-/-}$ mice.

![Figure 2.16: Histological analysis of CD34 expression in Cd34^{-/-} lung tissues](image)

CD34 is expressed in almost all cells within the lung except the airway epithelial cells, while CD34 expression is completely abrogated in $Cd34^{-/-}$ mice.
2.4.4 Endothelial cell adhesion to laminin and fibronectin is controlled by podocalyxin expression

One of the best-characterized functions of podocalyxin is in the regulation of adhesion. While it is able to act as an L-selectin ligand in a specific context of HEVs, there is ample evidence that, like other mucins, podocalyxin normally acts as an anti-adhesion molecule. Particularly in epithelial cells, podocalyxin has been reported to act as an “apicalizing” factor in establishing apical and basolateral domains (Takeda et al., 2000; Meder et al., 2005; Nielsen et al., 2007). In addition, forced expression of podocalyxin in epithelial cells is sufficient to inhibit cell-cell adhesion, induce apical bulging and shedding from monolayer cultures and decrease adhesion to matrix (Takeda et al., 2000; Somasiri et al., 2004; Nielsen et al., 2007; Graves, 2008; Meng et al., 2011; Cipollone et al., 2012). Therefore we undertook experiments to understand the role of podocalyxin in endothelial cell adhesion and spreading. In the lung, laminins provide the primary basement membrane for endothelial cells. When plated on laminin, \( \text{Podxl}^{\Delta EC} \) mEC behaved similar to control cells in the initial adhesion of cells, but exhibit a profound defect in spreading on laminin coated semi-rigid substratum. As a result, \( \text{Podxl}^{\Delta EC} \) mEC make initial contact with the substrate, and rather than form monolayers, they form clumps that remain adherent to the substratum. Endothelial binding to laminin is dependent on \( \alpha_3\beta_1, \alpha_6\beta_1, \) and \( \alpha_6\beta_4 \) and when we examined laminin specific integrins, \( \text{Podxl}^{\Delta EC} \) mEC increase mRNA expression of \( \text{Itga6} \) and \( \text{Itgb1} \), but not \( \text{Itga3} \) or \( \text{Itgb4} \) as though the cells were attempting to compensate for defective adhesion. We also observed increased expression of \( \text{Lama4} \) and \( \text{Lamc1} \) in \( \text{Podxl}^{\Delta EC} \) mEC, suggesting these cells may be secreting more laminin-8 as an adhesive substrate. In summary, these data argue that podocalyxin deficient endothelial cells have undergone selective alterations in the expression of their adhesion molecules and their ability to bind extracellular matrix, which clearly supports an important role for podocalyxin in regulating adhesive processes. Integrin mediated adhesion is an extremely complicated process that is regulated at multiple levels including gene expression (as shown here), protein expression, protein conformation (active versus inactive) and cellular localization (apical versus basal targeting). An in depth analysis
of the changes to these processes will be required to conclusively determine how the loss of podocalyxin leads to impaired endothelial cell spreading \textit{in vitro} and vessel patency \textit{in vivo}.

In the lung, fibronectin is typically used as a provisional matrix produced during angiogenesis (Hallmann et al., 2005). I found that loss of podocalyxin was sufficient to increase static adhesion, but did not affect spreading of mEC on fibronectin. $\text{Podxl}^{\Delta EC}$ mEC also increased both mRNA expression and surface expression of $\beta_1$ integrin over control cells. Recent experiments by Cipollone \textit{et al.} have shown that overexpression of podocalyxin in epithelial cells (OVCAR-3 cells, an ovarian carcinoma cell line) is sufficient to decrease adhesion to fibronectin (Cipollone et al., 2012). In these cells, adhesion is dependent on $\beta_1$ integrin, and podocalyxin was sufficient to alter the availability of surface $\beta_1$ integrin. Others have shown that forced expression of podocalyxin in epithelial cells is sufficient to exclude $\beta_1$ integrin from the apical surface and localize it to the basal surface (Economou et al., 2004; Somasiri et al., 2004). In summary, these data demonstrates that it is quite conceivable that loss of podocalyxin in endothelial cells leads to mistargeting of integrins, ECM proteins or other adhesion molecules to inappropriate subcellular domains that results in defective cell spreading.

In conclusion, my results are the first studies that establish that podocalyxin is required for normal structure and function of the lung through changes in vascular permeability, maintenance and regeneration, as well as adhesion to the endothelial ECM in the lung.
Chapter 3 : Endoglycan in the mouse vasculature

3.1 Introduction

Differentiated smooth muscle cells (SMCs) are typically quiescent during homeostasis, and provide many essential functions including contraction, regulation of blood vessel tone and control of blood flow. Mature, contractile, SMCs are distinguished from their progenitors by increased expression of contractile proteins including α-smooth muscle actin (αSMA), smooth muscle myosin heavy chain (SM-MHC), and calponin. However, SMCs also have a remarkable plasticity and are thought to undergo reversible changes in phenotype as a result of environmental stimuli, often as a result of injury or disease. This transition of mature cells to a “synthetic phenotype” results in reacquisition of characteristics present during development; namely, a high potential for proliferation and migration, as well as production of extracellular matrix proteins (Owens, 1995; Owens et al., 2004; Rzucidlo et al., 2007). Several endogenous and exogenous factors, including factors prominent in inflammatory lesions, have been shown to induce this phenotype transition. For example, platelet derived growth factor-ββ (PDGF-ββ) acts as both a potent growth factor and a chemoattractant of SMCs within atherosclerotic lesions (Heldin and Westermark, 1999). Furthermore, the addition of PDGFββ is sufficient to suppress differentiation of SMCs to a contractile phenotype through the downregulation of smooth muscle contractile proteins (Holycross et al., 1992). In addition, lipopolysaccharide (LPS) has also been shown to decrease αSMA expression through inhibition of transforming growth factor (TGF)-regulated transcriptional control elements (Sandbo et al., 2007).

The CD34 gene family (Cd34, Podxl, Podxl2) share genomic and structural features suggesting that they all evolved from a common ancestral gene and are likely to play a role in similar biological pathways. All three family members are markers of hematopoietic progenitors in the human and mouse but also have unique distribution on mature cell types and vascular cells that is dependent on the species examined. (Furness and McNagny, 2006; Nielsen and McNagny, 2008). CD34 family expression is often upregulated on activated cell types. CD34 is induced as hematopoietic stem cells are activated (Ogawa, 2002),
podocalyxin is upregulated during stress erythropoiesis (Maltby et al., 2009), while endoglycan is upregulated by Toll-like receptor ligands in B-cells (Lam, 2005). Functionally, both CD34 and podocalyxin are able to influence migration. Loss of CD34 in dendritic cells leads to a cell intrinsic migration defect that dampens the migration of these cells from the lung to draining lymph nodes and, correspondingly, confers resistance to hypersensitivity pneumonitis in CD34-deficient mice (Blanchet et al., 2011). Similarly, CD34-deficient eosinophils exhibit a defect in migration to eotaxin leading to protection from asthma (Blanchet et al., 2007). Furthermore, silencing of podocalyxin in A549 carcinoma cells decreases TGF-β induced migration (Meng et al., 2011), and podocalyxin expression on tumor cells demarcates those with highest metastatic potential (Somasiri et al., 2004; Sizemore et al., 2007; McNagny et al., 2012).

The principal aim of this study was to understand the role of endoglycan in the activation and migration of smooth muscle cells. We identified endoglycan expression in a number of mouse tissues, particularly those containing smooth muscle cells or large vessels and showed that smooth muscle cells, but not endothelial cells, express the highest levels of endoglycan. Our data show that endoglycan is upregulated with differentiation of primary smooth muscle cells to a contractile phenotype, but is not influenced by inflammatory stimuli or mitogenic factors. In addition, we indentified a mouse aortic smooth muscle line, MOVAS-1, that downregulates endoglycan expression after multiple passages. Ectopic expression of endoglycan in late-passage MOVAS-1 revealed a function in the attenuation of PDGFβ-dependent migration. In summary, we have found that endoglycan is a marker of differentiated SMCs and its expression is sufficient to enhance basal SMC migration.

3.2 Materials & methods

3.2.1 Animals

All animals were maintained in a pathogen-free facility at The Biomedical Research Centre at the University of British Columbia, and all experiments were performed in accordance with the UBC Animal Care Committee approved protocols. Unless otherwise indicated, adult C57Bl/6J (B6) mice (> 8 weeks old) were used in this study.
### 3.2.2 Generation of conditional endoglycan knockout, Endg1\(^{\text{floxed}}\) mice

A mutant endoglycan (Podxl2) genomic targeting construct was generated from a 129SvJ mouse genomic library clone by introducing two loxP recombination target sequences upstream of exons 3 and 5. A neomycin resistance cassette (Neo\(^{R}\)) was also inserted between exons 4 and 5 and flanked with Flp-recombinase target sequences (frt) to permit targeted deletion of the drug resistance cassette subsequent to the identification of drug resistant ES cell clones (Fig 3.6). When exposed to Cre-recombinase, the transgene should undergo deletion of the intervening DNA between loxP sites, removing Podxl2 exons 3 and 4. In addition to deleting these coding sequences, the product should result in a frame shift within the coding sequence and lead to a premature termination of any protein made from the residual transcripts.

The resulting vector was electroporated into R1 embryonic stem (ES) cells and selected with G418. Proper targeting of the Podxl2 locus in the resulting clones was confirmed in eight ES cell clones by PCR and Southern blot. Four clones were chosen for further analysis. Injection of these targeted ES cells into blastocysts and implantation into pseudo pregnant female mice was used to generate chimeras as per standard protocols. Four chimeric mice from one ES clone were born and then backcrossed with B6 mice to identify mice bearing a germline mutation of the Podxl2 allele. Two chimeras gave germline transmission, and produced two founders. Finally, the residual neomycin cassette was removed from the targeted-Podxl2 locus by breeding these mice to strains ubiquitously expressing the Flp-recombinase (Rodriguez et al., 2000). Of these two founders, only offspring from one mouse were able to delete the Neo\(^{R}\) cassette, identified by PCR screening. The strain arising from this founder was maintained for further analysis. All offspring, following deletion of the Neo\(^{R}\) cassette, were screened for the presence of the loxP-flanked (“floxed”) Podxl2 allele.

The diagnostic PCR strategy for identifying the conditional endoglycan allele in mice is shown in Figure 3.6 and relies on the PCR primers listed in Table 3.1. Briefly, successful integration of the Neo\(^{R}\) between frt sites in intron 4 was confirmed using a primers that bound within Neo\(^{R}\) cassette (Endo LAF2) and downstream of the final frt site in (Endo LAR1) resulting in a 319bp product to indicate germline transmission, while no product was
produced if the Neo<sup>R</sup> cassette was not integrated. Mice that underwent Flp-recombination to delete the Neo<sup>R</sup> cassette were identified using primers upstream of the second loxP site (Endo LAF1) and downstream of the final frt site (Endo LAR1) to generate a diagnostic 568bp product, otherwise a 465bp band was produced. Mice with successful deletion of exons 3 and 4 through Cre-mediated recombination were confirmed using primers upstream of the first loxP site (Endo SAF1) and downstream of the final frt site (Endo LAR1) in a diagnostic 444bp product.

Table 3.1: Genotyping primers for endoglycan conditional knockout mice

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo SAF1</td>
<td>TTC TAC AAA GGT AAC ACT AGA CTG C</td>
</tr>
<tr>
<td>Endo SAR1</td>
<td>CTT TGG AGT AAA AAA TAT GAC ATG TC</td>
</tr>
<tr>
<td>Endo LAF1</td>
<td>TTC AGT GTC GAG CAT CAG ACT C</td>
</tr>
<tr>
<td>Endo LAF2</td>
<td>CAG GGA TCG AAG TTC TAT TCC G</td>
</tr>
<tr>
<td>Endo LAR1</td>
<td>ACA ATG GTG CTT GAC GGT AGT C</td>
</tr>
</tbody>
</table>

3.2.3 Cells

MOVAS-1 cells were a kind gift from Dr. Mansoor Husain (University of Toronto) (Afroze et al., 2003). A20 cells (Kim et al., 1979), Y3 cells (Galfrè et al., 1979) and bEND.3 cells (Montesano et al., 1990) were purchased from ATCC. Cells were routinely maintained in complete DMEM media (DMEM supplemented with 10% fetal bovine serum (FBS), 1x penicillin/streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM L-glutamine). Cells were passaged using 0.25% Trypsin-EDTA (5 min at 37C) to lift adherent cells and maintained up to 30 passages. Primary mEC were cultured as previously described (see Chapter 2.2.3)

Primary mouse aortic smooth muscle cells (mSMC) were isolated as previously described with modifications (Cao et al., 2010). In brief, mice were sacrificed and descending aortas from heart to iliac bifurcation were excised, rinsed in phosphate buffered saline (PBS) and placed into biopsy media (Weymouth’s MB752/1 supplemented with antibiotic-antimycotic, L-glutamine, sodium bicarbonate, MEM non-essential amino acids and HEPES buffer) (He et al., 2006). Single aortas were minced into 1mm pieces and digested in 1.4mg/mL Collagenase type II (Worthington Biochemical) and 0.1875mg/mL Elastase type 1 (Sigma)
for 4 hours at 37°C. After digestion, complete DMEM was added to the cells, triturated to break up any remaining chunks, and centrifuged at 290xg for 5 minutes. The supernatant was removed and washed with SMC Media (DMEM with 1x penicillin/streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM L-glutamine, 20% FBS, 0.5ng/mL mouse recombinant epidermal growth factor (EGF), 5µg/mL insulin, 2ng/mL bFGF) and centrifuged again. Cells were resuspended in 2mL SMC Media and plate into T25 Flask. After 4 days, the media was changed and the volume increased to 4mL. Subsequently cells were passaged at confluence by re-seeding at 1/4 dilution. Cells were maintained until passage 12.

3.2.4 DNA constructs and endoglycan mutants

A full-length mouse endoglycan cDNA was identified in-house (Lam, 2005) and cloned into the pEF-IRESpuro6 expression vector (Hobbs et al., 1998). A sequence encoding a peptide recognized by an antibody to c-Myc (myc tag) was inserted at the 5’end of the endoglycan coding sequence (Endgl) using a SalI site introduced by site directed mutagenesis after the signal peptide (V31). PCR products were cloned into a pBS and pEF-IRESpuro6 plasmids and the final constructs were sequenced.

A20 and MOVAS-1 cells were transfected with pEF-IRESpuro6 alone (vector control) (Hobbs et al., 1998) or the same vector containing endoglycan cDNA using standard electroporation or calcium chloride transfection methods, respectively. Stable transfectants were selected by culturing cells in increasing concentrations of puromycin (up to 50µg/mL) and sorted for cells expressing myc-tagged endoglycan on the membrane surface (BD FACSVantage, BD Biosciences). Sorted, transfected cells were subsequently maintained in 10µg/mL puromycin.

3.2.5 Cell viability assay

Cells were cultured for 72 h in 96-well plates, and the number of viable cells was determined using the ProMega CellTiter 96 AQassay non-radioactive cell assay (MTS) according to the manufacturer’s instructions.
3.2.6 Antibodies & reagents

All media components unless otherwise stated were obtained from Gibco (Life Technologies). Human PDGFββ, murine bFGF, and murine EGF were obtained from PeproTech. LPS, insulin and cell culture grade BSA was obtained from Sigma. Primary and secondary antibodies used in this study are listed in Tables 3.2 and 3.3, respectively.

Table 3.2: Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Concentration</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-mouse Endoglycan (mEndo)</td>
<td>gIgG</td>
<td>4µg/mL, 10µg/mL, 1µg/mL</td>
<td>FC, IHC/IF, WB</td>
<td>R&amp;D Systems AF3534</td>
</tr>
<tr>
<td>α-human Endoglycan (hEndo)</td>
<td>gIgG</td>
<td>4µg/mL, 1µg/mL</td>
<td>FC, WB</td>
<td>R&amp;D System AF1524</td>
</tr>
<tr>
<td>α-Endoglycan F4B10-biotin</td>
<td>rIgM</td>
<td>3µg/mL</td>
<td>FC</td>
<td>In-house</td>
</tr>
<tr>
<td>α-Endoglycan (S19)</td>
<td>gIgG</td>
<td>4µg/mL, 1µg/mL</td>
<td>FC, WB</td>
<td>Santa Cruz sc-54194</td>
</tr>
<tr>
<td>α-Endoglycan (C16)</td>
<td>gIgG</td>
<td>1µg/mL</td>
<td>WB</td>
<td>Santa Cruz sc-54192</td>
</tr>
<tr>
<td>α-mPDGFRβ</td>
<td>rIgG2a</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 14-1402</td>
</tr>
<tr>
<td>α-mCD31 PECy7</td>
<td>rIgG2a</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 25-0211</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Isotype</td>
<td>4µg/mL</td>
<td>FC</td>
<td>R&amp;D Systems AB-108-C</td>
</tr>
<tr>
<td>Rat IgG2a PECy7</td>
<td>Isotype</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 25-4321</td>
</tr>
<tr>
<td>Rat IgG2a</td>
<td>Isotype</td>
<td>1µg/mL</td>
<td>FC</td>
<td>eBiosciences 14-4321</td>
</tr>
<tr>
<td>Rat IgM-biotin</td>
<td>Isotype</td>
<td>3µg/mL</td>
<td>FC</td>
<td>eBiosciences 13-5890</td>
</tr>
</tbody>
</table>

g – goat species, r – rat species, m – mouse species, h – human species
FC – flow cytometry, IHC – immunohistochemistry, IF – immunofluorescence, WB – western blot
Table 3.3: Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-goat IR800</td>
<td>66ng/mL</td>
<td>WB</td>
<td>Odyssey 926-32214</td>
</tr>
<tr>
<td>Donkey anti-mouse AF555</td>
<td>4µg/mL</td>
<td>IF</td>
<td>Invitrogen A-31570</td>
</tr>
<tr>
<td>Donkey anti-rabbit AF488</td>
<td>20µg/mL</td>
<td>IF</td>
<td>Invitrogen A-11070</td>
</tr>
<tr>
<td>Donkey anti-goat AF488</td>
<td>20µg/mL 5µg/mL</td>
<td>IF  FC</td>
<td>Invitrogen A-21222</td>
</tr>
<tr>
<td>Streptavidin – AF488</td>
<td>4µg/mL</td>
<td>IF</td>
<td>Invitrogen S11223</td>
</tr>
<tr>
<td>Goat anti-rat AF488</td>
<td>20µg/mL</td>
<td>IF</td>
<td>Invitrogen A-11006</td>
</tr>
<tr>
<td>Goat anti-rabbit IR700</td>
<td>66ng/mL</td>
<td>WB</td>
<td>Odyssey 926-32221</td>
</tr>
</tbody>
</table>

FC – flow cytometry, IHC – immunohistochemistry, IF – immunofluorescence, WB – western blot

3.2.7 Wound migration assays

Wound-induced migration was performed as described previously with modifications (Lee et al., 2007). 2x10^5 cells were added to 6-well tissue culture plates and allowed to grow to confluence. Media was replaced with serum free DMEM (including 0.5% BSA, penicillin/streptomycin and L-glutamine) for 24hr. Three parallel, linear defects per well were then made in the monolayer using a 200µl pipette tip. The wounded culture was washed with PBS and then incubated for 18hr in serum free media with or without 10ng/mL PDGFββ. Images were obtained immediately after wounding and 18hr later using Openlab 4.0 and the % area of wound was determined using ImageJ software. For each condition, 3 wells were analyzed per experiment.

3.2.8 Western blotting

Cells were washed with cold PBS and detached from plates using Tris buffered 10mM EDTA, pH 7.5 (TEN buffer) or lysed directly in the plate and scraped. Cells were lysed in RIPA buffer containing 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 1x protease inhibitor cocktail, 1x PMSF and 20mM β-glycerol (Schaafsma et al., 2007). Cell
lysates were centrifuged (13 000xg for 15min at 4°C) and the supernatants were collected as protein samples. Protein concentrations were determined using Pierce BCA Protein Assay (Thermo Scientific) (Tan et al., 2006). The protein homogenates were diluted with 4x sample buffer, and then boiled for 5min. Proteins were resolved on 8-10% polyacrylamide SDS gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 10% BSA for 1hr at room temperature and incubated overnight at 4°C with primary antibodies. Immune complexes were detected with fluorescently labeled secondary antibodies and visualized using the Odyssey Imager system (LI-COR Biosciences).

3.2.9 Genomic PCR, RT-PCR and real-time qPCR

For the purposes of genotyping mice, ear clips or isolated cells underwent proteinase K digestion followed by purification to isolate genomic DNA. Primers used to identify the various genotypes are listed in Table 1.1.

RNA isolation for cultured cells was performed using RNeasy mini kits (Qiagen), and isolation from tissue was performed using Trizol (Invitrogen). RNA quantification was performed using a ND1000 spectrophotometer (Nanodrop). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Gene expression of endoglycan by RT-PCR was performed using the primer sets 5’-CTGGGAAGAAGAGGAACTAAAC-3’ (Forward), 5’- TTGGAGGCACTGTTTGTG-3’ (Reverse) which generates a 662bp fragment.

Real time qPCR (qRT-PCR) gene expression analysis was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences), on a 7900HT Real Time PCR system (Applied Biosystems). Sequence information for the primers is listed in Table 3.4. Data was acquired using the SDS 2.0 software. The relative mRNA expression was normalized to simultaneous amplification of Gapdh gene and the results represent the mean of the three primer sets listed in Table 3.4.
Table 3.4: Mouse primer sequences for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endgl 99 Fwd</td>
<td>CCC ACT GCA GAC TAC GTC TTC C</td>
<td>99</td>
</tr>
<tr>
<td>Endgl 99 Rev</td>
<td>CAG GAT GGA GGG GAG GTT TG</td>
<td></td>
</tr>
<tr>
<td>Endgl 126 Fwd</td>
<td>GCC CAG TGA GAA AGA GCA AC</td>
<td>126</td>
</tr>
<tr>
<td>Endgl 126 Rev</td>
<td>TTC TGG ATG CCA ATC TCC TC</td>
<td></td>
</tr>
<tr>
<td>Endgl 151 Fwd</td>
<td>GAC TGT GAA GTA TTT CGG CGG C</td>
<td>151</td>
</tr>
<tr>
<td>Endgl 151 Rev</td>
<td>CAA GGG TCA TCA GCA GGT GTT G</td>
<td></td>
</tr>
</tbody>
</table>

3.2.10 Flow cytometry

Adherent cells were detached with TEN buffer and then washed with FACS buffer (PBS containing 2 mM EDTA and 10% FBS), incubated with primary antibodies (Table 3.2) for 20 min at 4 °C, washed, incubated with secondary antibodies (Table 3.2) for 20 min at 4°C and washed again before analysis. Data was collected with a FACSCalibur or BD LSRII using CellQuest or FACSDiva software, respectively. FlowJo software (Tree Star Inc.) was used for analysis of FACS data.

3.2.11 Immunohistochemistry and immunocytochemistry

For immunocytochemistry, cells were cultured on glass coverslips, fixed in 4% paraformaldehyde (PFA) for 15 min, washed and permeabilized with 0.1% Triton X-100 in PBS for 15 min. These were then incubated with primary antibody for 2 hr, washed in PBS with 1% BSA, stained with the secondary antibodies for 1 hr, washed and mounted with Fluoromount.

3.2.12 Statistical analysis

Data is expressed as means ± standard error of the mean. Statistical analysis was performed using Student’s t test for comparisons between pairs of groups, and by ANOVA with a Bonferroni post-test for multiple comparisons. P < 0.05 was considered as a statistically significance.
3.3 Results

3.3.1 Endoglycan is expressed on mouse smooth muscle cells but not endothelial cells

Expression profiling of endoglycan required a robust set of reagents to be able to identify the protein in multiple settings including immunohistochemistry, immunocytochemistry, western blot and flow cytometry. Commercially available antibodies for both human and mouse endoglycan are listed in Table 3.5. R&D Systems have generated goat polyclonal antibodies using a recombinant peptide encoding the extracellular domain of either mouse or human endoglycan (mEndo and hEndo, respectively), while polyclonal antibodies with reactive epitopes mapped to either the N-terminus (S-19) or C-terminus (C-16) of hEndo are available from Santa Cruz Technologies (Santa Cruz, CA). Fortunately, since the extracellular domain of mouse endoglycan shares 73% sequence identity with human, many of the polyclonal antibodies raised with the human peptide are expected to cross-react with mouse (and vice versa). To add to this pool, our lab also developed a rat IgM monoclonal antibody, hereafter designated F4B10, that reacts with both human and mouse endoglycan (Lam, 2005) and is now commercially available through Millipore.

Table 3.5: Endoglycan antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Immunogen</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-mouse Endoglycan (mEndo)</td>
<td>Polyclonal goat IgG</td>
<td>NSO-derived recombinant mouse extracellular domain</td>
<td>R&amp;D Systems AF3534</td>
</tr>
<tr>
<td>α-human Endoglycan (hEndo)</td>
<td>Polyclonal goat IgG</td>
<td>NSO-derived recombinant human extracellular domain</td>
<td>R&amp;D System AF1524</td>
</tr>
<tr>
<td>α-Endoglycan F4B10-biotin</td>
<td>Monoclonal rat IgM</td>
<td>non-glycosylated, N-terminal peptide from mouse protein</td>
<td>In-house</td>
</tr>
<tr>
<td>α-Endoglycan (S19)</td>
<td>Polyclonal goat IgG</td>
<td>N-terminal peptide from human protein</td>
<td>Santa Cruz sc-54194</td>
</tr>
<tr>
<td>α-Endoglycan (C16)</td>
<td>Polyclonal goat IgG</td>
<td>C-terminal peptide from human protein</td>
<td>Santa Cruz sc-54192</td>
</tr>
</tbody>
</table>
To validate the utility of these antibodies in each experimental procedure, we used the mouse B cell line, A20 that does not express endoglycan and an A20 transfectant expressing an epitope-tagged (myc) mouse endoglycan, A20-V31Myc as a negative and positive control respectively (Lam, 2005). In these analyses, we also included a mouse aortic smooth muscle cell line, MOVAS-1 that, in early passages, expresses low levels of endogenous endoglycan, as well as the same line transfected to express high levels the myc-tagged endoglycan construct (MOVAS-Endo). Only the mEndo and hEndo antibodies from R&D Systems detect endogenous endoglycan in parental MOVAS-1 by flow cytometry, but all antibodies in Table 3.5 were able to detect the A20 and MOVAS-1 cells transfected with endoglycan (Fig 3.1A). The C16 antibody was not tested in flow cytometry because its reactive epitope is cytoplasmic and therefore not surface exposed. Only A20 and MOVAS-1 cells transfected with Myc-tagged endoglycan produced reactive bands by Western blot, regardless of the antibody used (Fig 3.1). Membrane preparations containing protein levels of up to 75µg per lane were run, without any discernable specific bands in non-transfected cell lines. Non-specific bands detected with the C16 antibody in A20 cells (Fig 3.1B) are the heavy and light chain immunoglobulins of the B-cell receptor (BCR). Thus, the currently available panels of commercial endoglycan antibodies lack sufficient sensitivity to detect low levels of endogenous endoglycan expressed cell lines by western blot. Since the R&D Systems’ mEndo antibody appeared to be the most robust for flow cytometry applications, it was used in all of the following studies.
Figure 3.1: Evaluation of endoglycan-reactive antibodies

Evaluation of commercially available antibodies to endoglycan including R&D Systems polyclonal antibodies derived from recombinant extracellular mouse (mEndo) or human (hEndo) peptides; Santa Cruz Biotechnology Inc. antibodies with reactive epitopes in the N-terminal (S19) or C-terminal (C16) domain and the in-house F4B10-biotin.

(A) Evaluation of antibodies by flow cytometry. MOVAS-1 cells were detached from plates using TEN buffer and A20 and MOVAS-1 cells were stained in suspension. MOVAS-1 cells (centre right panel) expressing endogenous levels of endoglycan can be detected by mEndo (blue) and hEndo (orange) but not S19 (purple) or F4B10-biotin (red) when compared to the isotype control (green). Ectopically expressed endoglycan on A20-V31Myc (center left panel) and MOVAS-Endo cells (right panel) can be detected by all antibodies.

(B) Evaluation of commercially available antibodies by western blot. Cell extracts were immunoblotted with anti-endoglycan antibodies and visualized using the LI-COR Odyssey Imager system. Only ectopically expressed endoglycan could be detected by western blot.
We also examined the expression of mouse endoglycan message (*Podxl2*) in normal tissues (Fig. 3.2). We harvested a variety of tissues from naïve adult B6 mice for mRNA isolation and performed quantitative RT-PCR (qRT-PCR) using three mouse *Podxl2*-specific primers (see Table 1.4). Expression analyses using the relative quantification of all three primers products to *Gapdh* PCR products showed high expression of endoglycan message in the brain and testes (Fig 3.2, left panel), with moderate levels of expression in the bladder, colon, heart, spleen and small intestine (Fig. 3.2, right panel).

**Figure 3.2: Analysis of endoglycan expression in tissue**

Analysis of mouse endoglycan expression in adult, naïve B6 brain, testes, bladder, small intestine, colon, heart, aorta, and spleen. Complementary DNA (cDNA) was generated from mRNA isolated from each tissue. Gene expression by qPCR for each primer listed in Table 1.4 was normalized to a *Gapdh* control. The average of the normalized expression of three primer sets is shown for each tissue. Error bars = SEM

We were particularly interested in the expression of endoglycan in adult mouse vasculature. Human endoglycan expression has been reported on both endothelial cells and vascular smooth muscle (Sassetti et al., 2000), and mouse endoglycan expression has been reported on vascular smooth muscle (van der Zwaag et al., 2005). Furthermore, expression of endoglycan’s closest relatives, CD34 and podocalyxin, on endothelia has been found to regulate vascular lumen formation and vascular integrity (Strilić et al., 2009; Blanchet et al., 2010; Maltby et al., 2011).
To initiate our survey of vascular tissue, we used the previously described MOVAS-1 cells, and a mouse brain endothelial cell line, bEND.3, and found that endoglycan is expressed on smooth muscle but not endothelial cells by flow cytometry and qPCR. This finding was further validated in primary aortic smooth muscle cells (mSMC) and primary lung endothelial cells (mEC) isolated from naïve, adult B6 mice (Fig 3.3).

**Figure 3.3: Endoglycan is a marker of smooth muscle cells and not endothelial cells in the mouse**

Endoglycan expression on mouse endothelial cells and smooth muscle cells by flow cytometry and qPCR

(A) Primary lung mouse endothelial cells (mEC) and bEND.3 cells were labeled with antibodies against CD31-PECy7 and mEndo. Unstained cells in red, isotype controls (rIgG2a-PECy7/gIgG) in blue, and antibody staining in green. Endothelial cells stain positive for the endothelial cell marker CD31 but not endoglycan. Representative of three experiments.

(B): Primary mouse smooth muscle cells (mSMC) and MOVAS-1 cells were labeled with antibodies against PDGFRβ and mEndo. Unstained cells in red, isotype controls (rIgG2a/gIgG) in blue and antibody staining in green. Smooth muscle cells stain positive for both the smooth muscle cell marker PDGFRβ and endoglycan. Representative of three experiments.

(C): Primary mSMC and mEC along with MOVAS-1 and bEND.3 cells were profiled for endoglycan expression by qPCR. cDNA was generated from mRNA isolated from each cell type. Gene expression by qPCR for each primer listed in Table 1.4 was normalized to a Gapdh control. The average of the normalized expression of three primer sets is shown for each tissue. Error bars = SEM.
As transmembrane proteins, CD34-family sialomucins presumably are functionally expressed on the cell membrane surface. To confirm cellular localization of endoglycan on smooth muscle cells, we performed immunocytochemistry on primary mSMC cells and MOVAS-Endo\textsuperscript{hi}. Ectopic expression of endoglycan in MOVAS-Endo\textsuperscript{hi} cells clearly show the localization of endoglycan on the membrane surface, and the endogenous levels of endoglycan on mSMCs can be detected on the membrane, although in a much more diffuse pattern.

![Figure 3.4: Immunocytochemistry demonstrated membrane localization of endoglycan on smooth muscle cells](image)

(A) mSMC and (B) MOVAS-Endo\textsuperscript{hi} cells were grown on glass coverslips, fixed, permeabilized and stained with DAPI (blue) and antibodies against endoglycan (red).

### 3.3.2 Differentiation of smooth muscle results in upregulation of endoglycan expression

Primary mouse aortic smooth muscle cell differentiation and marker expression has been well characterized; therefore vSMC provide an excellent model for studying smooth muscle functions using \textit{in vitro} assays. Since stimulation of splenic B-cells \textit{in vitro} with PMA or LPS upregulates endoglycan cell surface expression (Lam, 2005), we hypothesized that endoglycan would be upregulated in smooth muscle cells through similar stimuli. While PMA produces mitogenic signals in B-cells, it regulates contractile response and inhibits mitogenesis in smooth muscle cells (Weiss et al., 1995; Trombino et al., 1998). Therefore, we used PDGF-ββ, a known mitogenic stimulus for smooth muscle cells.
Interestingly, under standard culture conditions, stimulation with PDGFββ or LPS for 24 or 48 hr had no effect on endoglycan expression by flow cytometry (Fig 3.5A). However, smooth muscle cells are well known to have a synthetic phenotype when under standard culture conditions. To generate cells with a contractile phenotype, primary SMCs must be cultured in serum-free conditions for 5-8 days (Owens, 1995; Heldin and Westermark, 1999; Owens et al., 2004). When differentiation was initiated by serum starvation of confluent SMC cultures, mSMCs upregulated endoglycan message within 3 days and surface protein expression within 5 days. Endoglycan surface expression continued to increase up to 8 days in serum-free conditions (Fig 3.5B). Notably, the upregulation of endoglycan expression in serum-free conditions paralleled the increased expression of the contractile phenotype marker, smooth muscle myosin heavy chain (SMMHC or myosin-11) (Fig 3.5C). Our data demonstrate that endoglycan can be used as an extracellular marker of differentiated smooth muscle cells and, furthermore, that endoglycan expression may be regulated by mechanisms similar to other markers of mature smooth muscle cells. To further elucidate the role of endoglycan in smooth muscle differentiation, we sought to generate aortic smooth muscle cells from endoglycan knockout mice.
Figure 3.5: Endoglycan is upregulated with differentiation of SMC to a contractile phenotype and not activation by PDGFββ or LPS

(A) Endoglycan expression profiles of PDGFββ and LPS stimulated cells. Cells were stimulated with 10ng/mL PDGFββ or 0.5ng/mL LPS in culture media for the time described. Cells were detached from plates using TEN buffer and stained in suspension with isotype (orange) or anti-endoglycan mEndo primary antibodies after 48hr (green), 24hr (red) or 0hr (blue) of stimulation.

(B, C) Endoglycan surface and mRNA expression profiles in differentiating smooth muscle cells. SMC were differentiated from a proliferative phenotype to a contractile phenotype through serum deprivation for 8 days. Endoglycan expression was assessed at times described with cells detached from plates using TEN buffer. To determine surface expression (B) cells were stained in suspension with isotype (orange) or anti-endoglycan mEndo primary antibodies after 1 day (red), 3 days (green), or 8 days (blue) of serum deprivation for FACS analysis. Mean fluorescence intensity (MFI) was determined by subtracting nonspecific background signal detected in by the isotype control in mSMC undergoing serum starvation. For mRNA expression (C), RNA was isolated using RNeasy kit and cDNA was generated for qPCR using primers indicated in Table 1.4. Data represented as fold change over control. * p<0.05 Bonferroni post-test, error bars = SEM.
A  LPS stimulation  

PDGFββ stimulation

Endoglycan

B  Serum deprivation

Endoglycan

C  Endoglycan gene expression with serum deprivation

SMMHC expression with serum deprivation
3.3.3 “Conditional endoglycan knockout mice” fail to lose endoglycan protein expression

To understand the function of endoglycan in vivo, we generated a conditional allele of the Podxl2 locus that can be inducibly deleted in specific tissues using the Cre/loxP system (Orban et al., 1992). The decision to make a deletable allele was based on several considerations including the broader expression pattern of endoglycan in the brain and smooth muscle, as well as our previous experience with germ-line deletion of its closest relative, podocalyxin. The analysis of podocalyxin function in Podxl/− mice was confounded by the fact that these mice die perinatally (Doyonnas et al., 2001). For clarity, we have identified the endoglycan conditional transgenic allele as Endgl rather than using the formal gene name Podxl2.

Following the generation of transgenic mice with the Endgl-floxed (Endglfloxd) allele (Fig 3.6), we crossed these mice to a number of Cre-deleter strain (Orban et al., 1992). While many tissue-specific and inducible-Cre mice are of interest to the lab, my focus was to develop and characterize the smooth muscle tissue-specific deletion of endoglycan. The most robust model used to delete floxed alleles in smooth muscle tissue is a transgenic strain that drives Cre-expression from the smooth muscle actin-22 (SM22α) (Tagln) promoter (Holtwick et al., 2002). Tagln-Cre efficiently deletes genes early in development in all smooth muscle of both large and small vessels (including the aorta), the intestines, bladder and heart (Holtwick et al., 2002; Carvalho et al., 2007; Hernando et al., 2007; El-Bizri et al., 2008; French et al., 2008; Arboleda-Velasquez et al., 2011)
Figure 3.6: Targeted deletion strategy for the endoglycan locus

(A) Schematic representation of wildtype and transgenic allele. Exons are depicted in red with corresponding exon numbers and introns are in white. loxP and frt sites are depicted with blue and green boxes with arrowheads, respectively. The Neo<sup>R</sup> cassette is represented by a green box. PCR primers with product sizes are identified by directional half-arrows. PCR primer sequences are listed in Table 1.1.

(B) Validation of the construct. In vitro Cre-mediated excision of the targeting construct was followed by transformation of the DNA into bacteria and subsequent analysis by restriction digest. The Cre-mediated excision event results in the loss of restriction fragments indicated by green boxes.

(C) Genotyping of mice carrying the conditional endoglycan locus. PCR genotyping using primers EndoLAF1 and Endo LAR1 confirmed deletion of the Neo<sup>R</sup> cassette to yield “floxed” allele. Deletion produced a 568bp product (*); otherwise a 465bp product was produced.
When Endgl$^{\text{flox}}$ mice were crossed to the Tagln-Cre mice, the resulting Endgl$^{\text{ASMC}}$ mice were viable, fertile and are indistinguishable from Endgl$^{\text{flox}}$ littermates. To characterize these mice more thoroughly we isolated aortic smooth muscle cells to validate functional deletion of the Endgl$^{\text{flox}}$ allele. Conditional knockout Endgl$^{\text{ASMC}}$ mice were identified by through two separate PCR reactions to have both a transgenic allele and the recombined product as expected, while only the transgenic allele but not the recombined product could be found in Endgl$^{\text{flox}}$ mice (Fig 3.7 A&B). These results, combined with Southern Blot analysis (Fig 3.6 B) suggests the correct integration site of the Endgl$^{\text{flox}}$ construct in the genome and that the loxP flanked sequence of this allele is appropriately deleted in the presence of Cre expression. However, to test the efficiency of deletion, we also examined protein expression in our cultured aortic mSMCs by flow cytometry (Fig 3.7 C). Surprisingly, we found similar levels of endoglycan protein expression by wild type B6, Endgl$^{\text{flox}}$ and Endgl$^{\text{ASMC}}$ mSMCs. While endogenous endoglycan expression could not be confirmed by western blot, mRNA expression by conventional RT-PCR confirmed the persistence of endoglycan message in all genotypes (Fig 3.7 D).

The flow cytometry and PCR data suggest that Endgl$^{\text{ASMC}}$ mice are not able to delete endoglycan despite the detection of a deleted locus by the genotyping. In a recent study, Turlo et al. summarized 5 possible mechanisms that can lead to the persistence of a protein in a conditional knockout mouse, namely; inappropriate targeting, heterozygous selection, recombination efficiency, protein half-life, and episomal expression (Turlo et al., 2010). In our current study, we have shown that, based on genotyping, there is appropriate targeting of the endogenous endoglycan locus but we could not test for heterozygous selection using Tagln-Cre mice due to the persistence of contaminating non-Tagln-Cre expressing cells. To more thoroughly evaluate the efficiency of the Tagln-Cre gene in vivo, we crossed this strain to a Rosa-rsYFP reporter mouse and evaluated its ability to induce YFP expression in smooth muscle. These data suggested that the mSMC isolated results in Cre-excision in 50% of the culture, however even sorting for YFP+ to select for active Cre excision was not sufficient to
see any changes in endoglycan expression. With regard to protein half life, while we have not formally tested the half-life of endoglycan, we would predict that endoglycan could have quite a long half-life especially in vSMC that are normally quiescent. Therefore it is possible that there is not sufficient protein turnover to be able to detect any differences. The episomal expression is an interesting possibility that we have not tested yet. The loxP-Cre system removes the DNA flanking the loxP sites, however it also forms a circular product of the excised DNA. Although episomal expression of a complete gene cassette is possible as outlined by Turlo et al., because we have excised only a small fragment of the Podxl2 gene, which lacks any regulatory elements, it is extremely unlikely that this explains the residual expression (Turlo et al., 2010). In summary, we have been unable to reconcile the residual protein expression in this strain. To circumvent this problem we have recently obtained a transgenic mouse from the Knockout mouse project (KOMP) repository, which carries a β-galactosidase (LacZ) reporter “knocked-in” to the coding region of the endoglycan gene – simultaneously deleting all protein-coding exons. Although this model results in conventional, germ-line deletion of endoglycan, the presence of the LacZ reporter under the control of the endogenous endoglycan promoter with allow us to characterize the pattern of endoglycan expression in vivo in hemizygous mice and, if viable, mice homozygous for the transgene will allow us to study the function of endoglycan in vivo.
Figure 3.7: Genotyping of Endgl\textsuperscript{flox}/Tagln-Cre (Endgl\textsuperscript{SMC}) smooth muscle cells

Smooth muscle cells isolated from individual naïve, adult mice were numbered in succession. Cells were isolated from wildtype B6 (SMC6), heterozygous Endgl\textsuperscript{flox}/wt (SMC5), homozygous Endgl\textsuperscript{flox} (SMC10, SMC13, SMC15) or conditional knockouts Endgl\textsuperscript{ΔSMC} (SMC8, SMC9, SMC11, SMC12, SMC14) Genomic PCR identifying wildtype (wt) or transgenic (Tg) alleles in Endgl\textsuperscript{flox} conditional knockout mice (A) Primers EndoSAF1 and EndoSAR1 listed in Table 1.1 were used. Mice carrying the transgenic allele produced a 568bp band, while those carrying a wildtype allele produced a 444bp band. (B) Genomic PCR indentifying the recombined transgenic allele. PCR primers EndoSAF1 and EndoLAR1 listed in Table 1.1 are designed to produce a 444bp product if recombinant; however if not recombinant, the primers span 2.2kb and therefore do not produce a PCR product. (C) Flow cytometry analyses of Endoglycan expression by mSMC. Cells were detached from plates using TEN buffer (unstained-red) and stained in suspension with isotype (blue) or anti-endoglycan mEndo primary antibodies (green). (D) Endoglycan expression by rt-PCR. cDNA was generated from mRNA isolated from cells directly from the dish using RNAeasy kit. Endoglycan cDNA was detected by rt-PCR, which produced a 662bp band. Y3 cells were used as a negative control and MOVAS-Endo as a positive control. Smooth muscle cells from multiple mice, whether Tagln-Cre positive or negative, show similar endoglycan expression.
A

Tg 568bp
wt 444bp

B

Tg Cre+ 444bp

C

mSMC5  mSMC6  mSMC7  mSMC8

Endoglycan

mSMC9  mSMC11  mSMC13  mSMC14

D

podxl2 662bp

Unstained  isotype  Endoglycan
3.3.4 Endoglycan dampens PDGFββ-mediated smooth muscle cell migration

Although we were unable to use primary smooth muscle cells that lack endoglycan to further understand the role of endoglycan in smooth muscle function, we found that MOVAS-1 cells lost endoglycan expression after multiple passages (>20) (MOVAS-Endo\textsuperscript{lo}). This is not unlike the other CD34-family members in endothelial cells (Horvat et al., 1986; Delia et al., 1993). Loss of endoglycan expression was rescued with ectopic expression of the V31mycEndo construct (MOVAS-Endo\textsuperscript{hi}) in the high passage cells with a log-increase in expression levels over the parental cell line (MOVAS-Endo\textsuperscript{mod}). Therefore, using this system we could compare levels of endoglycan MOVAS-Endo\textsuperscript{lo} < MOVAS-Endo\textsuperscript{mod} < MOVAS-Endo\textsuperscript{hi} in smooth muscle function.

![MOVAS-Endo\textsuperscript{lo}](image1)

![MOVAS-Endo\textsuperscript{hi}](image2)

**Figure 3.8: Endoglycan overexpression results in a spindle like-morphology**

MOVAS-Endo\textsuperscript{lo} cells display cuboidal morphology in both sub-confluent (top left) and confluent (bottom left) cultures, while MOVAS-Endo\textsuperscript{hi} cell display an elongated phenotype in subconfluent cultures (top right) that progress to spindle-like morphology that is aligned in confluent cultures (bottom right)
Interestingly, ectopic expression of endoglycan resulted in a spindle-like morphology, similar to that seen in differentiated, contractile smooth muscle cells (Fig 3.8). Since differentiated cells are less migratory, this finding suggests that endoglycan expression levels might alter the ability for these cells to migrate.

To test this hypothesis we used an *in vitro* wound (“scratch”) assay to assess the function of endoglycan in the migration of MOVAS cells in response to a known stimulus, PDGFββ. Compared to MOVAS-Endo<sub>mod</sub>, MOVAS-Endo<sub>lo</sub> cells displayed a 70% increase in PDGFββ-induced migration. When endoglycan is ectopically re-expressed in these cells, the moderate migration phenotype is regained (Fig 3.9). In addition, both cell lines expressing endoglycan (MOVAS-Endo<sub>mod</sub> and MOVAS-Endo<sub>hi</sub>), but not the Endo-deficient MOVAS-Endo<sub>lo</sub> cells migrate to close the wound in the absence of PDGFββ.

There are a number of factors that could induce the baseline migration in endoglycan expressing MOVAS-Endo<sub>mod</sub> and MOVAS-Endo<sub>hi</sub> cells. While primary cells will exit from the cell cycle with 24hr serum starvation, the immortalization of the MOVAS-1 cells prevents this. The observation that MOVAS cells maintain constant levels of phosphorylated Akt after serum starvation further argues that they have lost the normal cell cycle arrest response to serum starvation response (Fig 3.9). Even after 8 days of serum starvation, we were unable to induce a decrease in proliferation of the MOVAS cell line. We believe that the observed migration phenotype is not due to proliferation as there is no difference in proliferation between cells over the much shorter duration of the assay (Fig 3.9). This suggests that while proliferation is not a direct result of the migration, future work should mitigate this factor from the experimental design by treating with a cell cycle inhibitor such as mitomycin C.
Figure 3.9: Endoglycan expression dampens PDGFββ induced smooth muscle migration

(A) Effect of endoglycan on smooth muscle migration. Representative images of the quantification used to determine the area of migration.

(B) Effect of endoglycan on PDGFββ induced migration. Cells were serum starved for 24h before subjected to a wound by scratching with a pipette tip and treated with or without 10µg/mL PDGFββ for 18hr. Images were taken at t=0 and t=18hr and % fill of scratch calculated between time points and difference in area between untreated and PDGFββ-treated was reported. The scratched wound area was expressed as 100%. Data pooled from 3 experiments, with 9 scratches per condition.

(C) Effect of endoglycan on unstimulated migration. Cells were serum starved for 24hr before being subjected to a wound by scratching with a pipette tip. Images were taken at t=0 and t=18hr and % fill of scratch calculated between time points. The scratched wound area at t=0 was expressed as 100%. Data pooled from 3 experiments, with 9 scratches per condition.

(D) Effect of MOVAS cell proliferation on migration. 1000 cells were plated in a 96 well plate, and viable cells were assessed by Promega CellTiter MTS assay after 72hr to mimic the time cells are plated within the scratch wells from plating to the end of the assay. Data represents data from 3 experiments, with 3 replicates per cell type.

(E) Effect of PDGFββ activation of p-Akt in MOVAS-1. Cells were serum starved for 24hr and then treated with 10µg/mL PDGFββ for increasing time. The lysates from cells were immunoblotted for p-Akt.

* p<0.05 Bonferroni post-test, error bars = SEM. N.S.= no significance.
A

MOVAS-Endo\textsubscript{Lo} - DMEM

MOVAS-Endo\textsubscript{Mod} - DMEM

MOVAS-Endo\textsubscript{Hi} - DMEM

0hr 18hr

0hr 18hr

0hr 18hr

MOVAS-Endo\textsubscript{Lo} - PDGF

MOVAS-Endo\textsubscript{Mod} - PDGF

MOVAS-Endo\textsubscript{Hi} - PDGF

B

PDGF\textsubscript{ββ} mediated migration

% Fill of Scratch

\*

\*

C

Unstimulated mediated migration

% Fill of Scratch

\*

\*

D

Proliferation of MOVAS cells after 72hr

E

pAKT

0 5 10 20 60 120

N.S
3.4 Discussion

To date, endoglycan expression has been profiled in the mouse embryo and human tissues, as well as human and mouse hematopoietic cells. To characterize endoglycan expression in non-hematopoietic cells in the adult mouse, we profiled adult mouse vasculature and other tissues with smooth muscle cell content for endoglycan expression. We discovered that adult tissue expression in the bladder, colon, small intestines and the aorta persist from development, and have similar levels of endoglycan expression as the spleen, and to those found in the developing mouse (Lam, 2005; van der Zwaag et al., 2005; Kerr et al., 2008). This suggests that the level of endoglycan is maintained within smooth muscle cells throughout development. The isolation of smooth muscle cells from most organs is technically challenging, as is the in vitro culture of smooth muscle. However, we attempted the isolation and culture of primary aortic smooth muscle cells since these methods have been better characterized and vetted in the literature for the in vitro investigation of smooth muscle function.

Since the initial characterization of endoglycan in 2000, a number of antibodies that have been developed to help identify endoglycan in a variety of conditions and assays. Most of these antibodies are expected to recognize both the mouse and human forms due to high sequence identity in the extracellular domain. We profiled these antibodies in a number of cell types including cells transfected with full-length endoglycan (A20 and MOVAS-1), and cells that were reported to have endogenous endoglycan (MOVAS-1). As expected, all of the antibodies identified cells expressing ectopic endoglycan by both flow cytometry and western blot. These cells express endoglycan at very high levels and contain a similar form of endoglycan used to generate the antibodies. Conversely, only the polyclonal antibodies produced by R&D systems (mEndo and hEndo) were able to detect endogenous protein levels by flow cytometry, but not by western blot. Antibody affinity for endogenous endoglycan is likely influenced by a number of factors including changes in glycosylation or other post-translational modifications. As the R&D system antibodies are generated by cell based recombinant proteins, it is likely that these antibodies are able to account for more of these changes than those developed against synthetic peptides. As such, we have made use
of the polyclonal mEndo from R&D systems as our antibody of choice for the remainder of our studies.

In order to continue the study of endoglycan, further work is necessary to generate improved antibodies for analyses. One possibility would be to make antibodies against the endogenous protein isolated from cultured smooth muscle cells, rather than using recombinant protein from transfected cells in order to maintain any endogenous structural conformation or post-translational modifications. In addition, the use of endogenous tissues such as the brain, testes or smooth muscle would also be beneficial in the screening of antibodies to ensure that the affinity was sufficient to detect endogenous levels that are often 100-1000 fold less than transfected levels. Finally, once the KOMP endoglycan knockout is validated, cells derived from these mice would become the ideal screening tool to ensure that there is no cross-reactivity with other proteins. These knockout mice could also be immunized with wildtype cells in order to selectively generate antibodies against wildtype endoglycan.

The original paper describing endoglycan reported expression in endothelial cells including foreskin microvasculature and HUVECs. However, we were unable to detect endoglycan in mouse endothelial cells by flow cytometry using bEND.3 or primary endothelial cells isolated from the lung. Additionally, endoglycan is detectible in human but not mouse HEV (Sassetti et al., 2000; Kerr et al., 2008). Therefore, in endothelial cells and HEV, it appears that endoglycan exhibits a species dependent distribution. We have profiled a number of human cell lines including primary human airway smooth muscle cells and pulmonary artery smooth muscle cells and have found that endoglycan is expressed by these cells, suggesting that functions for endoglycan in mouse smooth muscle may be evolutionary conserved, and therefore data could be extrapolated between different model organisms.

Previous work by our group and others has shown that splenic B-cells upregulate endoglycan after stimulation with LPS or PMA stimuli relevant to inflammatory and mitogenic activation, respectively. As such, we investigated whether endoglycan could be upregulated in vSMC in a similar manner. When we stimulated mSMCs with LPS or PDGFββ, a well-characterized smooth muscle mitogen, we could not detect altered endoglycan expression.
In the vasculature, mature, contractile smooth muscle cells are essential for maintaining the stability of small vessels, and regulate constriction of large vessels, such as the aorta, during homeostasis (Carmeliet, 2000). This contractile phenotype can be mimicked in vitro by removing serum from culture media. Serum-starvation alters the structure and function of cultured smooth muscle cells from a synthetic to a mature, contractile phenotype. We have shown that endoglycan expression, both mRNA and protein, was increased in SMCs following serum starvation. Thus, endoglycan may serve as a marker of a contractile SMC phenotype together with contractile proteins such as smooth muscle actin, desmin, and smooth muscle myosin heavy chain. However, these other markers are intracellular proteins, while endoglycan is a cell surface marker. Thus, upregulation of endoglycan may provide a more convenient means to track the maturation status of SMCs. Possible applications may include live sorting of SMC populations using endoglycan surface expression, which currently relies on the expression of a transgene reporter such as SMA-GFP (Yokota et al., 2006). In addition, many of the contractile proteins used as markers for differentiated contractile proteins are regulated by CArG [CC(AT-rich)$_6$ GG] elements, the transbinding factor serum response factor (SRF), or transforming growth factor controlling elements (TCE) (Owens et al., 2004; Sandbo et al., 2007). Therefore it is tempting to speculate that since endoglycan is upregulated in serum-deprived vSMC displaying a contractile phenotype, it may also be controlled by similar transcriptional regulatory elements.

We next investigated the role of endoglycan in smooth muscle cell migration using the MOVAS-1 line where we manipulated endoglycan expression through passage and transfection. First, with regards to the loss of endogenous endoglycan expression with MOVAS cell passage, it is noteworthy that in normal vSMC, upregulated endoglycan expression correlated with increased differentiation to a contractile phenotype. Thus, loss of endoglycan by increased passage of MOVAS cells could reflect drifting of this cell line into a more immature, highly proliferative and less differentiated state. That caveat aside, it is intriguing that the baseline migration of endoglycan expressing cells (MOVAS$^{mod}$ or MOVAS$^{hi}$) was significantly higher than control, MOVAS$^{lo}$ cells and what is typically seen with primary smooth muscle cells (~60% vs. 30% and 20% respectively). While this clearly appears to be a gain of function due to endoglycan expression, it is somewhat paradoxical
since enhanced migration is not typically thought to reflect the behavior of contractile cells. This may however reflect a caveat in working with this transformed cell line. Although serum starvation of normal vSMC for 24-48hr drives these cells into Go, MOVAS-1 cells were published to have approximately 16% of cells still in S phase at this time point, likely reflecting their transformation by the middle T antigen of polyoma virus (Afroze et al., 2003). Phosphorylated AKT is also still present in these cells suggesting active cell growth and survival signals are being produced, and even starvation up to 8 days is unable to force quiescence in any of the MOVAS cell lines. In summary, it may be difficult to tightly correlate a change in behavior of this cell line with defined stages of normal vSMC differentiation.

Surprisingly, we found that endoglycan expressing MOVAS cells showed a decrease in PDGFββ-induced migration compared to non-expressing cells. Our MOVAS cells that express endoglycan at moderate and high levels only display a 1.3 fold increase in PDGFββ-mediated migration in MOVAS cells partially due to the significantly elevated level of baseline migration of endoglycan expressing clones. In most cases, cell migration is induced through a gradual increase in factor up until a threshold, and any amount over that threshold will actually inhibit migration. Therefore, an explanation for the observed decrease in PDGF-ββ dependent migration could be that endoglycan expression shifts the threshold necessary to induce vSMC migration. In addition, endoglycan is modified by chondroitin sulfate, and chondroitin sulfate is able to bind the long form of PDGFββ in order to retain it near the cell surface (Sassetti et al., 2000; Rolny et al., 2002). Consequently, it is possible that the overexpression of endoglycan could alter the amount of PDGFββ that is maintained at the cell surface at sufficient quantities to induce migration. However, we can observe a three-fold increase in PDGFββ-dependent migration in primary smooth muscle cells that express moderate levels of endoglycan (data not shown). Therefore it is arguable whether the MOVAS cells can provide any real insight into the role of endoglycan in vSMC migration. In summary, while it is clear that endoglycan drives enhanced vSMC migration at baseline in MOVAS cells, these experiments need to be repeated in primary vSMC and in vivo settings in order to confirm the role of endoglycan in migration and the possible molecular pathways involved.
In conclusion, our study demonstrates that endoglycan expression in the mouse vasculature is restricted to smooth muscle cells and not endothelial cells. Using smooth muscle cells, we found that differentiation, rather than activation, of the cells induced the upregulation of endoglycan. Finally, we found that cells expressing endoglycan exhibit enhanced baseline migration that can be increased with PDGF-ββ stimulation. This study provides novel insights into the regulation of endoglycan expression in smooth muscle cells and suggests a possible role for endoglycan in smooth muscle migration.
Chapter 4: Conclusions & discussion

My research has focused on understanding the expression and functional role of CD34, podocalyxin, and endoglycan in the adult vasculature. CD34 and podocalyxin are excellent endothelial cell markers however it is only recently that a role for CD34 and podocalyxin in endothelial cell function has been established. (Horvat et al., 1986; Fina et al., 1990; Schlingemann et al., 1990; Kalaria and Kroon, 1992; Delia et al., 1993; Ding et al., 2006; Pusztaszeri et al., 2006; Testa et al., 2009; Rose et al., 2011; Siemerink et al., 2012). Both podocalyxin and CD34 are important for lumen formation and CD34 is required for vessel integrity and vascular permeability during tumor-induced angiogenesis and under inflammatory conditions (Strilić et al., 2009, 2010; Blanchet et al., 2010; Maltby et al., 2011).

4.1 Podocalyxin summary and discussion

In this study, we have expanded our understanding of podocalyxin in endothelial cell function both in vitro and in vivo (Chapter 2). We established transgenic PodxlΔEC mice that interfered with the podocalyxin gene in a vascular specific manner. Using the lung vascular bed as our model, we demonstrated that loss of endothelial expression of podocalyxin results in a striking increase in basal permeability and inflammation-induced vascular permeability. Surprisingly, the loss of podocalyxin in the lung does not alter vessel density nor does it influence the expression of a select set of genes and cell surface markers characteristic of endothelial cells. Thus, my data suggest that loss of podocalyxin has a very specific effect on the mechanisms that regulate permeability rather than endothelial cell development per se. My work also revealed that expression of podocalyxin in vascular endothelia is required for normal lung architecture and function in adult mice since PodxlΔEC mice display increased lung volume, impaired lung maintenance and altered structural matrix composition. Surprisingly, in contrast to our hypothesis that podocalyxin and CD34 may have a similar function in the vasculature, PodxlΔEC Cd34−/− mice that lack both podocalyxin and CD34 from the vasculature exhibit no additional phenotypes over those observed in the PodxlΔEC mice. This suggests that CD34 and podocalyxin may indeed have independent functions in
endothelial cells, rather than play a compensatory role in vasculature function. To address the mechanisms responsible for the enhanced permeability, we isolated endothelial cells from wild type and knock out lungs and demonstrated that loss of podocalyxin alters adhesion and spreading in a matrix specific manner. Podocalyxin deficient cells plated on fibronectin exhibit enhanced static adhesion but normal spreading in comparison to control cells. More strikingly, although knock out cells appeared to attach normally to collagen type I and laminin, these cells exhibited a profound defect in spreading on laminin and, to a lesser extent, collagen. Podxl\textsuperscript{ΔEC} mEC make initial contact with the laminin substrate similar to control cells, but rather than form monolayers, they form clumps that remain as adherent clusters on the substratum. Intriguingly, I observed an increased gene expression of the integrin receptors required for binding to fibronectin and laminin, but not collagen type I, suggesting that loss of podocalyxin in endothelial cells leads to altered regulation of integrin expression. This data supports our hypothesis that podocalyxin is necessary for normal endothelial cell adhesion and spreading, but highlights that this is a selective process dependent on the substratum.

4.1.1 The role of podocalyxin in endothelial cell adhesion and spreading

Within the last 20 years, there has been mounting evidence to suggest that podocalyxin expression controls cell adhesion and spreading by defining the apical domain, and regulating the proper localization of integrins and the actin cytoskeleton (Takeda et al., 2000; Doyonnas et al., 2001; Economou et al., 2004; Schmieder et al., 2004; Nielseni et al., 2007; Kobayashi et al., 2009; Fernández et al., 2011; Fukasawa et al., 2011; Meng et al., 2011; Cipollone et al., 2012). In cell lines, forced expression of podocalyxin expands the apical domain of adherent cells at the expense of the basal lateral domain, thereby condensing the integrin in the basal lateral membrane domain into a diminished surface area, that has insufficient avidity to maintain adhesion (Economou et al., 2004; Somasiri et al., 2004). At the same time, since podocalyxin promotes the formation of actin-rich surface structures at the apical domain (microvilli), high levels of expression effectively titrates actin away from the cell-cell and cell-matrix contacts, and thereby weakens these interactions (Nielsen et al., 2007). Paradoxically, inhibition of podocalyxin expression leads to inappropriate “creeping” of integrins into the apical domain, effectively diluting their expression on the adhesive face.
of cells. Thus, podocalyxin expression appears to have the “Goldilocks” effect on cell adhesion; little to no expression permits dilution of integrin localization and weak adhesion, while too much expression leads to much apical domain on cells respective to the small basolateral domain and, again, weak adhesion. Therefore, the expression of podocalyxin has to be “just right” for normal adhesion and this phenomenon is likely the root of much of the controversy around podocalyxin as a pro- or anti-adhesive molecule (Nielsen and McNagny, 2008).

Figure 4.1: Levels of podocalyxin may explain the contradictory roles in cell adhesion

(A) Cells expressing normal levels of podocalyxin have well-defined cell-cell junctions and adhere well to the substratum. Overexpression of podocalyxin leads to recruitment of f-actin to the apical membrane for formation of microvilli along with apical domain expansion and weakening of cell-cell junctions.

(B) Low levels of podocalyxin allow integrins to creep toward the apical domain. Normal levels of podocalyxin establish apical domains and force integrins to the basal surface of cells, thereby enhancing cell adhesion. High levels of podocalyxin disrupting integrin mediated adhesion. Adapted with permission Nielsen and McNagny, Journal of Cell Science, 2008;121(Pt 22):3683-3692.

Tightly regulated levels of podocalyxin are necessary to define the apical domain and drive integrins to the basolateral domain to produce the optimal integrin density across the basolateral face to generate the cell-matrix forces required for adhesion. A model that could explain the defects in laminin-dependent spreading is that podocalyxin is able to regulate
homeotypic adhesion through charge repulsion or steric hindrance. Previous work has shown that podocalyxin regulates cell-cell contact through repulsion forces generated by its high negative charge. In suspension cell cultures, the ability for podocalyxin to block cell aggregation is dependent on the negative charge provided by the sialic acid residues (Takeda et al., 2001). CD34 can also regulate homeotypic adhesion. BMMCs cultured from \( Cd34^{-/-} \) mice exhibit increased homeotypic aggregation that can be rescued with re-expression of CD34 (Drew et al., 2005). This increased aggregation is also divalent cation-dependent suggesting that it may be mediated by integrins or cadherins. Interestingly, the naturally occurring splice variant of CD34 lacking the bulk of the cytoplasmic tail was more effective in reversing the aggregation phenotype than full-length CD34. This suggests that the bulky negative charge of the extracellular domain, rather than intracellular signaling, is responsible for inhibiting homeotypic adhesion. Therefore, it is conceivable that the laminin-dependent homeotypic adhesion we observe with the \( Podxl^{AEC} \) mEC could be caused by the loss of the charge repulsion or steric hindrance provided by podocalyxin (Fig 4.1A). While this model is attractive, a major caveat is that it fails to successfully explain why there is no defect in spreading to fibronectin and only a mild defect in spreading on collagen type I. If podocalyxin’s role was truly biophysical and charge related, one would expect that it would have a similar effect on adhesion to both of these substrates. Thus, our data would argue for a more subtle role.
Figure 4.2: Model demonstrating two possible mechanisms for Podxl

(A) Charge dependent model. The bulky, negatively charged extracellular domain of podocalyxin defines the apical domain blocks cell adhesion by charge repulsion and/or steric hindrance; thereby preventing the interaction of integrins with their ligands.

(B) Integrin/ECM localization model. Lack of podocalyxin leads to inappropriate integrin localization and laminin secretion thereby enhancing homeotypic adhesion.

One candidate model for this subtle role is that podocalyxin plays an additional role in regulating the deposition of extracellular matrix proteins and integrin ligands themselves. Interestingly, cultured endothelial cells are directed to secrete laminin to the basal surface, particularly when they are cultured under sparse conditions, as is the case in our endothelial spreading assay described in this thesis (Gospodarowicz et al., 1981). In our studies, we demonstrated that podocalyxin-deficient endothelial cells upregulate α6 and β1 integrins as well as laminin α4 mRNA expression, suggesting that the cells are increasing laminin secretion together with the laminin-related integrins. Thus, one possible explanation for the
striking adhesion phenotype of $Podxl^{\Delta EC}$ mEC to laminin is that the loss of podocalyxin may modulate integrin expression towards the apical domain as well as induce global surface secretion of laminin to promote the increased homeotypic adhesion seen in our adhesion assay. Taken together, we propose that podocalyxin could regulate laminin-dependent spreading by altering the localization of integrins, ECM proteins or other adhesion molecules (Fig 4.1B).

4.1.2 The elusive cause of the $Podxl^{\Delta}$ perinatal lethality

The initial publication characterizing the constitutive deletion of the $Podxl$ gene showed that 25% of the mice displayed severe edema and that all mice exhibited increased red cell ejection during severing of the umbilical cord suggestive of an increase in blood pressure. This coincided with a profound anuria as a result of defective kidney podocyte morphogenesis leading to the walling off of the urinary space in Bowman’s capsule (Doyonnas et al., 2001). Thus, these mice were presumed to die as a result of the kidney defect but direct confirmation of this as the principal lesion leading to this phenotype has not been established. In an attempt to rescue this defect, our lab generated a transgenic mouse line expression podocalyxin under the control of the podocyte specific dNPHS1 promoter and crossed these to the $Podxl^{\Delta}$ mice (Nielsen, 2006). This lead to a somewhat improved morphogenesis in podocytes, with increased numbers of foot processes and resolution of tight junctions but was still less than those found in wild type mice. Unfortunately these mice still exhibit suffer perinatal lethality. While this could be taken as evidence that there is other lesions (aside from the defective podocytes) that lead to perinatal lethality observed in ubiquitous podocalyxin knock out mice, definite proof would require selective ablation of podocalyxin in podocytes (in progress).

The development of the $Podxl^{\text{flox}}$ mice offers us the ability to question the role of podocalyxin in other specific tissues and delineates the tissues that require podocalyxin for survival. The work presented in this thesis demonstrates that loss of podocalyxin in the vasculature is not sufficient to cause the perinatal lethality, as $Podxl^{\Delta EC}$ mice are viable, fertile and display normal Mendelian frequency of mice and deletion patterns similar to the expression of $Cdh5$-
Similarly, deletion of podocalyxin specifically in hematopoietic cells using VAV-Cre mice also results in viable mice, and these mice can live to past one year of age (unpublished results). Additional studies by our collaborators have not identified any connection to the deletion of podocalyxin in neural tissue, using Nephrin-Cre mice, to the perinatal lethality observed in the \( \text{Podxl}^{-/-} \) mice (Perez and Soriano, unpublished observations). Furthermore, others have demonstrated that deletion of podocalyxin in the brain using the pan-neural Cre recombinase mouse, Cre3, also resulted in viable mice with only subtle defects in the ventricles and intraventricular capillary density (Nowakowski et al., 2010). However, on closer examination of the data presented by Nowakowski et al. it appears that podocalyxin expression is still detectable in neurons in the cortex and expression was decreased by 60% in the granular layer, but was not completely abrogated, which could be sufficient to support survival/viability if podocalyxin in the brain is indeed essential for postnatal life (Nowakowski et al., 2010). Taken together, these data do not determine the exact cause of the perinatal lethality exhibited by the \( \text{Podxl}^{-/-} \) mice. However, these data are also reliant on the efficiency of Cre expression in transgenic mice for the abrogation of podocalyxin expression, which is known throughout the field to be inherently variable. Therefore, due to the “patchy” or incomplete deletion seen in most Cre-driven deleter strains it is impossible to conclude that endothelial, hematopoietic or neural tissues do not play a role in perinatal lethality and only if a complete abrogation of podocalyxin specifically from these tissues is achieved will it be possible to determine where podocalyxin is critical for development.

### 4.1.3 \( \text{Podxl}^{\Delta \text{EC}} \) mice as a model for lung aging

The use of mice for modeling human disease has both benefits and drawbacks in the identification of clinically relevant treatments for disease. Human lungs begin to decline after the age 35, and slowly develop increased airspaces, increased compliance and fibrosis. As such, aging is an important factor in most human lung diseases. However, mouse lungs have the potential to regenerate well into adulthood. In a recent study that examined the role of aging in bleomycin-induced fibrosis, mice were considered “young” at 3 months and “old” at 24 months of age (Sueblinvong et al., 2011). It is also known within the field that if adult mice subjected to disease models are allowed sufficient time to recover after treatment, they exhibit marked improvements in lung pathology. Considering mice typically used in disease
models are 2-4 months of age, it is not surprising that the use of mouse models is insufficient to reveal the role of aging in lung pathology. A similar problem existed in the atherosclerosis field; most laboratory strains of mice were unable to develop atherosclerotic plaques without the use of high fat diets over numerous months. Nevertheless, when ApoE/ mice were characterized and determined to develop atherosclerotic plaques and fatty streaks on normal mouse chow very similar to that seen in human disease, these mice became an important tool for studying atherosclerosis in mice (Plump et al., 1992). As a result, there have been many discoveries of the genetic mechanisms contributing to atherosclerosis through the use of knockout or transgenic mice crossed onto the ApoE/ background. Interestingly, we find that podocalyxin mice have normal lung development, but present with defects in lung maintenance and regeneration, display localized increases in collagen content, and increased compliance, very similar to aged human lungs. Therefore, I propose that the PodxlΔ mice could become a very useful mouse as a “sensitized strain” for assessment of aging in lung disease in a fashion similar to the widespread use of ApoE deficient mice for discovering contributing factors in atherosclerosis.

4.1.4 Future directions

A key future direction for understanding the role of podocalyxin and CD34 in endothelial cell function will be to move the in vitro studies observed here into the human dermal microvascular endothelial cell (HDMEC) system. While the isolated mouse endothelial cells described here have provided novel observations that merit detailed follow up, primary mouse endothelial cells are technically challenging to work with in that they require very careful culture conditions and do not maintain a standard morphology between batches of cells. HUVECs are widely used in the field, much more so than mouse endothelial cells, and serve as a simplified and robust model because they have well characterized behaviours and tend to be very stable. They exhibit stereotypical cobblestone morphology and are able to form patent monolayers in culture. HDMECs also readily tolerate siRNAs or constructs to facilitate the knockdown of genes or to introduce gene reporters to facilitate visual investigation of processes such as actin cytoskeleton rearrangement. As a result, it would be desirable in future studies to knockdown podocalyxin or CD34 or both in these cultures and investigate a variety of cell processes that are technically challenging to evaluate with the
mouse primary cultures. A key experiment using the knockdown HDMECs would be to confirm the matrix dependent changes to adhesion and spreading, and investigate the localization of integrins and ECM protein secretion. In addition, since HDMECs are able to make patent monolayers through the formation of adherens and tight junctions, we could investigate the role of podocalyxin junctional localization both in basal conditions and with induced permeability by defined factors such as VEGF, angiopoietin, S1P and thrombin, among others. Similarly, endothelial cell permeability examined through in vitro studies favour cell-matrix dependent processes such as actin cytoskeleton rearrangements. This would allow us to study the role of podocalyxin in the regulation or localization of gene products involved with changes to the actin cytoskeleton including Rac1 and cdc42, as well as known binding partners of podocalyxin, namely, NHERF-1/2, the ERM proteins and cortactin that can mediate cytoskeleton rearrangements (Schmieder et al., 2004; Nielsen et al., 2007; Kobayashi et al., 2009).

While we have demonstrated a novel role for podocalyxin in vascular permeability, we still need to resolve the mechanisms responsible for the defects in basal permeability exhibited in Podxl\textsuperscript{ΔEC} mice. Basal permeability in lung capillaries is typically regulated by changes to the glycocalyx or transcytosis of albumin, although significant changes in junctional localization and stability would also be sufficient to alter basal permeability. We can determine the importance of the transcytosis pathway or junctional contribution by repeating the in vivo permeability assays with various sizes of labeled dextran or albumin. This has proven to be an effective method of determining more selective lesions in endothelial cell function. The transcytosis pathway can only accommodate the transport of proteins up to approximately 70kD, but that size of protein would be too large to be passed through normal junctions (Komarova and Malik, 2010). However, if there have been significant modifications to junctional localization or stability, the permeability could be altered such that large labeled proteins (~200kD) could be measured within the tissue. One intriguing observation from our lung studies is that loss of podocalyxin results in increased eNOS mRNA expression in lung tissue. Interestingly, increased NO production is sufficient to increase vascular permeability in the lung and can be abrogated using the NO synthase inhibitor L-NAME (Kavanagh et al., 1994; Predescu et al., 2005). Therefore further studies treating Podxl\textsuperscript{ΔEC} mice with L-NAME
could confirm whether podocalyxin regulates NO production to regulate basal permeability in the lung.

Another study that is required is to understand whether the sialic acids on podocalyxin are an important contributor of negative charge in the glycocalyx of the endothelium. The original studies that lead to the characterization of human podocalyxin in the kidney glycocalyx employ the use of cationic dyes such as alcian blue or labeled proteins known to bind sialic rich proteins could be used to test lung lysates in order to identify whether podocalyxin is the major sialoprotein in the lung and contributes significantly to the glycocalyx (Kerjaschki et al., 1984). The negative charge of the glycocalyx also mediates basal permeability by maintaining the balance of negatively charged albumin found in blood plasma and the positively charged albumin that must be sequestered within the glycocalyx (Michel and Curry, 1999). Therefore, by studying the role of podocalyxin in albumin binding to the glycocalyx, we could visualize any changes to the contribution of labeled albumin to the endothelium of both control and PodxlΔEC mice.

Another area of study regarding CD34 and podocalyxin that still requires extensive research is their role in angiogenesis. Tumor angiogenesis studies have demonstrated an important role for CD34 in maintaining vascular integrity in nascent vessels and future studies using a similar tumor model in the PodxlΔEC and PodxlΔEC/Cd34−/− mice could provide insight into whether podocalyxin and CD34 both play a role in tumor angiogenesis (Maltby et al., 2011). If podocalyxin and CD34 do mark stalk cells and tip cells respectively as suggested by Siemerink et al., then in a model of tumor angiogenesis, I would suspect that loss of podocalyxin would also impact vessel integrity and even vessel density in solid tumors, but that loss of both podocalyxin and C D34 would have severe impact in vessel integrity, permeability and likely inability to support large tumor growth (Siemerink et al., 2012). Additionally, tumor angiogenesis requires pericytes to stabilize the vasculature (Armulik et al., 2011). It is possible that podocalyxin could regulate the localization of signals that recruit pericytes, such as SDF-1 and HB-EGF. Therefore, targeting both CD34 and
podocalyxin within the tumor vasculature could be used a potential therapeutic to disrupt vessel integrity, and slow tumor growth.

Furthermore, retinal angiogenesis and development of the corpus luteum are both excellent in vivo models that could provide valuable insights into CD34 and podocalyxin function in normal angiogenesis (Reynolds et al., 2000; Gariano and Gardner, 2005). Similar to early aortic development, I would hypothesize that in normal angiogenesis, we would likely see defects early in vessel formation, but would be resolved in time to support the retina or placenta respectively. This is also supported by the fact that we observe normal litter sizes from female Podxl^{ΔEC} mice, which suggests that the corpus luteum in these mice is sufficient to effectively support embryo development. In summary, there are a number of interesting questions that need to be resolved in order to fully understand the roles of podocalyxin and CD34 in vascular function.

4.2 Endoglycan summary and discussion
A key step in understanding the role of any given protein is the characterization of that protein’s expression pattern. In Chapter 3, my objectives were to define the distribution of endoglycan in mouse tissues and to uncover a functional role for this molecule in the activation and migration of smooth muscle cells. We established that the highest level of endoglycan expression is in the brain, testes and tissues or large vessels that contain smooth muscle cells, but not endothelial cells of the mouse. Further study of smooth muscle cells demonstrated that endoglycan is upregulated upon differentiation of primary smooth muscle cells into a contractile phenotype, but this is not affected by inflammatory stimuli or mitogenic factors. This was somewhat surprising, as previous work in our laboratory had shown a selective upregulation of endoglycan on B cells in response to IL-1 or LPS stimulation. These differences likely reflect differences in the function of the molecule on these diverse cell types. Although I initially attempted to analyze candidate endoglycan knockout mice, detailed analysis of these mice suggest that our initial attempts at gene targeting failed. Therefore I shifted my focus to an immortalized mouse vascular smooth muscle cell line (MOVAS-1) as a model to investigate the role of endoglycan in smooth muscle cell migration. Expression of endoglycan by these cells was sufficient to permit
migration of MOVAS-1 cells in an in vitro wound assay without any stimuli at a similar level to that observed upon PDGF-ββ stimulation of MOVAS-1 cells that lack endoglycan. This suggests that endoglycan plays a functional role in facilitating cell migration, but further characterization will be required to reveal the exact mechanisms involved in this altered migration.

4.2.1 Endoglycan as a lineage marker for smooth muscle cells
Within this thesis, I determined that not only is endoglycan expressed on smooth muscle cells, but was also upregulated as smooth muscle cells were differentiated in culture. This suggests that endoglycan may be regulated in a similar manner to smooth muscle cell contractile proteins. Currently, these contractile proteins, which include αSMA and SMMHC, among others, are often used as lineage markers for vSMC, when they more accurately define the differentiation state of vSMC. The hematopoietic stem cell field originally coined the term “lineage markers” to describe the cell surface proteins that could be used to identify terminally differentiated cells. The ES cell and induced pluripotent stem (iPS) cell field has expanded this term to include stage specific markers to identify cells in intermediate or transition stages towards terminal differentiation. The use of stage- and lineage-specific markers, along with stage-specific growth conditions, facilitates the selection of ES or iPS cells undergoing differentiation to generate a relatively pure population of the desired differentiated cells. Unfortunately, the precise conditions to induce ES or iPS cell differentiation to vSMC fate have received little attention in comparison to those used in the derivation of vascular endothelial cells. As a result, a progenitor cell that gives rise to only vSMC has not been described to date, and the only available markers that are currently used to identify ES or iPS cells derived vSMC progenitors are the intracellular contractile proteins, αSMA and SMMHC (Cheung and Sinha, 2011). Therefore it is vital to identify a cell surface marker that could be used as a stage or lineage marker, specific for vSMC progenitors, to facilitate the purification of a homogeneous population of vSMC cells. Based on my data it would appear that endoglycan, which is expressed on the surface of smooth muscle cells and is regulated in a similar fashion to the contractile proteins currently being used as vSMC lineage markers, is a strong candidate for just such a marker. In short, we propose that endoglycan could be used as a cell-surface vSMC specific lineage marker.
While our work has focused on the role of endoglycan in vSMC, pericytes are also interact with the endothelium and are considered to be of the same lineage as vSMC. Pericytes have been proposed to be the resident vascular progenitor cells, as well as critical component of tumor vasculature, and therefore have become an alluring target for clinical applications (Armulik et al., 2011). Currently, the most conclusive method to identify pericytes requires ultrastructural analysis, however for practical reasons a combination of localization and gene expression is more commonly used. Nevertheless, no single molecular marker has been identified to distinguish vSMC from pericytes or other mesenchymal cells. Therefore while it would be an exceptional finding if endoglycan were actually unique to vSMC, it is quite likely that endoglycan could also be used as a pericyte marker. If indeed endoglycan is expressed by pericytes, understanding its functional role in pericytes could make endoglycan an interesting therapeutic target in managing vessel stabilization necessary to control vascular permeability and tumor growth.

4.2.2 Future directions

Although our initial studies of endoglycan have provided provocative suggestions of a role in smooth muscle activation and migration, we have been hampered in our ability to conclusively conclude these hypotheses due to the lack of an amenable animal model. It will now become crucial to develop an effective KO model. Toward that end we have recently acquired the transgenic endoglycan KOMP mouse, which will become an important tool to examine the expression profile of endoglycan, throughout development and into adulthood, by tracking the expression of the β-galactosidase (LacZ) reporter “knocked-in” to the coding region of the endoglycan locus. Early studies suggest that mice homozygous for the transgene, which results in conventional, germ-line deletion of endoglycan, are viable and would therefore give us the ability to study the importance of the loss of endoglycan in vivo. In addition, these “knockout” mice would allow us to isolate primary vSMC that lack endoglycan. This would greatly improve our ability to understand the role of endoglycan in smooth muscle differentiation and migration. Finally, these mice will be important in developing new robust, endoglycan antibodies that can detect endogenous levels of protein in a variety of assays, and facilitate the use of endoglycan as a marker for vSMC, pericytes or
vSMC progenitors. Until then, microarray profiling data from vSMC, pericytes or other mesenchymal cells could provide novel insights into the expression profile of endoglycan and allow us to speculate about potential functions for endoglycan in these cells.
References


diversity revealed by global expression profiling. Proceedings of the National Academy of Sciences 100, 10623–10628.


stable mammalian cell lines that express very high levels of recombinant proteins. Biochemical and Biophysical Research Communications 252, 368–372.


