Tissue Engineering Architectural Cues for \textit{in vitro} Models of Respiratory Epithelium

by

James Poon

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials & Biomedical Engineering
University of Toronto

© Copyright by James Poon, 2018
Tissue Engineering Architectural Cues for in vitro Models of Respiratory Epithelium

James Poon

Doctor of Philosophy

Institute of Biomaterials & Biomedical Engineering

University of Toronto

2018

Abstract

Diseases affecting the respiratory system are one of the major leading causes of death worldwide. Epithelial dysfunction is associated with many lung diseases and therefore tools for primary cell culture are important for generating models of human lung epithelium. Respiratory epithelium is comprised of many diverse cell types that inhabit structurally distinct regions in the lung and airways. In vitro approaches to generate or repair epithelium are inadequate as they do not incorporate the specific architecture that lung and airway tissues exhibit in vivo, resulting in disorganized and dysfunctional epithelial cells. We hypothesized micropatterned biophysical cues, in the form of stiffness and topography, will induce alignment of airway epithelial cells along a defined tissue axis. We also hypothesized that culture of distal lung epithelial cells in biomimetic architecture will extend the maintenance of viability and phenotype. We employed three scaffolding approaches with microengineered biophysical cues to control epithelial behaviour. In the first two approaches, we applied micropatterning techniques to create hydrogel systems that were compatible with air-liquid interface (ALI) culture of airway epithelium. Although our stiffness-patterning platform did not support primary culture, we found that
primary epithelial cells can be cultured on collagen vitrigel membranes and groove topography induces morphological alignment for up to 14 days of ALI culture. In the third approach, we generated a poly-dimethylsiloxane culture substrate with alveolar-mimetic curvature for culture of lung epithelial cells. Specifically, primary mouse cells grown in cavity culture conditions remain viable (96 ± 4% vs. 2 ± 1% on flat controls) and maintained expression of phenotypic markers (surfactant protein C, aquaporin-5) over one week. These findings demonstrate the profound influence of biophysical cues on epithelial behaviours including spreading, polarization and phenotype. Our rationally-designed biomaterial substrates are able to mimic numerous aspects of the extracellular matrix and direct the behaviour of adult primary cells derived from mammalian trachea and lung epithelial progenitors. Biomaterial scaffolds with defined architectural cues advance the capabilities of lung and airway epithelial models to instruct tissue function and provide platforms for understanding mechanisms of lung disease and repair.
Acknowledgements

The completion of this doctoral thesis was possible with the support of many people. First, I would like to express my sincere gratitude to my co-supervisors Drs. Alison P. McGuigan and Thomas K. Waddell for their continuous support of my PhD study. Their guidance was instrumental in all the time of research and writing of this thesis. I am deeply indebted to them for their patience, motivation, and wisdom. Their mentorship taught me to identify the right questions, how to find the answers to those questions, and allowed me to grow as a research scientist. My experience in your research program has taught me the lessons in persistence that I will carry for a lifetime.

I would also like to thank my committee members, Prof. Boris Hinz and Prof. Craig Simmons. Your insightful comments and suggestions were invaluable to the success of this thesis. Your critical review challenged me to widen my perspective.

I would like to acknowledge and thank Dr. Golnaz Karoubi for scientific discussion of manuscripts and for her input on the design of studies.

My special appreciation to my fellow graduate students and fellows in both the McGuigan and Waddell laboratory groups. In particular I would like to thank Dr. Takaya Suzuki for being an excellent role model and for his vital mentorship, support, and advice. Special thanks to John Soleas for his humour, encouragement, and for enduring the highs and lows with me. Thanks to Ratna Varma for her discussion and support regarding primary tracheal epithelial cells. Thanks to Dr. Lily Guo for her valued input and scientific advice. I would like to thank all the support staff at the Latner Thoracic Surgery Research Laboratories and BioZone for their scientific and technical input. All of you were there to support me when I collected the data for my PhD thesis.

I would like to thank Zhongfa (Felix) Liao and Prof. J. Stewart Aitchison for their fabrication expertise and ingenuity.

I thank Quynh Nguyen and Dr. Vito Mennella for their collaboration, discussion and insight regarding super-resolution microscopy and ciliated cells.

I thank Jenna Usprech for technical guidance and input on PEG chemistry and synthesis.

I thank Dr. Nadeem Moghal and Dr. Bo Ram Kim for providing access and insight on human tracheal epithelial cells.

I thank the Natural Sciences and Engineering Research Council of Canada for the Doctoral Program Postgraduate Scholarship that supported the majority my study. I thank the Institute of Biomaterials & Biomedical Engineering for their support through the Wildcat Graduate Scholarship.
I would also like to thank all of my friends who supported me throughout the years and for providing the emotional support I needed to strive towards my goal.

A special thanks to my family. My parents, Peter and Linda, and my brother and sister-in-law, Will and Jocelyn. Thank you for all the sacrifices that you’ve made for me and on my behalf. Your support and belief in me sustained me to complete this challenge.

At the end I would like to acknowledge and express my everlasting appreciation to my dear Miranda, who was there firsthand to put up with all the trials and tribulations. Thank you for your optimism, your encouragement, and for being a constant source of strength and inspiration that provided the hope I needed during many late nights of research. Your enduring and unwavering support made it possible for me to see this project through to the end.
Contributions

Chapter 3   Evaluation of UV-tunable Biomaterials as a Simple Tool for Cell Culture

James C.H. Poon\textsuperscript{1,2}, Sophie Mayrbaeurl\textsuperscript{3}, Hoda Maleki\textsuperscript{1}, Xian Wang\textsuperscript{4}, Craig Simmons\textsuperscript{1,4}, Alison P. McGuigan\textsuperscript{1,3}, and Thomas K. Waddell\textsuperscript{1,2,5}

1. Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto M5S 3G9, Canada

2. Latner Thoracic Surgery Research Laboratories and the McEwen Centre for Regenerative Medicine, Toronto General Hospital, 101 College St., Toronto, ON M5G 1L7, Canada

3. Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, M5S 3E5, Canada

4. Department of Mechanical and Industrial Engineering, University of Toronto, Toronto M5S 3G8, Canada

5. Institute of Medical Science, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada

J.C.H.P., A.P.M., and T.K.W. designed the study. J.C.H.P., S.M., H.M., and X.W. performed the experiments. J.C.H.P., S.M., H.M., and X.W. analyzed the data. J.C.H.P. wrote the chapter through discussion with and supervision by A.P.M. and T.K.W.

The XPS studies were performed by Rana Sodhi (Department of Chemical Engineering & Applied Chemistry, University of Toronto) at Surface Interface Ontario, University of Toronto. We thank Jenna Usprech (Institute of Biomaterials and Biomedical Engineering, University of Toronto) for technical assistance with PEG-NB and providing hMSCs. We thank Aileen Zhong (Institute of Biomaterials and Biomedical Engineering, University of Toronto) for providing VICs. We thank Emily Van de Laar (University Health Network, Toronto), Bo Ram Kim (Department of Medical Biophysics, University of Toronto), and Nadeem Moghal (Department of Medical Biophysics, University of Toronto) for providing tracheal epithelial cells and technical assistance with isolation. We thank Haijiao Liu (Institute of Biomaterials and Biomedical Engineering and Department of Mechanical and Industrial Engineering, University of Toronto) for technical assistance with AFM. We are grateful to Yu Sun (Department of Mechanical and Industrial Engineering, University of Toronto) for providing the Bioscope Catalyst AFM instrument for indentation experiments. The fluorescence studies were performed at the Advanced Optical Microscopy Facility, University Health Network, Toronto.
Chapter 4  Micro-grooved collagen vitrigel membranes align primary multiciliated airway epithelium during apical-basal polarization

James C.H. Poon\textsuperscript{1,2}, Quynh Nguyen\textsuperscript{3}, Zhongfa Liao\textsuperscript{4}, Ratna Varma\textsuperscript{1,2}, John P. Soleas\textsuperscript{1,2}, Golnaz Karoubi\textsuperscript{2}, J. Stewart Aitchison\textsuperscript{4}, Vito Mennella\textsuperscript{3,5}, Alison P. McGuigan\textsuperscript{1,6}, and Thomas K. Waddell\textsuperscript{1,2,7}

1. Institute of Biomaterials & Biomedical Engineering, University of Toronto, 200 College St., Toronto, ON M5S 3E5, Canada

2. Latner Thoracic Surgery Research Laboratories and the McEwen Centre for Regenerative Medicine, Toronto General Hospital, 101 College St., Toronto, ON M5G 1L7, Canada

3. Department of Biochemistry, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada

4. Electrical & Computer Engineering, University of Toronto, 10 King’s College Road, Toronto, ON M5S 3G4, Canada

5. Cell Biology Program, The Hospital for Sick Children, 686 Bay St., Toronto, ON M5G 1X8, Canada

6. Department of Chemical Engineering & Applied Chemistry, University of Toronto, 200 College St., Toronto, ON M5S 3E5, Canada

7. Institute of Medical Science, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada

J.C.H.P., A.P.M., and T.K.W. designed the study. J.C.H.P., Q.N., Z.L., and R.V. performed the experiments. J.C.H.P. analyzed the data. J.C.H.P. wrote the chapter through discussion with and supervision by G.K., A.P.M. and T.K.W.

We are grateful to Vito Mennella for providing the super-resolution microscope for 3D-SIM experiments. We thank Emily Van de Laar (University Health Network, Toronto), Bo Ram Kim (Department of Medical Biophysics, University of Toronto), and Nadeem Moghal (Department of Medical Biophysics, University of Toronto) for providing tracheal epithelial cells and technical assistance with isolation. The fluorescence studies were performed at the Advanced Optical Microscopy Facility, University Health Network, Toronto.
Chapter 5 Design of biomimetic substrates for long-term maintenance of alveolar epithelial cells


James C.H. Poon\textsuperscript{1,2}, Zhongfa Liao\textsuperscript{3}, Takaya Suzuki\textsuperscript{2}, Miranda M. Carleton\textsuperscript{2}, John P. Soleas\textsuperscript{1,2}, J. Stewart Aitchison\textsuperscript{3}, Golnaz Karoubi\textsuperscript{1,2}, Alison P. McGuigan\textsuperscript{1,4*}, and Thomas K. Waddell\textsuperscript{1,2,5*}

1. Institute of Biomaterials & Biomedical Engineering, University of Toronto, 200 College St., Toronto, ON M5S 3E5, Canada

2. Latner Thoracic Surgery Research Laboratories and the McEwen Centre for Regenerative Medicine, Toronto General Hospital, 101 College St., Toronto, ON M5G 1L7, Canada

3. Electrical & Computer Engineering, University of Toronto, 10 King’s College Road, Toronto, ON M5S 3G4, Canada

4. Department of Chemical Engineering & Applied Chemistry, University of Toronto, 200 College St., Toronto, ON M5S 3E5, Canada

5. Institute of Medical Science, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada

J.C.H.P., A.P.M., and T.K.W. designed the study. J.C.H.P., Z.L., T.S., and M.M.C. performed the experiments. J.C.H.P. analyzed the data. J.C.H.P. wrote the chapter through discussion with and supervision by G.K., A.P.M. and T.K.W.

The fluorescence studies were performed at the Advanced Optical Microscopy Facility, University Health Network, Toronto.
Table of Contents

Acknowledgements vi
Contributions vii
Table of Contents ix
Table of Figures and Tables xv
Table of Supplementary Figures and Tables xvii
List of Abbreviations xviii

Chapter 1 Rationale, Objectives, Aims and Hypotheses

1.1 Rationale 2

1.2 Specific Objectives and Hypothesis 5

Chapter 2 Literature Review

2.1 Introduction 8

2.2 Respiratory Epithelium 9

2.2.1 Tracheal epithelial biology 10

2.2.1.1 Terminally differentiated airway cells 10

2.2.1.1.1 Tracheal epithelial cell types 10

2.2.1.1.1.1 Multiciliated cells 11

2.2.1.1.1.1.1 Cilia ultrastructure 12

2.2.1.1.1.1.2 Ciliogenesis 14

2.2.1.1.1.1.3 Refinement of cilia polarity 16

2.2.1.1.1.1.4 Mucociliary clearance 17

2.2.1.1.1.1.5 Organization of MCCs 17

2.2.1.2 Apical-basal polarity 18

2.2.1.3 Planar polarity 21
2.2.2 Alveolar epithelial biology

2.2.2.1 Type 1 cells

2.2.2.2 Type 2 cells

2.2.3 Extracellular matrix components and structural organization

2.2.3.1 Tracheal ECM

2.2.3.2 Alveolar ECM

2.3 Current challenges for primary epithelial culture

2.4 Current in vitro culture models

2.4.1 Platforms and approaches relevant to in vitro airway models

2.4.1.1 Culture platforms

2.4.1.1.1 Transwells

2.4.1.1.2 Hydrogels

2.4.1.1.2.1 Naturally-derived hydrogels

2.4.1.1.2.1.1 Gelatin

2.4.1.1.2.1.2 Collagen

2.4.1.1.2.1.3 Collagen vitrigel membrane (CVM)

2.4.1.1.2.1.4 Silk fibroin

2.4.1.1.2.1.5 Matrigel

2.4.1.1.2.2 Synthetic hydrogels

2.4.1.1.2.2.1 PEG-NB

2.4.1.2 Tissue engineering approaches

2.4.1.2.1 Micropatterning

2.4.1.2.2 Engineered Stiffness

2.4.1.2.3 Engineered Topography

2.4.2 Platforms and approaches relevant to in vitro alveolar models
Chapter 3 Evaluation of UV-tunable Biomaterials as a Simple Tool for Cell Culture

3.1 Abstract
3.2 Introduction
3.3 Methods
  3.3.1 PEG-NB polymer synthesis
  3.3.2 Hydrogel fabrication
  3.3.3 Mechanical testing of substrates
  3.3.4 AFM Measurement and data analysis
  3.3.5 X-ray photoelectron spectroscopy (XPS)
  3.3.6 Visualization of CRGDS
  3.3.7 Cell Culture
  3.3.8 Quantification of cellular elongation and alignment
  3.3.9 Microscopy
  3.3.10 Statistical analysis
3.4 Results
  3.4.1 Stiffness varies with PEG-NB content and UV exposure
  3.4.2 Photopatterned PEG-NB produces a cell alignment response
  3.4.3 Alignment does not depend on cell density
  3.4.4 Patterning with UV light does not produce local stiffness differences in PEG-NB
  3.4.5 UV-patterned PEG-NB gels have no topography
  3.4.6 UV-patterned PEG-NB gels have differential adhesion
  3.4.7 Tracheal progenitor cells have RGD-responsive receptors
3.4.8 Cell adherence and spreading on PEG-NB gels under defined conditions varies for different cell types

3.5 Discussion

3.6 Conclusions

Chapter 4 Micro-grooved Collagen Vitrigel Membranes Align Primary Multiciliated Airway Epithelium during Apical-basal Polarization

4.1 Abstract

4.2 Introduction

4.3 Methods

4.3.1 Generation of microgrooves

4.3.2 Microgroove geometry characterization

4.3.3 Epithelial cell line culture

4.3.4 Isolation and culture of HTECs

4.3.5 Cell seeding and ALI culture

4.3.6 Immunocytochemistry

4.3.7 Microscopy

4.3.8 Quantification of cellular elongation and alignment

4.3.9 Statistical analysis

4.4 Results

4.4.1 Primary cells do not align on 3.14 μm deep grooves

4.4.2 Primary cells align on 5 μm deep grooves

4.4.3 Grooves induce HTEC morphological planar polarity in early ALI culture

4.4.4 Ciliation of polarized HTECs on CVM

4.5 Discussion

4.6 Conclusions
Chapter 5 Design of Biomimetic Substrates for Long-term Maintenance of Alveolar Epithelial Cells

5.1 Abstract

5.2 Introduction

5.3 Methods

5.3.1 Fabrication of hemispherical architecture masters

5.3.2 Manufacture of cavity culture substrates

5.3.3 Hemisphere geometry characterization

5.3.4 Isolation of mouse alveolar epithelial cells

5.3.5 Cell culture

5.3.6 Immunocytochemistry and microscopy

5.3.7 Quantification of nuclear area coverage, cell viability and phenotype

5.3.8 Statistical analysis

5.4 Results and Discussion

5.4.1 Characterization of biomimetic substrate manufacturing strategy

5.4.2 Optimization of seeding strategy to produce cavities with primary monolayers

5.4.3 Cell viability is maintained in cavity culture

5.4.4 Cavity culture maintains SPC and AQP5 expression over 7 days

5.5 Conclusions

Chapter 6 Conclusion, Discussion and Future Directions

6.1 Biomaterials with UV-tunable properties enable precise spatial control of cell alignment

6.2 Micro-grooved topography aligns primary airway epithelium during apical-basal polarization
6.3 Biomimetic architecture maintains alveolar epithelial cell utility 133

6.4 Overall Research Contributions 136

Chapter 7 Supplementary Figures and Tables 137

Chapter 8 References 143
## List of Figures and Tables

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Schematic diagram of cilia ultrastructure.</td>
<td>13</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Stages of ciliogenesis in HTEC culture.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Actin and microtubules organize cilia in multiciliated cells.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Interactions of polarity complex components in polarized epithelial cells.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Hierarchical organization of ciliated epithelium.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Cell types of the alveolus.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Basement membrane of rat tracheal whole mount.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Architecture of mouse lung alveoli.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Anatomy and epithelial histology of the adult human lung.</td>
<td>30</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Human tracheal progenitors possess proliferative and differentiation potential.</td>
<td>32</td>
</tr>
<tr>
<td>Table 1.</td>
<td>Hydrogel overview.</td>
<td>37</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Theoretical compositions of nitrogen in 8-arm PEG-NB hydrogels.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Stiffness varies with monomer content and UV exposure in PEG-NB gels.</td>
<td>56</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>hMSCs and VICs have different responses on photopatterned PEG-NB.</td>
<td>57</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Cellular alignment at varying cell densities.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>UV-patterned PEG-NB gels do not have anisotropic stiffness.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Photopatterning of PEG-NB gels does not introduce surface topography.</td>
<td>61</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Measured compositions of nitrogen in 8-arm PEG-NB hydrogels.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>Patterning does not create differential diffraction of light.</td>
<td>63</td>
</tr>
<tr>
<td>Figure 17.</td>
<td>UV-patterned PEG-NB gels have patterned adhesion.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 18.</td>
<td>HTECs express RGD-competent receptors.</td>
<td>65</td>
</tr>
<tr>
<td>Figure 19.</td>
<td>Cell morphology on TCPS and PEG-NB hydrogels.</td>
<td>67-68</td>
</tr>
<tr>
<td>Figure 20.</td>
<td>BEAS-2B and HTECs on 3.14 μm deep grooves.</td>
<td>86</td>
</tr>
<tr>
<td>Figure 21.</td>
<td>Screening optimal groove dimensions for epithelial cell culture.</td>
<td>87</td>
</tr>
<tr>
<td>Figure 22.</td>
<td>Primary HTEC elongation and alignment on PDMS microgrooves.</td>
<td>89</td>
</tr>
<tr>
<td>Figure 23.</td>
<td>Culture of primary HTECs on CVM microgrooves under ALI.</td>
<td>90</td>
</tr>
<tr>
<td>Figure 24.</td>
<td>Ciliation of polarized HTECs on CVM.</td>
<td>92</td>
</tr>
<tr>
<td>Figure 25.</td>
<td>CVM produces optical aberrations in 3D-SIM.</td>
<td>93</td>
</tr>
<tr>
<td>Figure 26.</td>
<td>Biomimetic substrate manufacturing strategy.</td>
<td>110</td>
</tr>
<tr>
<td>Figure 27.</td>
<td>Characterization of substrate cavities.</td>
<td>112</td>
</tr>
<tr>
<td>Figure 28.</td>
<td>Optimization of primary cell seeding to produce monolayers within the cavities.</td>
<td>114</td>
</tr>
<tr>
<td>Figure 29.</td>
<td>Assessment of cell viability maintenance on flat versus cavity substrates.</td>
<td>116</td>
</tr>
<tr>
<td>Figure 30.</td>
<td>Assessment of AT2 phenotype on flat versus cavity substrates.</td>
<td>117</td>
</tr>
<tr>
<td>Figure 31.</td>
<td>Assessment of AT1 phenotype on flat versus cavity substrates.</td>
<td>119</td>
</tr>
</tbody>
</table>
List of Supplementary Figures and Tables

SI Table 1. Characterization of microgrooved PDMS 138

SI Figure 1. BEAS-2B alignment on PDMS microgrooves 139

SI Figure 2. At higher groove depths (10 μm), monolayer formation is compromised 140

SI Figure 3. Optimization of cavity substrate manufacturing process 141

SI Figure 4. Optimization of cell seeding to achieve reproducible monolayer cultures within the cavities 142
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µCP</td>
<td>Microcontact printing</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>3D-SIM</td>
<td>Three-dimensional structured illumination microscopy</td>
</tr>
<tr>
<td>AEC</td>
<td>Alveolar epithelial cell</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALI</td>
<td>Air-liquid interface</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>AQP5</td>
<td>Aquaporin-5</td>
</tr>
<tr>
<td>AT1</td>
<td>Type 1 alveolar epithelial cell</td>
</tr>
<tr>
<td>AT2</td>
<td>Type 2 alveolar epithelial cell</td>
</tr>
<tr>
<td>BB</td>
<td>Basal body</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth media</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BOE</td>
<td>Buffered oxide etcher</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCSP</td>
<td>Club cell secretory protein</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CVM</td>
<td>Collagen vitrigel membrane</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>DMAP</td>
<td>(dimethylamino)pyridine</td>
</tr>
<tr>
<td>DRIE</td>
<td>Deep reactive ion etching</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EBL</td>
<td>Electron beam lithography</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>Fzd6</td>
<td>Frizzled 6</td>
</tr>
<tr>
<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>HTECs</td>
<td>Human tracheal epithelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropyl alcohol</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induce pluripotent stem cells</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>Lgl1</td>
<td>Lethal giant larvae homolog 1</td>
</tr>
<tr>
<td>Lgl2</td>
<td>Lethal giant larvae homolog 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCCs</td>
<td>Multiciliated cells</td>
</tr>
</tbody>
</table>

xviii
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MIBK</td>
<td>Methyl isobutyl ketone</td>
</tr>
<tr>
<td>MIRO</td>
<td>Microscope image registration and overlay</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mPADs</td>
<td>Micropost array detector systems</td>
</tr>
<tr>
<td>NB</td>
<td>Norbornene</td>
</tr>
<tr>
<td>NEBs</td>
<td>Neuroepithelial bodies</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PATJ</td>
<td>Protein associated with tight junctions</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEG8NB</td>
<td>PEG-octa-norbornene</td>
</tr>
<tr>
<td>PEGdiPDA</td>
<td>PEG di-photodegradable acrylate</td>
</tr>
<tr>
<td>PEG-NB</td>
<td>PEG-norbornene</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PNECs</td>
<td>Pulmonary neuroendocrine cells</td>
</tr>
<tr>
<td>POC1B</td>
<td>POC1 centriolar protein homolog B</td>
</tr>
<tr>
<td>SAGM</td>
<td>Small airway growth media</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPA</td>
<td>Surfactant protein A</td>
</tr>
<tr>
<td>SPB</td>
<td>Surfactant protein B</td>
</tr>
<tr>
<td>SPC</td>
<td>Surfactant protein C</td>
</tr>
<tr>
<td>SPD</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vangl1</td>
<td>Van Gogh-like 1</td>
</tr>
<tr>
<td>VICs</td>
<td>Valvular interstitial cells</td>
</tr>
<tr>
<td>Wt%</td>
<td>Weight percent</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
Chapter 1

Rationale, Objectives, Aims and Hypotheses
1.1 **Rationale**

Respiratory disease is the third leading cause of death in the developed world [1]. Attempts to repair injured respiratory epithelium with autologous tissues, cryopreserved homografts or allogeneic tissues have so far proven challenging [2, 3]. In these cases epithelialization was inadequate both in terms of the number of cells and the formation of the appropriate histological subtype. Lung and airway tissues exhibit specific architecture *in vivo* that is linked to cell maintenance and function however these cues have not been previously incorporated into engineered epithelium strategies. Therefore, there is an urgent need to explore the role of architectural factors controlling tissue maturation and organization for generating highly functional tissues for repair of respiratory epithelium. Recent advances in biomaterials science enable the rational design of biomimetic scaffolds to instruct cell function and maintenance. However scaffolds are not yet optimized to support epithelial cell attachment, growth and maturation and this remains an important challenge that needs to be addressed. In the following paragraphs we describe recent progress for the construction of trachea and lung replacements, the key challenges that remain and which of these challenges are addressed in this thesis.

In the case of generating tracheal replacements, in recent years there has been an effort to create tissue-engineered constructs for large airways, using decellularized cadaveric donor matrices seeded with recipient-derived cells [4, 5]. Graft survival is high but these few cases were performed on compassionate grounds and formal clinical trials remain to be completed. To date, the implantation of synthetic-based scaffolds in patients results in poor survival rates and remains controversial [2, 6-8]. Recipients were susceptible to recurrent infections and mucous impactions postoperatively, resulting in re-hospitalization and high mortality rates [2, 3]. In animal models it has been shown that part of the reason for graft failure is due to incomplete,
delayed, or dysfunctional epithelialization of the airway constructs [9]. In patients that received tracheal allografts mucosal coverage was slow (>1 month), and restoration of mucociliary clearance took up to 6 months after surgery [3, 4, 6]. This highlights a major limitation for trachea replacement: scaffolds must not only permit epithelialization, but must also facilitate the organization and maturation of highly functional epithelium. This challenge is addressed in Chapters 3 and 4.

In the case of generating engineered lung replacements, available sources of alveolar epithelial cells (AECs) are limited. Primary cells isolated from lung tissue represent the most relevant model of mature lung biology but alveolar epithelial models do not perfectly capture the in vivo situation as primary cells lose their phenotype and have limited utility after isolation [10, 11]. Studies characterizing lung organoids or cystic structures where cells were embedded in three-dimensional (3D) hydrogels report higher maintenance of cell viability but not phenotype. These limitations effectively restrict the number of cells available for assembling engineered lung models and highlights a major limitation for lung replacement: in vitro culture models of alveolar epithelium do not adequately present the architectural cues that cells require to maintain AEC identity. This challenge is addressed in Chapter 5.

Thesis Overview

The overall aim of this thesis is to explore the utility of micro-scale architectural cues to manipulate the organization and maturation of airway epithelium. We defined architecture as physical or structural features in tissue (e.g. curvature, topography). In Chapter 2 of this thesis we provide a summary of the current literature and describe the state-of-the-art models available to engineer airway epithelium in vitro. In the rest of the thesis we utilized three tissue-engineered
scaffolding approaches to augment the current methods used for primary culture of adult respiratory epithelial cells in models of trachea and distal lung.

The first approach investigates the role of local substrate stiffness on epithelial progenitors. Mechanical stiffness studies are commonly carried out on isotropically stiff substrates or surfaces with unidirectional stiffness gradients [12, 13]. Many native tissues are however anisotropically stiff and it is unknown whether controlled presentation of stiff and soft axes on the same substrate governs cell alignment during repair and differentiation. To our knowledge, systems do not yet exist to produce subcellular-scale anisotropic stiffness that is compatible with air-liquid interface (ALI) culture. Such a system would permit the generation of aligned mature airway epithelium. Therefore in Chapter 3 we describe the development of a spatially-patterned hydrogel culture platform to direct cell alignment.

The second approach to tissue engineering respiratory epithelium involves imprinting biomaterials with substrate topography for the alignment of primary ciliated epithelium. Topographical cues are present in the ECM of many tissues [14], and their influence has been observed for many types of cells [15]. Recent work from our labs has shown that the bronchial epithelial cell line BEAS-2B grown on 3.14 µm deep gelatin grooves exhibit cell and cytoskeletal alignment along the direction of the grooves [16]. However, BEAS-2B cells are a limited model for tracheal epithelium as they do not form the multiciliated cells (MCCs) that line the tracheal lumen. Therefore in Chapter 4 we optimized an alignment strategy with conditions that would enable simultaneous planar polarization and ciliogenesis for primary human tracheal epithelial cells (HTECs). Specifically we describe the adaptation of microgrooved topography to generate hydrogel inserts that were compatible with transwell culture.
The third approach relates to the role of biomimetic architecture in preserving cell viability and phenotype. Traditionally, AECs are cultured on flat plastic or transwell surfaces, which do not recapitulate the physical microenvironment that cells experience \textit{in vivo}. Adult type 2 AECs (AT2) cells cultured on these substrates have a reduction in cell survival, asymmetric division and rapid loss of phenotype [10, 11]. The tissue engineering literature suggests differences in phenotype between cells cultured in 2-dimensional (2D) and 3D systems. Architectural cues have been shown to modulate proliferation, cell survival, and differentiation in many cell types including epithelial and stem cells [17-21]. The hollow acinar architecture of the alveolus could be an important physical cue for the preservation of cell phenotype [22-25]. Therefore in \textit{Chapter 5} we describe the development of a biomimetic culture platform and characterize the effect of 3D culture towards maintaining cell utility.

Finally in \textit{Chapter 6} of this thesis we discuss the conclusions and limitations of the work and suggest areas for further investigation in the future.

1.2 \textbf{Specific Objectives and Hypothesis}

This thesis is comprised of the following three specific objectives:

\textbf{Objective \#1}: To develop a simple patterning technique for generating hydrogel cell culture substrates with subcellular-scale anisotropic stiffness.

\textbf{Hypothesis}

PEG-based hydrogel polymers have UV-tunable properties that can modulate stiffness, crosslinking, and peptide content. We hypothesize simple photopatterning of poly(ethylene glycol)-norbornene (PEG-NB) hydrogels will produce significant differences in the local
stiffness of the gel surface that generates alignment of primary tracheal epithelial tissue (tested in Chapter 3).

**Objective #2:** To customize grooved culture systems that support the expansion and organized differentiation of primary HTECs.

**Hypothesis**
We hypothesize that culture of primary human airway epithelial cells on grooved substrates will induce cytoskeletal and nuclear alignment, leading to cilia alignment along a defined tissue axis (tested in Chapter 4).

**Objective #3:** To generate highly-reproducible surfaces of biomimetic architecture that maintains the viability of lung epithelial cells, and use this platform to investigate the role of architecture on the preservation of AEC phenotype.

**Hypothesis**
We hypothesize that culture of primary AECs on alveolar-mimetic architecture will prolong the maintenance of AEC viability and phenotype (tested in Chapter 5).
Chapter 2

Literature Review
2.1 Introduction

The respiratory tree can be divided into three structurally distinct regions: i) the large airways (trachea and primary bronchi), ii) the small airways (bronchioles), and iii) the alveoli [1, 26]. Each region is lined by a different epithelial subtype and supported by a dense vascular network important for nutrient-waste exchange [27]. The trachea and primary bronchi form the beginning of the cartilaginous airways, whose function is to conduct and condition the air. The columnar epithelium in this region plays an important defensive role by removing particulates and pathogens in the air. As the epithelium progresses towards the distal small airways, it changes from pseudostratified to simple cuboidal epithelium [28-31]. At the terminal end of the respiratory tree is the alveolar region. Alveoli are the smallest functional unit of primary gas exchange. They consist of peripheral sacs separated by primary septa; the structure of an alveolus maximizes the surface area for fast diffusion of oxygen and carbon dioxide.

Attempts to repair injured respiratory epithelium with decellularized tracheal scaffolds have been achieved with mixed results. One of the major limitations is that epithelialization by seeded cells within the engineered construct is poor and cells do not quickly re-establish the pseudostratified epithelial tissue important for mucus clearance [4]. Evidence from in vivo injury models suggests that if the basal lamina is not adequately re-epithelialized, the underlying stromal fibroblasts undergo extensive proliferation and give rise to granulation tissue that block the airways [32]. These data indicate that there is a tight interplay between the airway epithelium and underlying stroma that keeps fibrosis in check. The main challenge in generating tissue-engineered grafts with highly functional epithelium is that the factors governing cell maturation and tissue level organization have yet to be explored and clearly defined. The assembly of component cells into an artificial tissue provides a platform to explore the relationship between lung cell properties and tissue architecture.
This review is organized to highlight the current progress of tissue-engineered airways and outstanding challenges within the field, such as poor epithelialization of constructs and how this might be enhanced by architectural cues. We review some of the discoveries and current understanding of lung epithelial cells derived from two of the epithelial stem/progenitor cell populations in the adult lung: basal cells and type 2 alveolar epithelial (AT2) cells. We discuss their function, organization, and the current challenges for their primary culture in vitro. We consider how cell matrix architecture can influence lung biology, such as the mechanisms by which lung progenitors organize and maintain homeostasis, and how engineered scaffolding approaches could harness these cues to manipulate epithelial behaviour. Finally, we conclude with some of the current state-of-the-art in vitro models, their advantages and limitations, and future directions for designing more physiologically-relevant epithelial culture models.

2.2 **Respiratory Epithelium**

The lung and airways are thought to be composed of as many as 40 different cell types [33]. The airway epithelium possesses secretory, ciliated, basal and neuroendocrine cells. Secretory cells produce mucins and antimicrobial peptides, whereas ciliated cells produce the propulsion necessary to clear debris [34]. Basal cells are found in proximal airways where they act as epithelial progenitors to regenerate lost secretory and ciliated cells. Neuroendocrine cells function as sensors of the external environment. By contrast, the distal lung contains alveolar epithelium that is comprised of type 1 (AT1) and AT2 cells. AT1 cells have functions in gas exchange while AT2 cells produce surfactant that reduces the work of breathing. This thesis focuses on the epithelial compartment of the trachea and distal lung, with particular emphasis on ciliated and AT2 cells.
2.2.1 **Tracheal epithelial biology**

The tracheal epithelium is characterized by its pseudostratified morphology and serves several functions including maintaining homeostasis, selective transepithelial transport, ion and fluid balance, coordinating fluid movement, mucus secretion, and acting as a physical barrier [35]. Basal, club, goblet and multiciliated cells (MCCs) populate the epithelial lining. These cells each perform different and sometimes overlapping functions. All cells have direct connections with the basement membrane (BM) and form highly specialized contacts with neighbouring cells and the extracellular matrix (ECM) [36-39].

2.2.1.1 **Terminally differentiated airway cells**

2.2.1.1.1 **Tracheal epithelial cell types**

In the tracheal epithelium, terminally differentiated airway cells have specialized functions, do not move or change position, and do not undergo mitosis (cell division). In humans, the terminally differentiated cell types found in most of the large airways (down to 1.0-1.5 mM in diameter) are MCCs, goblet cells, club cells and rare neuroendocrine cells [40].

Goblet cells are most abundant in the proximal airways and generally not found in the distal bronchiolar region. They are molecularly characterized by expression of MUC5AC and MUC5B [36]. This secretory cell type is responsible for producing the mucin proteins, glycoproteins, and antimicrobial peptides that comprise mucus, which functions to trap inhaled foreign material to clean the air [41, 42]. Mucus secretion can be stimulated by chemical irritants, immune secretions and airflow [43-45]. The controlled production of mucus is critical for mucociliary clearance and airway health.

Club cells are scattered throughout much of human airway epithelium, with a higher concentration in the distal respiratory bronchioles [46]. They are molecularly characterized by
the expression of club cell secretory protein (CCSP; also known as CC10; in humans) and SCGB1A1 (in mice) [35]. Club cells are secretory with short microvilli [46, 47]. These dome-shaped cells secrete CCSP, uteroglobin, secretoglobin 1A, and glycosaminoglycans (GAGs) to protect the bronchiole lining and degrade mucus [1, 48-51]. Furthermore, club cells are also involved in xenobiotic metabolism via cytochrome P450s such as CYP4B1 [50].

Pulmonary neuroendocrine cells (PNECs) are rare, basally-localized cells present in small numbers in the conducting airway [1, 26, 52-54]. They can be found as single cells or as organized clusters that are in close contact with nerve fibres and surrounded by variant club cells [35]. In early generations of the airway tree they are found as single cells. PNECs also cluster near branch points in the intralobar airways where they are known as neuroepithelial bodies (NEBs) [36]. Molecularly, PNECs are marked by the expression of CGRP (calcitonin gene-related peptide; CALCA), chromogranin A and ASCL1 (achete-scute homolog 1) [52, 55]. The role of PNECs is to sense stimuli in the airways (e.g. oxygen, chemical, mechanical); they are thought to communicate with the immune system and the nervous system. [35, 56-58]. Lineage-tracing studies indicate that PNECs self-renew, but do not contribute to other epithelial cell lineages under steady state conditions [55].

2.2.1.1.1 Multiciliated cells

A fourth terminally differentiated cell type found throughout the large airways are multiciliated cells (MCCs). MCCs possess specialized organelles known as motile cilia, which can be found on the apical cell surface in clusters of 100-300 [59]. Cilia beat cooperatively, in a coordinated wave to generate directional fluid flow (known as ‘metachronal synchrony’). This cell type possesses a high number of mitochondria to satisfy the energy demands of cilia beating [60]. Molecularly, MCCs are characterized by expression of the nuclear transcription factor
Because much of the work presented here concerns MCC, cilia will be discussed in further detail.

2.2.1.1.1.1 Cilia ultrastructure

Cilia are anchored at their base by a tubulin-based structure known as the basal body (BB). BBs are critical for cilia assembly [61] as they serve as the nucleation sites for the axoneme, which emerges through the ciliary pocket [62]. The BB originates from the mother centriole (from the previous round of mitosis) and is essentially a modified centriole comprised of 9 microtubule triplets with accessory structures including transition fibres, basal feet, and ciliary rootlets [63]. In multiciliated epithelia, many BBs are generated de novo from a special protein structure known as the deuterosome [64]. The centriole core of the basal body is approximately 250 nm in diameter, near the physical limit of traditional fluorescence optical microscopy [65]. BBs can be reliably marked with fluorescently tagged centrin, gamma tubulin or POC1 centriolar protein homolog B (POC1B) [66-68]. Important structures associated with the BB that are relevant for this thesis include the basal foot and the striated rootlet [66]. The basal foot projects asymmetrically off the BB in the direction of cilia beating [69, 70]. This structure can be marked by centriolin [71]. The striated rootlet projects off the opposite direction [72, 73] and can be marked by fluorescently tagged CLAMP [66].

The axoneme is a microtubule-based structure that makes up the internal cytoskeleton of the cilia (Fig. 1) [60]. It is comprised of a ring of 9 microtubule doublets [74]. Motile cilia have two additional central microtubules in the center of the 9 doublets. The 9 microtubule doublets are made up of one complete A-tubule and an attached but incomplete B-tubule [75]. The A- and B-tubules are made up of protofilaments, which themselves are comprised of α- and β-tubulin monomers [76]. The outer doublet microtubules are connected to adjacent doublets by nexin.
Figure 1. Schematic diagram of cilia ultrastructure. Cilia are anchored to the cell through the BB. Axonemes are comprised of 9 microtubule doublets; motile cilia have an additional microtubule doublet in the centre of the nine outer doublets. Radial spokes connect the inner doublets to the outer doublets. Dynein and nexin proteins connect the outer doublets to each other. Reprinted with permission from [62].
linkers, which function to maintain cilia structure and rigidity, preventing the movement of outer doublets with respect to each other. Inner microtubule doublets are connected to outer doublets through radial spokes [77]. These multi-protein complexes provide structural support for the axoneme. To drive cilia movement, the axonemes of motile cilia have 2 sets of dynein arms (inner and outer) along the length of the microtubule doublets that connect them with each other [78]. Axonemal dyneins are ATPase-driven unidirectional motor proteins that “walk” along adjacent microtubule doublets. The combined configuration of the central doublet, radial spokes, nexin linkages and dynein arms produces the inter-doublet sliding that translates into ciliary beating [78, 79]. Two phases comprise ciliary beating: the vertical power stroke and the horizontal recovery stroke. The power stroke produces the movement that propels fluid, while the recovery stroke primes the cilium for the power stroke. Both sets of dynein motors are required to drive both phases of beating [60].

2.2.1.1.1.2 Ciliogenesis

In primary culture of mammalian airway epithelial progenitors, primary cilia are present in the submerged cells prior to the emergence of motile cilia (see Current in vitro culture models section) [80, 81]. After induction of air-liquid interface (ALI), cells destined to become MCCs reabsorb their primary cilia and the constituent proteins are recycled for the generation of motile cilia. These results recapitulate observations in developing mouse lungs whereby primary cilia are detected in lung buds at embryonic day (E) 9.5 and replaced by motile cilia at E15.5.
Figure 2. Stages of ciliogenesis in primary culture. Schematic representation of primary airway epithelial cells and the four stages of ciliogenesis. Adapted with permission from [82].

Ciliogenesis occurs through several ordered steps (Fig. 2) [82]. Initially Foxj1− cells have short primary cilia (~1-2 µm) with two centrioles at the apical surface (either submerged or early ALI cultures). In the next stage (Stage I), Foxj1− cells nucleate a long (5-10 µm) primary cilium and amass ciliary components into pericentrosomal aggregates (the deuterosome) [64, 83]. These proteins are waiting to be incorporated into the BB and axoneme (~ALI D2 in mice, decrease in abundance until ALI D10). In Stage II cells, centrioles undergo replication to give rise to many BBs ("centriologenesis") [81]. These Foxj1+ cells exhibit a tight cluster of nascent centrioles positioned which are positive for many ciliary proteins and tubulin. At this stage, cells are actively generating centrioles (~ALI D3 in mice and decrease in abundance until ALI D12). Stage III cells are strongly Foxj1+ and characterized by the outward and upward spreading of centrioles from the cluster. Acetylated α-tubulin and centriolar proteins do not perfectly overlap, representing their localization to distinct regions of the BB. Centrioles are undergoing apical trafficking and docking at this stage (~ALI D4 in mice, persisting until ALI D14). In the final stage (Stage IV), cells are characterized by evenly distributed centrioles at the apical surface, which function as BBs for motile axonemes (1-5 µm long). Stage IV cells are mature MCCs with
motile cilia that have complete ciliogenesis. Cells at this stage are strongly Foxj1+ and axonemes are strongly positive for acetylated α-tubulin (~ALI D4, represent all MCCs after ALI D14).

EM studies indicate some cells have both replicating and trafficking BBs, suggesting overlap exists between Stages II and III. The timing of ciliogenesis is tightly controlled by the age of the centriole. Older centrioles function as axoneme nucleation sites much sooner than younger, newly-created centrioles [84], possibly explaining the overlap between Stage II and Stage III ciliogenesis. BB transport is thought to be mediated by effectors of the planar cell polarity (PCP) pathway and a Rho GTPase, which involves BBs attaching to membrane-bound vesicles and vesicle-trafficking machinery [73]. During BB docking, the BB/vesicle complex fuses with the cell plasma membrane forming a ciliary membrane that is contiguous with the cell membrane.

2.2.1.1.1.3 Refinement of cilia polarity

Many multiciliated epithelia, including tracheal epithelium, Xenopus laevis larvae skin and brain ependymal cells have highly conserved mechanisms to generate planar polarity and cilia alignment. Refinement of cilia orientation is essential for coordinating MCCs across a tissue to create tissue-level alignment and generating the directional fluid flow for effective mucociliary clearance. Cilia have orientation both within cells (rotational orientation) and across a tissue axis (tissue-level orientation). It is important to note that once BBs have docked and cilia are generated, the cilia beat direction is largely determined; the initial docking determines the gross orientation of the cilia beat direction [73]. This mechanism is likely patterned and biased, as BBs in E17.5 mouse tracheas show significant polarity immediately after docking [69]. After docking, refinement of BB orientation occurs in response to cilia-derived fluid flow [85, 86]. The small local shear forces act as a positive feedback loop to refine neighbouring cilia to all point towards the same direction [87]. PCP protein signaling has been shown to be necessary and sufficient for
initiating the weakly directional flow that polarize and refine cilia [69, 86, 88]. MCCs across a tissue are able to refine their beat direction within a specific time-window prior to complete maturation, at both the rotational and tissue-level [69, 86, 89]. The refinement of cilia is consistent with the timing of PCP signaling contributing to initial cilium orientation [69, 86, 87]. Fully mature cilia have locked orientations and do not respond to externally-applied flow [86].

2.2.1.1.1.4 Mucociliary clearance

MCCs work together to produce the primary airway defense known as mucociliary clearance. Coordinated ciliary beating generates the propulsive force that moves mucus towards the hypopharynx for elimination [90, 91]. Ciliary beating is organized as a metachronal wave, which visually appears as the propagation of a wave-like motion across the epithelial surface [92-94]; the beating of motile cilium in sequence produces more effective mucus clearance than synchronized beating. In the trachea, the baseline clearance rate is approximately 12-16 Hz and varies depending on the mucus viscosity [95, 96]. Dysfunction of mucociliary clearance is a feature of many chronic lung and airway diseases [97, 98].

2.2.1.1.1.5 Organization of MCCs

Within individual cells, cilia protein components are arranged in well-ordered structures with high molecular density. The BBs of individual cilia are linked to each other by an intricate network of microtubules and actin filaments (Fig. 3) [66], which are additionally linked to the proximal membrane by cortex-associated microtubules. The microtubules function to directly connect and space neighbouring BBs. Microtubule dynamics are essential for establishing local coordination of cilia polarity [66]. There exist two distinct pools of actin near the apical surface of MCCs [66]. The most apical pool of actin forms a mesh-like network in same plane as the BBs and functions to space the BBs. A second pool of sub-apical actin, 0.5 µm below the cell
surface, links the BB to the distal tip of the striated rootlet of neighbouring cilia. The sub-apical actin network creates a highly organized matrix that is important for global coordination of cilia polarity within a cell. Disruption of either actin (Cytochalasin D) or microtubules (nocodazole) produces disorganized ciliary beating within MCCs. Within ciliated epithelium, actin and microtubules together form highly complex apical structures that directly interact to govern the direction and coordination of ciliary beating along the proximal-distal axis.

![Figure 3](image-url)

**Figure 3. Actin and microtubules organize cilia in multiciliated cells.** (A) A polarized cell depicting the highly organized subapical pool of actin. Striated rootlets stained with GFP-CLAMP (green), actin stained with phalloidin (white) and BBs with centrin-RFP (red). (B) A diagram of the high degree of polarized cytoskeletal organization in ciliated cells and the proposed model for the propagation of a physical signal involved in metachronal synchrony. Adapted with permission from [66].

### 2.2.1.2 Apical-basal polarity

Morphologically, mucociliary epithelium is apical-basally polarized such that the lumenal (apical) face is exposed to air and airway surface liquid, and the basolateral side forms attachments with the underlying matrix [99, 100]. Polarity is crucial for tissue maturation during
the development and repair of the airway epithelium [101, 102]; mature mucociliary epithelium is characterized by tight junction formation, the appearance of cilia (in MCCs), and secretory vesicles (in goblet and club cells).

Figure 4. Interactions of polarity complex components in polarized epithelial cells. The diagram shows the main epithelial polarity factors and the interactions between them. The apical factors are depicted in blue, the junctional domain is shown in pink, and the lateral domain in green. Reprinted with permission from [103].

There are 3 highly conserved polarity complexes that each define a cellular domain: Crumbs, PAR-3, and Scribble (Fig. 4) [99, 100, 104]. The apical cell domain is mediated by the Crumbs proteins, which together with PALS1, PAR-6, PATJ (protein associated with tight junctions) and aPKC (atypical protein kinase C) form a complex [105]. The Crumbs complex functions to form tight junctions through PATJ [99]. Through mutual inhibition with the Scribble complex, PAR-6 excludes lateral proteins in order to maintain the apical domain [100, 104]. In the junctional
domain, tight and adherens junction formation are critical for epithelial function; they create the physical separation of the apical-basal polarity domains within the cells [99, 106]. In this domain, the PAR-3 complex initiates the formation of the cortical actin ring characteristic of adherens junctions and restricts expansion of the apical boundary [99, 107]. Proteins found in this complex include PAR-3, PAR-6 and aPKC. Scribble, lethal giant larvae homolog 1 (Lgl1), Lgl2, Discs large (Dlg) and PAR-1 comprise the Scribble complex, which localizes to the lateral domain [104]. Basally, epithelial cell attachment is mediated by integrin binding to the BM and ECM components. How cell-matrix interactions effect cytoskeletal organization, differentiation and cell function is not well understood [99, 104].

The epithelial barrier function is achieved through a network of tight junctions, gap junctions, and adherens junctions. These junctions function to occlude water and solutes (tight junctions) [108] or anchor the cells for structural cohesion (adherens junctions). The density and makeup of these junctions modulate the ‘leakiness’ of the fluid barrier [47, 109]. Small molecules, ions, and macromolecules excluded by junctions must be transcellularly transported; the transport of ions allows the epithelium to regulate fluid flow. Changes in osmolarity are achieved by chloride and sodium-potassium pumps [110, 111].

Cells secrete the matrix components that form the BM, a thin, fibrous matrix of dense extracellular material that separates the epithelium from the interstitial tissue (connective tissue) and mesenchyme [112, 113]. In mucociliated epithelium, basal epithelial cells form specialized contacts with the basal lamina via α6β4 integrin [1, 114]. These interactions allow the BM to orient the epithelia to establish polarity, and also provide cells with survival, proliferation and differentiation signals [115].
2.2.1.3 Planar polarity

Mucociliated epithelia also experience polarization in the planar direction (perpendicular to the apical-basal axis). Polarity can be manifested at the morphological (e.g. cell elongation) or molecular level (e.g. asymmetric localization of proteins) [69, 116]. Planar polarity is important for physically orienting cilia towards the proximal direction along the oral-lung axis. During polarization, cellular actin and microtubule-based structures reorganize to produce asymmetric distribution of planar cell polarity (PCP) proteins and structural features in different regions of the cell [69]. The PCP signaling pathway functions by aligning neighbouring cells with two groups of core PCP proteins that are localized to the proximal and distal apical cell junctions (Fig. 5) [70]. This mechanism has been demonstrated to orient neighbouring MCCs in tracheal epithelia, which are intracellularly coordinated by a sub-apical network of actin and microtubules [66, 69, 70]. The intracellular network is linked by additional microtubules to the PCP complex in the proximal domain. In order to generate effective mucociliary clearance, an individual cilium needs to be aligned with respect to all the other cilia within the same MCC, with respect to cilia in neighbouring MCCs and with respect to the proximal-distal axis. Coordination of the beating between cilia in multiple MCCs is necessary for producing directional motility that eliminates mucus [70].
The signal(s) that govern tissue-wide directional alignment have yet to be defined. Two PCP complexes coordinate and propagate cell polarities across adjacent cell membranes to create intercellular alignment. In airway epithelium, the proximal complex contains Fzd6 and the distal complex contains Vangl1. Cytoskeletal elements are responsible for linking the basal feet of the cilia to the proximal cortex for intracellular alignment. Reprinted with permission from [70].

Recent work in the developing mouse trachea has helped researchers understand how cells acquire and polarize motile cilia. Intercellular communication via PCP signaling has been shown to precede motile ciliogenesis [69, 70]. The core PCP proteins Frizzled 6 (Fzd6) and Van Gogh-like 1 (Vangl1) localize to the proximal-distal domains (“crescents”) beginning at embryonic day 14.5 (E14.5). These events occur prior to the emergence of motile cilia at E16.5. The PCP crescents persist throughout the life of the organism and are restored following injury to the airway epithelium. Nascent cilia are immature and initially poorly aligned, then gradually refine their alignment to produce robust, directional fluid flow by postnatal day 9 (P9). The signal that establishes the directionality of the PCP crescents remains unknown. It is possible that, like in *Drosophila*, another set PCP proteins may be responsible for determining the position of the crescents [117]. As mentioned above, directional hydrodynamic forces from cilia-derived fluid flow have been shown to contribute to cilia orientation refinement [85, 86].
2.2.2 Alveolar epithelial biology

Alveoli form the basic units of respiration. These thin-walled, hollow cavities are lined by epithelial cells that carry out vital gas exchange [118]. To facilitate this function the alveoli are surrounded by a dense capillary network [36]. The alveolar epithelial cells (AECs) are comprised of two cell types: type 1 cells and type 2 AEC (AT1 and AT2) [119]. AECs are intimately associated with mesenchymal cells such as fibroblasts (including myofibroblasts and lipocytes) and pericytes [120]. These cell populations are thought to play important roles in forming and maintaining vascular networks, sensing injury, recruiting inflammatory cells, and remodelling the ECM. In addition, alveolar macrophages reside within the bronchioles and alveolar ducts. They function as mobile scavengers to engulf and remove foreign particles such as dust, bacteria and damaged red blood cells [121, 122].

![Figure 6. Cell types of the alveolus.](image)

The alveoli are lined by squamous AT1 and cuboidal AT2 cells. AECs are intimately associated with mesenchymal cells such as fibroblasts, endothelial cells, and pericytes. Adapted with permission from [36].

2.2.2.1 Type 1 cells

AT1 cells comprise approximately 40% of AECs and 8% of all lung cells [119]. This cell type plays a primary role in gas exchange and regulation of fluid homoeostasis. These cells
possess a squamous morphology and have a large surface area that covers 93-96% of the alveolar surface [119, 123, 124]. Their extremely thin morphology (<200 nm) facilitates efficient diffusion of gases. Molecularly, AT1 cells are characterized by expression of Hopx, T1α (also known as podoplanin) and aquaporin-5 (AQP5) [11, 35, 36]. Intercellular adhesion molecule 1 (ICAM-1) has also been shown to be expressed at high levels in AT1 cells and minimally expressed in AT2 [125, 126].

The replication of AT1 cells is thought to be low or non-existent. Recently, Hopx-CreER-based lineage labeling experiments have shown that, in rare cases, AT1 cells can replicate and generate AT2 cells in vivo following pneumonectomy (resection of the left lung) [127]. This pneumonectomy-induced compensatory lung growth requires a complex signaling loop that includes fibroblast growth factor receptor (FGFR) activation in AT2 and VEGFR (KDR) signaling activation in endothelial cells [128]. Currently, the signals that regulate plasticity mechanisms have yet to be determined. Future work will require single-cell analysis to assess whether de-differentiation is possible in all AT1 cells or a special sub-population.

2.2.2.2 Type 2 cells

AT2 cells are cuboidal pneumocytes that cover only ~4% of the alveolar surface area but constitute 60% of AECs and 10-15% of all lung cells [119]. This cell type is often found in the corners of the alveoli where neighbouring alveoli meet [129]. AT2 cells produce surfactants to lower the cell surface tension. This functions to reduce the work of breathing and prevent alveolar collapse [35]. The clearance of surfactant contributes to pathogen removal and the maintenance of host defense [130, 131]. Abnormalities in surfactant homeostasis underlie the pathogenesis of inflammatory responses and diffuse alveolar diseases such as idiopathic pulmonary fibrosis (IPF) and pulmonary alveolar proteinosis [130, 131]. AT2 cells are
molecularly characterized by the expression of NKX2.1, LAMP3, and proteins associated with surfactant production and secretion (surfactant protein A (SPA), SPB, SPC, and SPD) [1, 132]. In addition to secreting surfactant, AT2 cells function as the progenitor cell population in the alveolus.

AEC turnover in normal conditions is slow and estimated to be on the order of 2-3 weeks [36]. AT2 cells give rise to daughter cells, some of which remain as AT2 cells and some of which transdifferentiate to become AT1 cells. In studies of alveolar injury models, hyperoxic agents such as bleomycin or NO\textsubscript{2} are used to injure AT1 cells [124, 133]. In a classical NO\textsubscript{2} injury model where rat AT2 cells were labeled with tritiated thymidine and followed for 14 days, Evans et al. showed that the proportion of labeled AT1 cells increased over time, suggesting that AT2 cells give rise to AT1 cells [124]. These observations were confirmed with rigorous lineage-tracing studies that demonstrated AT2 cells maintain the homeostatic turnover of AT1 cells and also contribute to the AT1 population following bleomycin-induced lung injury [36, 132, 133]. Moreover, these experiments also qualified AT2 cells as progenitors in the adult lung alveoli after it was shown that AT2 cells can clonally generate both AT1 and AT2 cells [133].

2.2.3 Extracellular matrix components and structural organization

Chapters 3-5 of this thesis will be focused on designing biomaterials with specific biophysical properties to achieve more physiologically-relevant \textit{in vitro} models of adult respiratory epithelium. Specifically, our \textit{in vitro} tissue engineering approach consists of tailoring scaffolds to mimic the native extracellular matrix (ECM) with micropatterned stiffness, topography, or architecture. This section of the literature review will describe in detail the structural components and organization of the proximal and distal airway ECM.
2.2.3.1 **Tracheal ECM**

The tracheal basement membrane (BM) has functions in cell adhesion, sequestering growth factors and ions, as well as acting as a mechanical barrier between the external environment and the deep tissues [27, 134-137]. It serves to mediate cell-cell interactions, particularly in the exchange of information between epithelium and fibroblasts during growth, inflammation, and in response to injury [138]. Specifically, the epithelium attaches to the lamina densa which together with the lamina reticularis forms the BM [112, 138]. The lamina densa is a dense layer of matrix comprised of type IV collagen, laminins, entactin and heparin sulfate proteoglycans [136]. The lamina densa is in turn connected to the type I and type III collagen fibres of the lamina reticularis through type VII collagen anchoring fibrils, which loop through collagen strands in the lamina reticularis and then reattach to the lamina densa [136, 139, 140]. The lamina reticularis is considered to be a specialized extension of the ECM consisting of collagen fibrils. It functionally represents the ECM that lies between the epithelium and a layer of attenuated fibroblasts lining the airways. Non-structural proteins in this layer include tenascin, fibronectin and proteoglycans [27, 136]. Beneath the BM is the ECM. This region is composed of a dense network of collagens, elastins, laminins, polysaccharides and GAGs which mechanically support the vascular and pulmonary trees [27]. ECM components are generally conserved among species [141].

Fluorescent imaging and scanning electron microscopy (SEM) of whole-mount rat tracheas reveal that structural proteins have a complex and highly organized structure immediately beneath the lamina densa. The BM is arranged as a 1-2 µm thick mat of entwined and parallel fibres oriented along the longitudinal axis of the airway (Fig. 7). These large fibres are composed of collagen III with some collagen I and V [136]. Smaller fibres are oriented at approximately right angles to the large fibres and crosslinked with them to complete the
framework [142, 143]. Immunohistochemical studies indicate that the crosslinking fibres are covered with bands of elastin and elastin-associated microfibrils (fibrillins, microfibril-associated glycoproteins, emilin, fibulin-2) [138]. The fibrous framework is covered by amorphous proteins, which may contain proteoglycans (e.g. hyaluronan, chondroitin sulfate, and heparin sulfate) and other non-structural proteins reported to be in the lamina reticularis [27, 136]. Findings from these studies were used to inform our bioengineering strategy using substrate stiffness and topography (discussed in Chapters 3 and 4).

Figure 7. Basement membrane of rat tracheal whole mount. (A) Scanning electron micrograph of the longitudinally oriented lamina reticularis (LR). In some areas basal cells (BC) and columnar cells (CC) remain attached. Bar: 42 µm. (B) In areas where the lamina densa is missing, the smaller crosslinking fibres are clearly visible (dark arrows). Bar: 10 µm. (C) Higher magnification micrograph reveals a network of fine fibrils surrounding the large longitudinally oriented fibres (arrows). Bar: 3 µm. Reprinted with permission from [138].
2.2.3.2 Alveolar ECM

The alveolar basement membrane is fused from the basal lamina of the lung alveoli and the basal lamina of the lung capillaries [144]. This forms an extremely thin (2 µm-400 nm) semipermeable membrane known as the blood-air barrier (also known as the alveolar-capillary barrier) [145]. This barrier prevents air bubbles from forming in the blood, and prevents blood from entering the alveoli. This interface has functions in gas exchange, fluid homeostasis, cell-cell crosstalk, and defense [144]. In addition to facilitating diffusion of oxygen and CO₂, the blood-air barrier is also permeable to CO and many other gases.

![Figure 8. Architecture of mouse lung alveoli.](image)

Figure 8. Architecture of mouse lung alveoli.

(A) Scanning electron micrograph of perfusion-fixed mouse lung showing transitional bronchiole (trb) that opens into the acinus (arrow). Double-headed arrows mark inner diameter of alveolar ducts (2r). Solid white line marks the outer diameter of the alveolar sleeve (2R). Adapted with permission from [23].

The microstructure of the distal alveolar region is distinct from that of the large airways (Fig. 8). In vivo, the lung alveolus is a saccular structure with three-dimensional architecture and an approximate diameter of 200 µm (in humans) or 50 µm (in mice). These structural cues were used to guide our bioengineered alveolar models in Chapter 5. The alveolar BM is comprised of specific ECM proteins such as laminin, type IV collagen and proteoglycans. Together these
molecules form an interconnected mesh-like network that allows for selective transfer of cells and signaling molecules [146]. Co-culture studies using SV40-T2 cells, a rat model epithelial cell line, have shown that the assembly of a continuous basement membrane by alveolar epithelium requires signaling of soluble factors from pulmonary fibroblasts [147]. Immunohistochemical analyses revealed the components of the alveolar BM include various laminins (−α1, −α3, −α5, −β1, −γ1), collagen type IV, entactin, and perlecan [148, 149]. Further work with both SV40-T2 and murine MLE-15 cells demonstrated that synthesis of these constituents is significantly enhanced by TGF-β1, which is presumably one of the soluble factors secreted by pulmonary fibroblasts [148, 150]. Consistent with these findings, AT2 epithelial cells express α1, α2, α3, α6 and β1 integrin subunits, which are necessary to mediate normal epithelial-matrix interactions via binding of collagen and laminin [151-153].

2.3 **Current challenges for primary epithelial culture**

The region-specific architecture that exists in the lung and airway is linked to cell maintenance and function (Fig. 9); however these cues have not been incorporated into engineered epithelial constructs. The materials used in these constructs can be designed with specific biomechanical properties to promote desirable phenotypes. Therefore there is a crucial need to explore the architectural parameters that govern tissue organization and homeostasis.

Contributing to the limitations in airway and distal lung epithelial models is the need to establish efficient methods for expanding cell populations while maintaining their capacity for differentiation. In practice, the current sources of epithelial cells include i) cell lines, ii) primary cell isolated from donor tissue, and iii) stem cells (either induced or derived from elsewhere). Cell lines are often immortalized or derived from lung tumours and do not recapitulate healthy
**Figure 9. Anatomy and epithelial histology of the adult human lung.** The trachea and large bronchi (>1 mm in diameter) are lined by a pseudostratified epithelium with basal, multiciliated, and secretory cells. Mucous goblet cells predominate in the larger airways, and club cells predominate in the smaller airways. The alveoli are lined by squamous AT1 cells and cuboidal AT2 cells. Adapted with permission from [1].

Respiratory biology [16, 154-156]. Specifically, model cell lines for large airways do not form multiciliated epithelium (BEAS-2B). For distal lung models, tumour-derived cell lines (MLE-12, A549) continually proliferate and constitutively express surfactant protein C (SPC), unlike the in vivo situation where AT2 progenitors are normally quiescent but are activated to replicate and transdifferentiate to replenish AT1 cells after injury. Primary cells isolated from animal or human tissue provides more physiologically-relevant cell phenotypes, but can be technically challenging to obtain and are limited by low cell numbers [10, 157, 158]. Moreover, primary cell phenotypes often change in culture, especially cells derived from stem/progenitor populations [11]. For cells generated from human embryonic stem cells (ESCs), ethical concerns of access
and the burden of proof for demonstrating a lack of malignant transformation is high which undermines their utility as a consistent cell source [159]. Epithelium can also be differentiated from autologous fibroblasts (i.e. induced pluripotent stem cells (iPSCs)). iPSCs are a promising source of epithelium as they promise large expansion capability and fibroblasts can be harvested through non-invasive means. However, iPSC-derived epithelial cells may not fully function as their reprogrammed cell type and may display ‘memory’ of former cell types [160, 161]. Furthermore, this source risks of tumorigenic mutations induced by reprogramming. All of the aforementioned sources will require optimization to generate large numbers of fully functional cells for TE applications.

2.4 Current in vitro culture models

In this section of the literature review we provide an overview of the strategies and tools currently available to engineer lung and airway epithelium in vitro. Cellular function and tissue formation in vivo are regulated by diverse biological factors including cell-matrix interactions, cell-cell communication, and soluble factors. Tools to modulate these factors in vitro are enhancing our understanding of concepts such as tissue hierarchy, complexity, dynamics, adaptation and healing [162].

2.4.1 Platforms and approaches relevant to in vitro airway models

2.4.1.1 Culture platforms

2.4.1.1.1 Transwells

Investigators commonly use porous transwell membrane cultures to generate adult airway epithelium in vitro. This ALI culture system allows for apical-basal polarization of primary basal cells over an approximately four-week maturation period (Fig. 10) [163]. Although ALI culture allows for differentiation of tracheal basal cells into mature ciliated and secretory cell types, it lacks the instructive cues required to planar polarize ciliated epithelium [95, 164, 165]. Other
major limitations for these cells are the long culture times necessary for terminal differentiation (at least 21 days for human cells) and their differentiation potential decreases with passaging (beyond 2 passages). Cell lines such as BEAS-2B are a limited model for tracheal epithelium as they do not differentiate into columnar ciliated cells that populate the airway.

It should be noted that the timescales for the differentiation of primary airway cells are different for mouse and human. In mouse ALI cultures, ciliogenesis is complete with mature MCCs at ALI D14 [82, 163]. Human tracheal cells generally have a longer timescale (~31-45 days) before completion of ciliogenesis and complete maturation of MCCs [166].

Figure 10. Human tracheal progenitors possess proliferative and differentiation potential. Primary p63+ basal progenitor cells are isolated from tracheal tissue by DNAse I and Pronase digestion and expanded under liquid-liquid interface until confluence. Media is removed from the upper compartment of confluent sheets and cells are fed only basolaterally to establish ALI. In human cells, terminal differentiation is achieved after 31 days.

2.4.1.1.2 Hydrogels

Hydrogels are a class of biomaterials, often with tunable properties, typically used to mimic the ECM found in soft tissues [167-169]. A variety of polymers exist with cytocompatible chemical crosslinking strategies that permit cells to be cultured in 2D or 3D. Gels have high water content and are porous, making them suitable substrates for transwell culture (to allow nutrient diffusion from the basolateral compartment in ALI). The bulk mechanical properties of a hydrogel can be tuned by the monomer content (weight percent) and crosslink density. Furthermore, hydrogels are compatible with soft lithography and can be imprinted with topographical features [16]. Hydrogel products can be classified on different basis (e.g.
polymeric composition, physical configuration, type of crosslinking). We have classified them below based on their natural or synthetic origin [170, 171] (Table 1):

2.4.1.1.2.1 Naturally-derived hydrogels

Naturally-derived matrices contain native ligands for cell adhesion and signaling that more closely replicate the in vivo ECM than synthetic polymers [167]. However few techniques are available to modify the structural and mechanical properties of naturally-derived hydrogels after the matrix has gelled [172-174]. Recently methods have been developed to functionalize natural biopolymer side-chain chain hydroxyl, carboxyl, and amine groups with acrylate or methacrylate groups to render them crosslinkable [175-179]. The mechanical properties of natural hydrogels can be tuned by the degree of functionalization, the molecular weight of the gel monomer, and its concentration during gel formation.

2.4.1.1.2.1.1 Gelatin

Gelatin is a widely used material with wide applications ranging from medicine and pharmaceuticals to the food industry [180]. The popularity of gelatin stems from its numerous favourable characteristics such as biocompatibility, biodegradability, low cost and ease of manipulation [181]. It is a proteinaceous material comprised of denatured and hydrolyzed collagen (mainly type I) [182, 183]. Gelatin retains the bioactive sequences of collagen such as RGD for cell adhesion and matrix metalloproteinase (MMP)-sensitive sequences for cell-mediated enzymatic degradation [184]. As such gelatin has been used to study cellular functions including migration, proliferation and differentiation [177, 185]. Gelatin is liquid at 37°C and needs to be chemically crosslinked (often with glutaraldehyde) in order to be used as a biomaterial [186].
2.4.1.1.2.1.2 Collagen

Collagen-based hydrogels are another widespread substrate for cell culture due to its abundance in the natural ECM [187, 188]. Generally most collagen hydrogels are prepared from type I collagen (which comprises 90% of protein in human connective tissues) by polymerization at 37°C [189-192]. Comparisons between studies using customized collagen concentrations is difficult due to significant variation in hydrogel fabrication and characterization protocols between different research groups. In addition to the commonly reported parameters (e.g. collagen concentration), the material properties of collagen are highly dependent on numerous other factors such as collagen source, polymerization temperature, gelation pH and ionic strength [190, 193-196].

2.4.1.1.2.1.3 Collagen vitrigel membrane (CVM)

Collagen hydrogels can be limited by opacity and weak mechanical properties due to loose packing of collagen fibrils [197]. Collagen gel transparency and increased fibril density can be achieved by a processing method known as vitrification [198, 199]. Vitrification allows water in the hydrogel to evaporate in a controlled manner that results in collagen fibrils reorganizing and forming crosslinks between each other. This produces a collagen vitrigel membrane (CVM) that is highly transparent, mechanically strong (tensile strength 797 to 8697 kPa) [200] and elastic. CVM has been shown to support culture of the conjunctival epithelium of the eye and limbal stem cells [201].

2.4.1.1.2.1.4 Silk fibroin

Silk refers to protein fibres produced by the domesticated Bombyx mori silkworm [202]. Silk fibroin is a natural fibrous protein with favourable material properties, biocompatibility and easy processing into several forms, making it a suitable material for biomedical applications [203]. Silk fibroin is advantageous in that it has high mechanical strength (in the mPa range) [204] and
maintains high stability in response to heat and humidity. Furthermore, silk fibroin has low immunogenicity and is permeable to oxygen and other molecules [205]. Adhesion and proliferation studies have demonstrated that silk fibroin films support the growth of both human and animal cell lines [206-208]. The disadvantage of silk hydrogels are their slow gelation rate [209]. For example, 2% silk fibroin solution at 37°C forms a gel after ~30 days. This process can be sped up by increasing incubation temperature to 60°C [210] or decreasing the pH toward the isoelectric point of silk (3.8).

2.4.1.1.2.1.5 Matrigel

Matrigel is a mixture of ECM proteins extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells [211-213]. This matrix is heterogeneous and its main components are laminin, entactin, collagen and heparin sulfate proteoglycans [214]. Growth factors such as transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) are also present which function to promote the proliferation of many cell types. Matrigel is noted for its ability to retain stem cells in an undifferentiated state but how it accomplishes this is poorly understood [214, 215]. Other proteins are present in small amounts and the exact composition can vary from lot to lot making Matrigel not well chemically defined.

2.4.1.1.2.2 Synthetic hydrogels

Hydrogels have been synthesized from a range of synthetic polymers such as poly(ethylene glycol) (PEG), poly(vinyl alcohol) and poly(propylene fumarates) [216]. PEG-based macromers terminated with either acrylate or methacrylate end groups were used to develop some of the first bio-inspired synthetic hydrogels that remains widely used today [145, 217]. These hydrogels were formed using conventional chain-growth polymerization mechanisms. PEG hydrogels are advantageous because their non-fouling, biologically inert chemical backbone allows researchers
to take a bottom up approach to engineer specific moieties of interest [218]. This results in low levels of protein and cellular adsorption, allowing for the isolation and study of key functionalities of ECM [219, 220].

2.4.1.1.2.2.1 PEG-NB

One of the most promising alternatives to chain-growth polymerization are photoinitiated thiol-norbornene (thiol-ene) addition reactions [220, 221], which were first utilized in poly(ethylene glycol)-norbornene (PEG-NB) hydrogels for the study of human mesenchymal stem cells (hMSCs) in 3D culture. The PEG-NB gel network forms by UV photo-crosslinking between thiol and norbornene moieties; the mechanical properties of the matrix can be tuned over a wide range by varying the PEG concentration, molecular weight, and the degree of crosslinking (crosslinker concentration and UV dose). This falls under the class of ‘click’ chemistry: reactions that are fast, efficient, specific and proceed under mild conditions to produce non-toxic byproducts [222, 223]. One important advantage of click crosslinking chemistry is the specific and facile incorporation of bio-inspired peptides to mimic native ECM [224]. As a consequence, PEG-NB hydrogels are highly biocompatible and modular; investigators are afforded precise control over the localization of biological and biophysical properties of these materials. Many studies have investigated cellular responses to isotropic stiffness variations but relatively few studies have produced stiffness changes at the level of single cells or small groups of cells. Ongoing work in the hydrogel field is contributing to a growing library of functional monomers that allow for stiffness patterning at the micron level (see Stiffness below).
<table>
<thead>
<tr>
<th>Naturally-derived hydrogels</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>Biocompatible, ease of use</td>
<td>Requires chemical crosslinking</td>
<td>[181-183]</td>
</tr>
<tr>
<td>Collagen</td>
<td>Biocompatible, found in high concentrations in natural ECM</td>
<td>Opaque, weak mechanical properties</td>
<td>[187, 189-191]</td>
</tr>
<tr>
<td>Collagen vitrigel membrane</td>
<td>Transparent, strong mechanical properties</td>
<td>Thin (difficult to handle)</td>
<td>[198-200]</td>
</tr>
<tr>
<td>Silk fibroin</td>
<td>High mechanical strength, low immunogenicity</td>
<td>Slow gelation rate</td>
<td>[203-206]</td>
</tr>
<tr>
<td>Matrigel</td>
<td>Supports clonal expansion of stem/progenitor cells</td>
<td>Lot to lot variation, not chemically defined</td>
<td>[211-215]</td>
</tr>
<tr>
<td>Synthetic hydrogels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-norbornene</td>
<td>Photocrosslinkable, incorporation of specific peptide moieties</td>
<td>Requires optimization to support culture of specific cell types</td>
<td>[220-224]</td>
</tr>
</tbody>
</table>

Table 1. Hydrogel overview.

2.4.1.2 Tissue engineering approaches

2.4.1.2.1 Micropatterning

There have been a variety of approaches to micropattern cells on stiff substrates (e.g. tissue culture polystyrene (TCPS), glass coverslips, polydimethylsiloxane (PDMS)) [225, 226] and hydrogels [227, 228]. These approaches include fabrication strategies such as photolithography, microcontact printing (µCP), micromolding (soft lithography), printing and dip-pen spotting [229-232]. Most of these approaches function by localizing cells to patterned regions on a substrate and allow for patterning of two or more cell types in spatially defined co-cultures [233]. Surfaces can be patterned with cell-adhesive (e.g. ECM proteins) or cell-repulsive stimuli (e.g. BSA, hydrophilic polymers) [226]. These approaches have been used to study the effects of cell-matrix interactions and cell-cell contact on various cell behaviours [19, 233]. Studies have also utilized the spatial control of adhesive patterning to study the effect of colony size and distance between colonies on activation of cell signaling and paracrine signaling, respectively [225, 228, 234, 235]. Micropatterning approaches provide a facile approach for controlled cell microenvironments but are limited to spatially patterning cell matrix proteins in a 2D surface and...
do not recapitulate the microenvironmental architecture or mechanical cues that cells experience in vivo.

2.4.1.2.2 Engineered stiffness

Cells respond to external physical cues such as matrix stiffness; in seminal work Pelham and Wang modulated bulk stiffness by culturing cells on polyacrylamide hydrogels to direct cell spreading [236]. Approaches have been extended to other naturally-derived [175, 177] and synthetic [220, 224] hydrogel substrates with varying functionalities. Stiffness sensing through cell-matrix interactions are regulated largely by mechanotransduction mechanisms such as RhoA/ROCK signaling and Rac activation [19, 237]. Signaling through these pathways regulate myosin phosphorylation, actin network assembly and cell contraction. Substrate stiffness can greatly influence cell behaviours such as adhesion, migration, proliferation and differentiation in many cell types including stem cells, cardiomyocytes, vascular smooth muscle cells, fibroblasts, human umbilical artery endothelial cells, neurons, and cervical epithelial cells [238-244]. Relatively little work has explored the role of substrate stiffness in airway and lung epithelium but substrate stiffness likely plays an influential role in epithelial cell behaviour.

The presentation of matrix stiffness also guides cell behaviour. Many tissues are heterogeneous and regional differences in stiffness exist. Airway epithelium is supported by a basement membrane comprised of aligned collagen fibres [138, 245] that likely has greater stiffness in the longitudinal direction than the transverse direction [135, 246]. It follows that cells experience local variations in stiffness, whereby the rigidity of the material (tissue) differs in different directions (anisotropic stiffness). This has been demonstrated in 3T3 fibroblasts [247] and hMSCs [248], where cells grown on micromolded elastomeric micropillar substrates exhibit more elongated and branched shapes compared with cells on flat substrates. These micropost
array detector systems (mPADs) impose physical constraints to spatially reorganize the cellular cytoskeleton and have been shown to impact cell morphology, focal adhesions, cytoskeletal contractility and even differentiation. Oval-shaped micropillars can be produced so that the stiffness of the substrate is greater along the long axis of the posts [248, 249], and this anisotropy was found to induce directional growth and migration of Madin-Darby canine kidney (MDCK) epithelial cells. The advantage of mPADs are their ability to spatially prescribe micron-scale topographic signals for analysis of cell behaviour, independent of adhesion and other material surface properties. However, mPADs have not been used with primary airway epithelial cells as the soft lithography for creating microposts is not compatible with imprinting the hydrogels necessary for ALI culture.

Efforts to spatially pattern local stiffness at the scale of the cells in hydrogels mainly involve UV light-mediated crosslinking of photocrosslinkable polymers. Light affords spatial control of substrate mechanics without introducing changes in topography or surface chemistry. Photomasks have been used to pattern stiffness (2.5 kPa to 25 kPa) in methacrylated and norbornene-modified hyaluronic acid hydrogels down to 50 µm resolution [250, 251]. Atomic force microscopy (AFM) indentation experiments revealed that stiffness changes were achieved within the same gel surface and that stiffness patterning with 25 µm photomasks was not observed. Stiffness patterning at this scale is relevant to patterning groups of cells but not for introducing multiple stiffness cues to single cells.

Another method for introducing oriented stiffness in porous substrates is stretching of collagen. Uniaxial stretch has been applied on collagen hydrogels to align collagen fibres, which was shown to direct the extensions of neurons cultured in 3D [252]. Similarly, hMSCs cultured on stretched electrocompacted collagen sheets exhibited cell alignment, upregulation of markers
and matrix deposition associated with an anisotropic tissue type (tendon) [253]. This stretching approach could be adapted to create hydrogel platforms with anisotropic stiffness cues for ALI culture of airway epithelial cells. One challenge that would need to be addressed is the decoupling of anisotropic mechanical properties from mechanical stretch or topographical cues so that their contributions to cell responses can be isolated individually.

2.4.1.2.3 Engineered topography

In the airways, the general ECM structure is composed of collagen fibres that run along the longitudinal axis of the windpipe. These fibres have inherent topography that provides biophysical cues to the overlying epithelium. Cell spreading along the direction of the topography is guided by the contact guidance response [254-256]. Cortical tension is transmitted through the cytoskeleton which is translated to regulation of cell morphology [257]. To probe this phenomenon, researchers have developed substrates with topographical features such as nano- and microgrooves, microposts or islands [135, 246, 258, 259]. These features can be microstamped into elastomeric and porous materials with varying properties, allowing for studies of epithelial cell interactions with complex biomaterials. Another method for recapitulating the native ECM includes electrospinning fibres to closely mimic the fibrous matrix nanostructure [260-262]. This technique is advantageous in that it allows for the tunability of fibre diameter and orientation to the desired dimensions. In order to enhance the utility of electrospun fibres several practical limitations will need to be addressed including poor cell adhesion, potential toxicity of the electrospinning process or chemical residues, and slow production rates [263]. Defined topographical structures can exert effects on cell behaviours such as adhesion, elongation, alignment, migration and differentiation [264-271].
2.4.2 Platforms and approaches relevant to in vitro alveolar models

Much of the current work in alveolar epithelial cells use immortalized cell lines such as SV40-T2 (rat), MLE-12 (mouse), and A549 (human) [148, 150, 154]. In contrast to primary cells, these tumour-derived cell lines expand rapidly in culture and constitutively express AT2 markers such as SPC. One of the major limitations regarding primary lung AECs are that they cannot be expanded efficiently in culture as there is a significant cell death after isolation and the surviving cells are non-proliferative [10, 158, 272]. Moreover, the cells that survive in culture are not representative of the in vivo situation as cells lose expression of AT2 markers and there is increased expression of AT1 markers [11]. This is thought to be due to transdifferentiation of AT2 cells towards an AT1-like phenotype. Much of the lung tissue engineering literature has focused on identifying the biological factors that are important and characterizing their contribution to AEC utility. Many of these studies look at the effect of various matrix coatings (e.g. Matrigel, collagen) and culture media supplements (e.g. keratinocyte growth factor (KGF), rat serum) on cells cultured on 2D surfaces. There is relatively little work that rigorously analyzes the role of matrix architecture in maintaining AEC utility. The alveolus is a saccular structure with three-dimensional shape and this architecture likely regulates cell signaling and phenotype. Studies have cultured cells in biological scaffolds (derived from decellularized tissues), however these systems are not optimally suited for isolating the contribution of microenvironmental cues from biological effects [17, 18].

2.4.2.1 Engineered geometry and architecture

How physical forces and tissue architecture orchestrates mechanical and paracrine/autocrine factors remains poorly defined but are beginning to emerge as important parameters for regulating cell behaviour [273, 274]. The geometric organization of cells varies the mechanical stress in different regions of the tissue [275]. Forces from intercellular adhesion and cortical
tension integrate into a tissue surface tension signal [276] to regulate the local behaviour of groups of cells [277]. Tissue geometry also modulates other microenvironmental parameters including cell-matrix interactions, cell-cell interactions and soluble factors; together these local parameters govern cell behaviour.

Manipulating tissue geometry with substrate architecture has been demonstrated to modulate proliferation, cell survival, and differentiation in many cell types including epithelial and stem cells [20, 278, 279]. Bovine endothelial cells and rat kidney epithelial cells seeded onto adhesive islands of differential geometries display differential BrdU incorporation at the edges where mechanical stress was highest [20]. Cells have been shown to switch to a apoptotic state when cell spreading is restricted by culture on adhesive islands of different sizes, indicating that local geometric control provided by the tissue microenvironment may represent a fundamental mechanism for the regulation of cell viability [280]. MSCs preferentially adopt an adipogenic or osteogenic lineage when their culture is restricted on different star-shaped geometries [21]. Furthermore, geometric micropatterning experiments to pattern cancer cells in 2D and 3D hydrogels indicated high levels of cancer stem cell signaling and tumorigenicity correlated most often at the perimeter of the shapes, with regions of high mechanical stress and enhanced α5β1, MAPK (mitogen-activated protein kinase) and STAT (signal transducers and activators of transcription) activity [281]. The cellular response to geometry is likely a universal phenomenon as it has been shown to guide cell behaviours in many cell types including A549 lung cancer cell lines [281]. The hollow acinar architecture of the alveolus could be an important physical cue for the preservation of cell phenotype [22-25]. Micropatterning experiments allow experimenters to test the impact of concave and convex shapes in 2D but facile fabrication methods to produce curvatures or contours in 3D do not yet exist.
2.5 Summative Statement

Lung epithelium is comprised of many diverse cell types that inhabit structurally distinct niches in the respiratory tree. Epithelium dysfunction is implicated in many lung diseases therefore organized, functional epithelial constructs remains an important unmet milestone. One major limitation of epithelial culture models is that they are conducted on stiff 2D substrates that do not contain the topographical or architectural cues that cells sense in vivo. Biophysical cues from the surrounding matrix have profound effects on many epithelial behaviours including cell organization and phenotype. The rational design of materials could produce engineered scaffolds to direct primary cells towards desired phenotypes to improve their utility as in vitro culture models or engineered epithelial replacements.
Chapter 3

Evaluation of UV-tunable Biomaterials as a Simple Tool for Cell Culture
3.1 Abstract

In Chapter 3, we used a simple photomasking approach to produce patterned adhesive properties in a hydrogel scaffold. Initially our goal was to customize differential stiffness in a culture platform for the planar polarization of airway epithelial cells. Many tissues are heterogeneous and present local differences in stiffness at the microscale. Anisotropic stiffness cues are known to govern behaviours such as cell morphology and migration \textit{in vitro}, but this has not been tested in a system that presents these cues under air-liquid interface conditions. We attempted to adapt photocrosslinkable poly(ethylene glycol)-norbornene (PEG-NB) hydrogels for transwell culture to spatially control the presentation of stiffness signals. Although we were able to tune the mechanical properties of the gel at the macroscale level, micron scale differences in stiffness were not observed with our photopolymerization strategy. We did however observe an alignment response with patterned gels and determined that the photopatterning approach produced spatially-patterned adhesive regions. Our hydrogel platform supported a limited number of mesenchymal cell types (human mesenchymal stem cells and valvular interstitial cells) but did not support the adhesion of primary airway epithelial cells. Adherent cell types displayed differing morphological and proliferation responses on our PEG-NB hydrogels, suggesting this platform could be a useful tool for manipulating adhesion to engineer desired mesenchymal tissues. A major advantage of this simple photomasking approach is the ability to pattern adhesion peptides with single-cell resolution, without the need for complex techniques such as multi-photon confocal laser patterning. Additional optimization for other cell types will be necessary for patterning other tissue types.
3.2 **Introduction**

Airway epithelium contains multiciliated cells (MCCs) that each contain hundreds of motile cilia [59]. The coordinated beating between multiple cilia eliminates mucus and particles [90, 91], which is critical for the prevention of infection and mucous compaction [9, 70]. Cilia formation and coordination requires appropriate polarization of the epithelial lining. Polarization in the apical-basal direction is necessary for airway tissue maturation and ciliogenesis [163]. Airway epithelial cells are also polarized in the planar direction. Planar polarization produces cues that induce cytoskeletal reorganization along the proximal-distal (oral-lung) axis essential for coordinating a sub-apical network of actin and microtubules within MCCs [66, 69]. This mechanism coordinates cilia alignment within individual cells and ultimately multiple MCCs across the epithelia. Current *in vitro* culture systems for human airway epithelial progenitors produce disorganized multiciliated epithelium. Primary airway epithelial cells are commonly grown on permeable transwell membrane inserts under air-liquid interface (ALI). This system promotes apical-basal polarization but lacks the instructive cues to generate planar polarized epithelium [95, 164, 165]. This limitation results in cultures with disorganized cilia beating and mucus clearance.

One approach to control cell organization is to modulate the mechanical properties of its microenvironment. The ability to manipulate cell spreading and proliferation has been demonstrated on isotropic substrates with varying stiffness. Mechanical stimuli and tension from the external environment is transmitted from the extracellular matrix (ECM) through transmembrane integrin receptors to the intracellular cytoskeleton of the cell. This mechanotransduction mechanism is mediated in part by signaling through the Rho/ROCK pathway, which in turn has downstream effects on myosin phosphorylation, actin polymerization, and actomyosin contraction [19, 237]. However, to better reflect the
microenvironments that cells experience \textit{in vivo} it is necessary to design more sophisticated matrices with spatial control of these behaviours. Many tissues are heterogeneous in nature and display complex presentation of mechanical properties. Airway epithelium rests on a basement membrane of aligned collagen fibres and significant local variation in mechanical stiffness likely exists at the scale of the individual cells [138, 245]. This elastic property is termed anisotropic stiffness, where the mechanical properties of the material differ in different directions (e.g. in trachea, the direction of greatest stiffness is along the length of aligned collagen fibres).

Tissue engineering approaches to produce anisotropic stiffness include the micropost array detector system (mPADs), where polydimethylsiloxane (PDMS) oval micropillars are fabricated by soft lithography so that stiffness of this substrate is greater along the long axis of the posts [248, 249]. Using the mPADs, Saez et al. showed that anisotropic rigidity can induce directional growth and migration in Madin-Darby canine kidney (MDCK) epithelial cells. Anisotropic stiffness has been shown to orient actin stress fibres and focal adhesions along the direction of greatest stiffness in several cell types including epithelial cell sheets, fibroblasts, and human mesenchymal stem cells (hMSCs) [247-249, 282]. This alignment response correlates with high tractional stresses concentrated at the long axis edges of the cell. Surfaces with stiffness anisotropy thus present a strategy to induce planar alignment in airway epithelial cells. In the case of epithelial cells, such an engineered scaffold with defined mechanical properties must be permeable (i.e. a hydrogel) in order to facilitate the basal diffusion of nutrients in ALI culture of airway epithelium (apical-basal polarity). Hydrogels however are not compatible with the soft lithography required to generate mPADs (microposts are too small to be imprinted into hydrogels). Therefore, we set out to design another approach to produce stiffness anisotropy on hydrogel surfaces for the alignment of ciliated epithelium in ALI culture.
Few examples exist where hydrogel properties are controlled spatially. Most systems that afford spatial control of mechanics are dependent on the use of light due to the control that it affords. Photopolymerization with UV light is a commonly employed technique that involves radical polymerization using thiol-ene functionalized polymers [219, 220]. By restricting light to specific regions, complex patterns can be imparted onto hydrogels to spatially control cell behaviour [283-285]. We selected PEG-NB as our cell culture substrate because this polymer has more biochemical and mechanical tunability than gelatin or collagen gels [219, 220].

In this study, we set out to develop a culture platform that aligns cells, but also supports the culture and differentiation of primary human tracheal epithelial cells (HTECs). We were unable to spatially pattern the surface mechanics of PEG-NB by regionally restricting light exposure. However, our patterning strategy produced corresponding patterns of cell adhesion. We were able to utilize this system for the alignment of hMSCs and valvular interstitial cells (VICs). Adhesion and support of primary HTECs however was not supported by this hydrogel. Although this represents only a preliminary step towards developing an effective tissue engineering strategy, this system allows for the spatial control of matrix adhesion for the purposes of understanding how differences in peptide concentration modulate the behaviour of hMSCs and VICs.

3.3 Methods

3.3.1 PEG-NB polymer synthesis

PEG-NB was not currently commercially available and synthesized in-house. PEG-octa-norbornene (PEG8NB) was synthesized by reacting PEG-OH (20 kDa) (JenKem, Allex, TX, USA) with 5-norbornene-2-carboxylic acid (Sigma-Aldrich, St. Louis, MO, USA) as described previously [219, 220, 286]. In a round-bottom flask, norbornene acid was converted to
norbornene anhydride in dichloromethane (DCM) using \(N,N'\)-Dicyclohexylcarbodiimide (DCC) as a catalyst. Norbornene anhydride was reacted with 8-arm PEG-OH, in the presence of (dimethylamino)pyridine (DMAP) and pyridine, to yield PEG8NB. All reactions were performed under argon. After overnight reaction, the product was precipitated in diethyl ether. The product was characterized with proton NMR (Agilent DD2 500 MHz NMR Spectrometer, Department of Chemistry, University of Toronto) and the degree of functionalization was 99%.

3.3.2 Hydrogel fabrication

PEG-NB hydrogels were prepared by step-growth photo-polymerization using 8-arm PEG-NB (20 kDa) and PEG-dithiol (PEG-DT; 3.4 kDa) cross-linker. Thiol-ene photo-polymerization was initiated using 0.05% Irgacure 2959 (Sigma-Aldrich, St. Louis, MO, USA) photoinitiator (dissolved in phosphate buffered saline (PBS)). Pre-polymer solution containing PEG8NB, PEG-DT, CRGDS (or CRGDSK) (cell adhesion peptide) (Genscript, Piscataway, NJ, USA), and photoinitiator was exposed to collimated UV light (365 nm, 10.5 mW/cm\(^2\)) using an OmniCure® S2000 (Lumen Dynamics, Mississauga, ON, CAN) for varying times. This step could be performed to uniformly expose entire gels or create gels with spatially controlled mechanics by restricting UV light with a photomask. Patterned gels were formed by an initial curing step with short uniform UV exposure (20 s), followed by a second step where UV was restricted over the surface of the gel to create different exposure times and crosslinking across the gel surface. For patterning experiments, photomasks with line arrays of varying widths and spacing (13-50 μm) were designed with AutoCAD software and commercially printed on acetate paper (Pacific Arts & Designs, Inc., Markham, ON, CAN). CDDGEAG peptide was a gift from Jenna Usprech (Institute of Biomaterials and Biomedical Engineering, University of Toronto). For all PEG-NB hydrogels, a stoichiometric ratio between norbornene and thiol groups was maintained. Gels were sterilized with 70% ethanol for 30 min, hydrated with PBS and
maintained under sterile conditions afterward. After polymerization, gels were incubated at 37°C in PBS and washed three times daily for two days to remove any unreacted thiolated molecules.

Hydrogel discs produced for mechanical testing were fabricated using 1 mL syringe as molds (BD Canada, Mississauga, ON, CAN) with the top removed. Gel inserts for transwell cell culture were cast within circular PDMS molds between two glass slides. Various mold diameters were fabricated using Biopunch® tools (Ted Pella, Inc., Redding, CA, USA). After fabrication, gel inserts were sterilized by overnight incubation with 70% ethanol.

3.3.3 Mechanical testing of substrates

Hydrogel discs (approximately 5 mm diameter, 2 mm thick) were loaded in compression using a low force fatigue test machine (Test Resources Model: 840LE2). A 25 g load cell was used for the compression tests. Prior to a test, the loading plate was lowered to a point just touching the sample. Samples were tested at a rate of 20% strain/min, in accordance with previous studies [177]. The samples were compressed until a load of 20 g was reached, after which the test was terminated. Force displacement data were collected and converted into an engineering stress/strain graph. The compressive modulus (Elastic Modulus) was taken as the slope of the tangent to the stress/strain curves at elastic deformation region.

3.3.4 AFM Measurement and data analysis

Atomic force microscopy (AFM) was performed in collaboration with Hoda Maleki (Institute of Biomaterials and Biomedical Engineering, University of Toronto) and Xian Wang (Department of Mechanical and Industrial Engineering, University of Toronto). Individual AFM silicon tipless cantilever (MLCT-O10, nominal cantilever spring constant k = 0.03 N/m for cantilever D; Bruker, Santa Barbara, CA, USA) were used. The AFM indenter tip was a polystyrene microsphere, with a radius, R, of 5 µm, which was glued by epoxy to the AFM
cantilever. The cantilever spring constant was calibrated as 0.0227 N/m using thermal spectroscopy (Nanoscope 8.10) and the deflection sensitivity was calibrated as 29.2 nm/V. Force measurements for PEG-NB hydrogels were made under fluid conditions using PBS at room temperature. AFM force measurements were made using microscope image registration and overlay (MIRO) and AFM indentation with a constant loading speed of 15 µm/s, Bioscope Catalyst AFM (Bruker), over the surface of a uniform gel or the center of a stripe in a photopatterned gel. The trigger force was set as 5 nN and the ramp size was set as 10 µm. For each gel 3 locations were tested (3 technical measurements were made per location). For patterned gels (made with 50 µm photomasks), points were chosen at 50 µm regular intervals along the distance of the gel. Captured force curves were used to estimate the Young's modulus of the gel surface. The Hertz model for a cylindrical tip (assuming the AFM tips approximate a cylindrical shape) was applied to fit the rising slopes of the approach curve before relaxation [287].

Force-indentation curves were fit to the Hertz contact model for a spherical tip with radius \( R \), given as:

\[
F = \frac{4}{3} E^* \sqrt{R} \delta^3
\]

where \( \delta \) is the indentation depth and \( E \) is given in with \( v = 0.5 \) for gels. The apparent Young’s moduli for PEG-NB gels are reported as mean ± SEM.

3.3.5 X-ray photoelectron spectroscopy (XPS)

XPS was used to identify the presence of nitrogen (and consequently CRGDS peptide incorporation) on the surfaces of PEG-NB gels because it is the only unique element present in amino acids and the PEG backbone. The following equation was used to calculate theoretical nitrogen content:
Percent nitrogen(%) = \(100\% \times \frac{\text{# of arms functionalized with peptide} \times \text{# of nitrogens}}{\text{Total MW of molecule}}\)

Theoretical compositions of nitrogen content for PEG-NB gels are outlined in table 2.

<table>
<thead>
<tr>
<th>Number of PEG-NB arms functionalized</th>
<th>Percent arms functionalized (%)</th>
<th>Percent CRGDS (%)</th>
<th>Percent nitrogen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>25</td>
<td>4.83</td>
<td>1.14</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>9.21</td>
<td>2.17</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>13.2</td>
<td>3.11</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>16.9</td>
<td>3.97</td>
</tr>
</tbody>
</table>

Table 2. Theoretical compositions of nitrogen in 8-arm PEG-NB hydrogels.

Spectra were collected on a ThermoFisher Scientific K-Alpha XPS (ThermoFisher Scientific, E. Grinstead). Data acquisition and work-up were obtained using the software supplied with the instrument (Avantage). Monochromatic Al K\(\alpha\) X-ray radiation (1486.65 eV) was used with a nominal spot size of 400 \(\mu\)m. Charge compensation was supplied using the combined Ar+/e- floodgun supplied with the instrument. The value of the main C-C peak was shifted to place it at 285.0 eV. Survey spectra were obtained (Pass Energy - 200 eV). Low energy spectra were also obtained (PE - 150 eV) for certain samples, from which the relative atomic % were obtained from the peak areas after a Shirley Background was subtracted, and using the sensitivity factors supplied with the instrumentation. (C 1s - 1; N 1s - 1.676; Na 1s - 10.588; O 1s - 2.881; P 2p - 1.353; Si 2p - 0.9). Finally, high resolution C 1s peaks were obtained (PE - 25 eV) which were then curve fitted using the supplied software (Avantage).

3.3.6 Visualization of CRGDS

PEG-NB gels were incorporated with FITC-CRGDS (Genscript) or CRGDSK as described above (Genscript). CRGDSK was labeled with Alexa Fluor 488 5-SDP ester (Invitrogen, Burlington, ON, CAN) according to the instructions of the supplier. SDP reacts
with the primary amine group in lysine. PEG-NB gels were incubated with 3 μM Alexa Fluor 488 5-SDP ester for 15 min at room temperature, washed with PBS, and mounted for imaging. Samples with no peptide or no Alexa Fluor 488 5-SDP ester were run as negative controls.

3.3.7 Cell Culture

Normal human tracheal tissue was obtained as surgical waste from lung transplant operations with written informed consent from lung donors, and with the approval of the University Health Network Research Ethics Board (Study 08-0318-T). The tracheal tissue was healthy and was obtained from lungs deemed to be of suitable quality for lung transplants, but was not necessary for the transplant procedure. Basal cells were isolated following the procedure of Van de Laar et al. [166]. Briefly, tracheal tissue was digested with 0.1 mg/mL DNAse (DN25, Sigma Aldrich) and 1.4 mg/mL Pronase (P5147, Sigma Aldrich) in minimal essential media (11140050, Thermo Fisher, Burlington, ON, Canada) at 4°C for 24–48 hours. After digestion, tracheal epithelial cells were pelleted, rinsed with minimal essential medium, resuspended in bronchial epithelial growth media (BEGM; Lonza, Missisauga, Ontario, Canada) with 10% fetal bovine serum (FBS; PAA laboratories) and 10% dimethylsulfoxide, and stored in cryovials in a liquid nitrogen freezer.

Primary human tracheal epithelial cells (HTECs) were expanded in BEGM containing additional supplements as described by the manufacturer (Lonza), on tissue culture polystyrene (TCPS) Petri dishes coated with 60 μg/ml PureCol (Advanced BioMatrix, Carlsbad, CA, USA) diluted in 0.01 N hydrochloric acid. Cells were grown at 37°C in a 5% CO₂ incubator, and media was changed every other day. To subculture basal cells, cells were treated with 0.025% trypsin/EDTA, which was subsequently neutralized with an equal volume of BEGM (Lonza).
Primary porcine valve interstitial cells (VICs) were a gift from the Simmons lab (Institute of Biomaterials and Biomedical Engineering and Department of Mechanical and Industrial Engineering, University of Toronto). Following the procedure of Chen et al. [288], VICs were isolated from hearts from 8 month old pigs immediately after death at a local abattoir.

Normal human bronchial epithelial cells (BEAS-2B), VICs, human fetal foreskin fibroblastic cells (HFFF2 and BJ) and mouse embryonic fibroblasts (3T3) were from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were maintained in low glucose DMEM (Invitrogen), supplemented with 10% FBS and 1 µg/mL penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Low passage hMSCs (passages 2-4) were obtained from Lonza Corporation (Walkersville, MD, USA) cultured in αMEM (Gibco, USA) containing 10% FBS (Gibco), 1% PenStrep (Corning, USA) and 1% L-glutamine.

For cell adhesion studies, PEG-NB substrates were cast in 96-well plates and seeded with 200 µL of media at a density of 5,000-15,000 cells/cm². The cells were incubated at 37°C in 5% CO₂ and the culture media (low glucose DMEM, αMEM or BEGM) were refreshed every 2 days (3-4 days for hMSCs). For RGD competition assays, HTECs were cultured in 24-well plates with 500 µL BEGM at a density of 25,000 cells/cm². The media was supplemented with 0.5 mM CRGDS (Genscript) for 4 h and imaged by phase contrast.

3.3.8 Quantification of cellular elongation and alignment

Cells on gel substrates were assessed daily by phase contrast using an Olympus IX81 inverted light microscope (Olympus Canada, Richmond Hill, ON, CAN). Cell adhesion and time to confluence were determined. We quantified the elongation and alignment of plasma membranes by tracing fluorescent images by hand and using the fit ellipse and angle measurement tool of ImageJ. Within each image 20-30 random cells were traced and measured.
Cells without complete borders or touching the edge of the image were excluded from the analysis. Elongation was defined as the ratio of the cellular major axis to the minor axis. Cells with a ratio greater than 1.4 were defined as elongated while cells with elongation < 1.4 were deemed to be indistinguishable from perfect circles.

3.3.9 **Microscopy**

Fluorescence microscopy was performed with a 20x/0.75 UplanSApo or 40x/1.3 Oil UplanFl objective, using an Olympus IX-81 inverted confocal microscope. Images represent either individual confocal sections or projections of sections that were chosen from Z-stacks (1-2 µm intervals), which were routinely acquired for all studied gels and cultures. Image analysis, cell counts and cell shape measurements were done by evaluating Z-stacks, using the FV10-ASW viewer (Olympus) and ImageJ software (NIH). Images were processed with the FV10-ASW viewer and ImageJ.

3.3.10 **Statistical analysis**

Unpaired, two-tailed Student’s t-tests or one-way ANOVA followed by Tukey’s (to compare among groups) post hoc tests were performed as appropriate. For all tests, p-values < 0.05 were considered significant.

3.4 **Results**

3.4.1 **Stiffness varies with PEG-NB content and UV exposure**

We first set out to test whether stiffness could be modulated in our hydrogel by varying PEG-NB monomer content and UV exposure time. Using bulk compression testing, we determined the elastic modulus of these gels. We observed that the bulk elastic modulus of PEG-NB hydrogels increases with PEG-NB monomer content (Fig. 11A). Elastic moduli ranged from 13.2 kPa (4% Wt PEG-NB) to 33.9 kPa (20% Wt PEG-NB) for gels with 100% crosslink
density. Stiffness also varied with the total UV dose (Fig. 11B). The stiffness of hydrogels had a rapid growth phase before plateauing near a maximum elastic modulus for a given gel composition. The kinetics and maximum rigidity of the hydrogel network varied with the crosslink density; elastic moduli reached approximately 50% of the peak elastic modulus at 45, 20, and 5 s for 4% Wt PEG-NB gels at crosslink densities of 25%, 50%, and 100%, respectively. 4% Wt PEG-NB gels had maximum stiffnesses of 8.44 kPa, 13.7 kPa, and 15.3 kPa for crosslink densities of 25%, 50%, and 100%, respectively. We therefore concluded that the bulk mechanical properties of PEB-NB hydrogels could be modulated.

![Figure 11. Stiffness varies with monomer content and UV exposure in PEG-NB gels.](image)

(A) Material stiffness varies with PEG-NB content. All PEG-NB gels were crosslinked for 60 s. Values represent mean ± SEM (n = 4). (B) Differential stiffness generated by differential UV dosing was validated by bulk compression testing in unpatterned (uniform UV exposure) PEG-NB gels. Values represent mean ± SEM (n = 4).

3.4.2 Photopatterned PEG-NB produces a cell alignment response

We first wanted to test whether this hydrogel be utilized to support cell culture and produce an alignment response. Specifically we seeded hMSCs and VICs as positive controls on PEG-NB gels; either unpatterned (uniform) or photopatterned with 13 µm photomasks (Fig. 12A, 12D). Using F-actin staining and cell counting, we assessed cell elongation (aspect ratio) and proliferation. hMSCs displayed marked elongation compared to isotropically soft (7.4 kPa) and stiff (13.7 kPa) gels (Fig. 12B) but no proliferation (Fig. 12C). VICs did not have enhanced
elongation compared to isotropic cultures (Fig. 12E), but we observed marked proliferation and ability to form aligned tissues after 7 days on patterned PEG-NB (Fig. 12F). These results indicate that photopatterned PEG-NB gels can support and direct the growth of hMSCs and VICs in culture. We therefore concluded that photopatterned PEG-NB could be a useful \textit{in vitro} tool for aligning tissues.

\textbf{Figure 12. hMSCs and VICs have different responses on photopatterned PEG-NB.} (A) Representative fluorescent image showing cellular alignment of hMSCs on 13 µm patterned PEG-NB after 7 days in culture. (B) Aspect ratio of hMSCs on TCPS and hydrogels at day 1 and day 7 in culture. (C) Cell number per field of hMSCs on TCPS and hydrogels at day 1 and day 7 in culture. (D) Representative brightfield image showing cellular alignment of VICs on 13 µm patterned PEG-NB after 7 days in culture. (E) Aspect ratio of VICs on TCPS and hydrogels at day 1 and day 7 in culture. ‘Soft gels’ = 7.4 kPa; ‘stiff’ gels = 13.7 kPa. Data represent mean ± SEM and * indicates p<0.05 (n = 3).
3.4.3 Alignment does not depend on cell density

We next evaluated whether cellular alignment is diminished by cell crowding. We seeded hMSCs onto photopatterned hydrogels at cell densities ranging from $15 \times 10^5$ to $10 \times 10^6$ cells/cm$^2$ and quantified cell angular alignment (fraction of cells within $20^\circ$ of pattern) at 24 h using ImageJ. Cell number was plotted against its alignment fraction (Fig. 13). We found no observable correlation between the two variables, suggesting the alignment response is independent of cell density. We therefore concluded this tool can be used to align both single cells and confluent cell sheets.

![Graph showing no correlation between cell alignment and density](image)

**Figure 13. Cellular alignment at varying cell densities.** No correlation was found between the fractions of cells aligned within $20^\circ$ of the pattern and cell density. Each data point represents one image (20 cells analyzed per image).
3.4.4 Patternning with UV light does not produce local stiffness differences in PEG-NB

We set out to validate anisotropic stiffness in photopatterned PEG-NB gels (Fig. 14A). We observed that the resultant hydrogels had visible patterns under transmitted light, of comparable scale to the photomasks, which consisted of alternating gaps and lines (Fig. 15A). AFM force indentation measurements were performed to assess local changes in stiffness at the micron-scale level. We observed that gels that had been uniformly exposed to 20 s of UV (0.71 ± 0.37 kPa) exhibited lower stiffness compared to gels exposed for 120 s (5.45 ± 2.81 kPa), validating our prior results with bulk stiffness (Fig. 14B). Photopatterned PEG-NB gels, however, displayed uniform stiffness at the local scale. Regions

Figure 14. UV-patterned PEG-NB gels do not have anisotropic stiffness. (A) Schematic depicting photopatterning strategy by differential UV dosing. Left column depicts cross-sectional view. Right column is the top view. (B) Experimental data comparing modulus values for gels exposed to 20 s and 120 s of UV light (uniform exposure). (C) Young’s modulus values for photopatterned gels. Error bars are standard error of the mean and * indicates p<0.05 (n = 3).
exposed to 20 s UV light did not have a statistically significant difference in stiffness compared to areas that received 120 s UV (2.04 ± 0.25 kPa vs. 2.06 ± 0.32 kPa) (Fig. 14C). These results suggest that contact photolithography does not produce local stiffness differences at locations that are within 50 µm of each other. We therefore concluded photopatterning was not an appropriate strategy for generating anisotropic stiffness with sub-cellular resolution.

3.4.5 UV-patterned PEG-NB gels have no topography

Although it was determined that stiffness was not altered by photopatterning PEG-NB, we wanted to identify the cue that was producing cell alignment in these hydrogels. It was observed that patterned PEG-NB gels consistently displayed an optical refraction pattern under transmitted light (Fig. 15A). We assessed whether our patterning strategy introduced substrate topography into the cell surface. Specifically we incubated patterned PEG-NB with FITC-labelled dextran to visualize the gel side profile by confocal microscopy. We found that there was no apparent topography in the surfaces of our patterned PEG-NB hydrogels (Fig. 15B). In comparison, PEG-NB surfaces cast against grooved masters displayed obvious microgrooved topography (Fig. 15C). We therefore concluded that photopatterning of PEG-NB did not create topographical signals in the gel surface.
Figure 15. Photopatterning of PEG-NB gels does not introduce surface topography. (A) DIC image of PEG-NB surface patterned with 13 µm photomask. (B) XZ confocal reconstruction of photopatterned PEG-NB labelled with FITC-dextran (green). (C) Side profile of PEG-NB gels cast against 5 µm deep microgrooves (green) (n = 3).

3.4.6 UV-patterned PEG-NB gels have differential adhesion

Ruling out changes in local stiffness and topography, we next assessed the possibility that our photopatterning strategy produced patterned localization of adhesion molecules. We first wanted to confirm the presence of CRGDS peptide in our hydrogel substrates. We used XPS to determine the nitrogen content at the surface of PEG-NB gels, which acted as a surrogate metric for amino acids. We observed detectable levels of nitrogen (1.14 ± 0.09%) in gels with 5 mM CRGDS in the polymer network (Table 3). XPS of PEG-NB gels incorporated with 0 mM CRGDS had no detectable levels of nitrogen. These results are consistent with the expected theoretical value of PEG-NB with 2 peptide functionalized arms (1.14%) (Table 2). We therefore concluded that CRGDS peptide was present at the PEG-NB hydrogel surface at detectable levels.

<table>
<thead>
<tr>
<th>PEG-NB arms available for functionalization (%)</th>
<th>CRGDS content in gel (mM)</th>
<th>Measured N1s % ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0</td>
<td>0.40 ± 0.087</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>0.50 ± 0.085</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>1.14 ± 0.096</td>
</tr>
</tbody>
</table>

Table 3. Measured compositions of nitrogen in 8-arm PEG-NB hydrogels. Quantification of nitrogen is expressed as a mean percentage of total composition ± S.D. (n = 3).
Patterned PEG-NB gels incubated with FITC-dextran did not diffract light in the FITC channel (Fig. 16). This result allowed us to visualize RGD with fluorescence. To specifically visualize RGD localization, we conjugated PEG-NB hydrogels (containing CRGDSK) with Alexa Fluor 488 5-SDP ester, which reacts with primary amines. We observed that uniform gels with fluorescently-tagged RGD had consistent levels of fluorescence across the gel surface (Fig. 17C, 17E). In contrast, patterned gels displayed alternating bands of high or low fluorescent signal (Fig. 17D). There were bands of higher intensities in regions that received less UV (Figure 17F). The width of these bands was approximately on the same order as the features in the photomasks (13-50 µm). Fluorescence was not detected in samples with no peptide present or without Alexa Fluor 488 5-SDP ester. These results were replicated with hydrogels that incorporated FITC-RGD in lieu of fluorescently-tagged CRGDSK. Together, these data suggest that our patterning strategy produces variations in adhesion peptide incorporation. We therefore concluded that photopatterning PEG-NB is a useful strategy to generate cell culture substrates with patterned adhesion with subcellular-scale resolution.
Figure 16. Patterning does not create differential diffraction of light. (A) Brightfield image of patterned PEG-NB gel surface. (B) Corresponding fluorescent image of (A) with PEG-NB stained with FITC-dextran. Profile plots of PEG-NB surface depicting changes in pixel intensity vs. position in brightfield (C) and FITC channel (D) reveal that fluorescent labelling is not diffracted by photopatterning.
Figure 17. UV-patterned PEG-NB gels have patterned adhesion. (A) DIC image of uniform (A) and patterned (B) PEG-NB gels. CRGDSK localization in uniform (C) and photopatterned (D) PEG-NB gels labelled with Alexa Fluor 488 5-SDP ester (green). (E) Corresponding confocal side profile of (C). (F) Corresponding confocal side profile of (D) \((n = 3)\).

3.4.7 Tracheal progenitor cells have RGD-responsive receptors

We were particularly interested in supporting HTEC culture with RGD-patterned PEG-NB hydrogels, therefore we assessed the ability of HTECs to recognize and bind the RGD sequence \textit{in vitro}. To do this we incubated HTECs cultured on TCPS with 0.5 mM FITC-labelled RGD for 30 min. We observed primary HTECs specifically bind fluorescently-tagged RGD,
indicating that these cells possessed RGD-competent receptors (Fig. 18B). We next evaluated the responsiveness of HTEC binding sites with a RGD competition assay by supplementing the culture media with CRGDS peptide for an extended period of time (4 h). Competitive binding to HTECs by freely available CRGDS resulted in fewer cells with spread morphology (Fig. 18D-E). These results indicate that HTECs have receptors that bind to RGD.

**Figure 18. HTECs express RGD-competent receptors.** Immunofluorescence of HTECs incubated with FITC (A) or FITC-RGD (B) on TCPS (*n* = 3). Phase contrast images of HTEC cultures supplemented with (D) or without (C) 0.5 mM CRGDS peptide. Scale bars: 100 µm. (E) Cells incubated with competitive CRGDS displayed a fewer number of cells with spread morphology. Data represent mean ± SEM and * indicates p<0.05 (*n* = 4).

**3.4.8 Cell adherence and spreading on PEG-NB gels under defined conditions varies for different cell types**

We next screened the utility of PEG-NB hydrogels in supporting the culture of various cell types. Cell adhesion on PEG-NB substrates was assessed by comparing cell morphology on isotropically stiff hydrogel substrates (ranging 7.4-13.7 kPa) and tissue culture polystyrene (TCPS). hMSCs and VICs display comparable cell morphology on both substrates (Fig. 19A-D). In contrast, other epithelial (BEAS-2B, HTEC) and fibroblast (HFFF2, BJ, and 3T3) cell types
displayed rounded morphologies on the hydrogel compared to TCPS (Fig. 19E-L). Furthermore, HTECs were unable to adhere to PEG-NB gels that were coated with various ECM ligands (fibronectin, gelatin, collagen Types I, III, and IV) (data not shown). These results indicate that hMSCs and VICs are able to form the cell-substrate contacts necessary to adhere and spread on the hydrogel. We therefore concluded that PEG-NB may be optimized for some mesenchymal cell types (hMSCs and VICs) but not epithelial cell types.
Figure 19. Cell morphology on TCPS and PEG-NB hydrogels. Representative brightfield images displaying the morphology of hMSCs, VICs, HFF2, BJ, 3T3, and BEAS-2B cells on (A,C,E,G,I,K) TCPS and (B,D,F,H,J,L) PEG-NB (n = 3).
3.5 **Discussion**

Here, we present initial steps towards generating substrates for advanced TE approaches as well as to understand alignment responses of single cells and cell sheets. We initially set out to produce anisotropic stiffness at the local (subcellular) scale using viscoelastic hydrogel substrates. We found that this could not be achieved with our simple photopatterning strategy. UV-patterned PEG-NB did not display mechanical properties that varied across the surface. One possible explanation for this result is that the chemical thiol-ene reaction initiated by UV light generates complex secondary reactions that propagate throughout the gel network, which functionally reduces or eliminates the regional differences in crosslink density.

We instead discovered that PEG-NB displayed differences in adhesion peptide concentration in photopatterned regions of 13 µm-wide stripes. After 24 h, cells acquire morphologies that are elongated and robustly aligned in the direction of the adhesion pattern (Fig 12). We showed using confocal analysis that this response was not due to changes in surface topography. After 7 days, VICs but not hMSCs became confluent on patterned PEG-NB gels. This difference in confluence could be due to cells proliferating, as well as cells migrating from the high adhesion to low adhesion regions (or vice versa). Cells that feature smaller or fewer focal adhesions have been demonstrated to migrate more rapidly than cells that feature large focal adhesions [289, 290]. Further investigation is needed to understand how this behaviour is translated to processes such as differentiation and to understand how cells in different local environments affect each other through mechanisms such as cell-cell or paracrine signaling. Based on these results we conclude that our photopatterning strategy can be applied on hydrogel surfaces for the investigation of spatial variations in peptide concentration on cell behaviour.
Studies using transparency-based photolithography have produced patterns with minimum feature sizes down to 40 µm [291, 292]. Using higher resolution printer in mask fabrication and a collimated light source, we report a further reduction in minimum feature size (13 µm) which is below the scale of a whole cell. Stripe patterns are rapidly generated using this technique (Fig. 17). Modulating the spatial concentration of bioactive moieties has been shown to influence cell morphology, alignment and migration [293, 294]. Consequently, our tool is useful for studying the impact of exposing single cells to surfaces with multiple adhesion properties at the local scale.

Based on our results we conclude that PEG-NB hydrogels are suitable substrates for the culture of hMSCs and VICs but not other epithelial and fibroblast cell types. Primary basal cells from human trachea were not supported by PEG-NB even though the data suggests that there was sufficient incorporation of CRGDS into the PEG-NB network and HTECs are responsive to the fibronectin-derived RGD sequence, which is consistent with literature stating HTECs are α6β4 integrin-positive basal cells [1, 166]. One possibility to customize PEG-NB for other cell types would be to incorporate other peptide sequences such as YIGSR (Tyrosine-Isoleucine-Glycine-Serine-Arginine) and DGEA (Aspartic Acid-Glycine-Glutamic Acid-Alanine), into the polymer network. Activation of specific receptors by different ECM-derived peptide sequences has been demonstrated to regulate behaviours such as adhesion and differentiation [295, 296]. Another explanation could be that the PEG-NB gels were not in the correct stiffness range. The elastic modulus of rabbit tracheal subepithelial connective tissue samples has been reported to be 4.1 kPa [297], which falls within the stiffness range of PEG-NB. However, epithelial cells are capable of growing on stiffer substrates in vitro. In fact, it may be the preferred stiffness as primary cells adhere on PDMS and the polyester membranes of transwell inserts (mPa stiffness
range). ECM stiffnesses that are too soft result in unstable, dynamic focal adhesions and low cell densities post-seeding [236].

3.6 Conclusions

We have developed a highly reproducible fabrication method and cell culture platform for growing cells on surfaces with spatially-patterned adhesion. The ability to spatially control adhesion was possible by varying the UV light exposure time in different regions of the gel. hMSCs and VICs cultured on hydrogel substrates display varying morphology and proliferation responses on patterned CRGDS surfaces. Cells remain spread and aligned in the direction of the CRGDS pattern, in contrast to cells grown on unpatterned substrates. Our photopatterned culture system is however limited as we were unable to generate local stiffness variations or support the adhesion of primary HTECs. We concluded that this approach was not feasible for assessing the impact of stiffness anisotropy on organization of ciliated airway epithelium. At present, this patterned gel system will enable the exploration of numerous novel biological questions in the future such as how can adhesion be systematically varied with photopatterning to evaluate tissue level organization. We speculate that this model will provide a valuable tool for those studying the biology of hMSCs and VICs. Future work to expand the utility of this tool will require optimization of PEG-NB for adhesion of primary cell types. Specifically, complex combinations of adhesion molecules known to bind basal epithelial cells could be presented in a physiologically-relevant manner to enhance cell binding.
Chapter 4

Micro-grooved Collagen Vitrigel Membranes Align Primary Multiciliated Airway Epithelium during Apical-basal Polarization
4.1 Abstract

In Chapter 4, we utilized a tissue-engineered scaffolding approach to produce grooved hydrogel substrates for the planar polarization of airway epithelial cells. Topographical cues are known to influence cell organization both in native tissues and in vitro. In the trachea, the matrix beneath the epithelial lining is composed of collagen fibres that run along the long axis of the airway. Previous studies have shown that grooved topography can induce morphological and cytoskeletal alignment in epithelial cell lines. In the present work we assessed the impact of substrate topography on primary cell organization. Grooves with optimized dimensions were imprinted into collagen vitrigel membranes to produce gel inserts for air-liquid interface (ALI) culture. We show that these substrates were able to produce an alignment response in both submerged and ALI culture, demonstrating that our grooved cue was robust enough to induce planar polarization that was compatible with apical-basal polarization. Using cell morphometric analysis, it was found that grooved architecture could direct alignment for 14 days in ALI but this effect was lost at later timepoints. Critically, primary cells were able to terminally differentiate into a multiciliated epithelium on both flat and groove substrates. Groove architecture represents a promising scaffolding approach to manipulate the organization of epithelial cell types with a tissue-scale directional cue; however future work is necessary to determine the impact of early morphological planar polarization on cilia basal body orientation.
4.2 Introduction

There is a clinical need for airway replacements. Injuries and lesions affecting short segments of the trachea can be resected and reconnected, but to date there are no acceptable treatments for the reconstruction of long regions of damaged or diseased upper large airways [298]. Efforts are ongoing to optimize scaffolds to support cell adhesion and maturation, but tissue-engineered constructs possessing a mature epithelial lining that confers long-term organ function is a critical milestone that has yet to be met [4, 5]. Clinical trials to treat large airway defects by implantation of decellularized tracheal allografts or synthetic scaffolds had poor patient survival rates and remains controversial [2, 6-8]. In particular recipients were susceptible to recurrent infections and mucus impaction postoperatively, resulting in re-hospitalization and high mortality rates [4, 9]. One suggested reason for this high infection rate was delayed or dysfunctional re-epithelialization of the airway constructs. For example, in patients that received tracheal allografts, mucosal coverage was slow (≥1 month), and restoration of mucociliary clearance took at least 6 months after surgery [3, 4, 6]. Novel methods to generate functional airway epithelium are therefore required to improve the clinical translatability of this tissue engineering approach.

Airway epithelium is made up of a pseudostratified monolayer of columnar epithelial cells. Within this pseudostratified layer, are multiciliated cells (MCCs) that each contain hundreds of motile cilia. Cilia are microtubule-based protrusive structures, the coordinated, concerted beating of which generates an elevator function that eliminates mucus and particles that cause infection [9, 59, 70, 90, 91]. Coordinated beating between multiple cilia in MCCs is critical for producing the mucociliary elevator function and prevention of mucous compaction [9, 70]. Cilia formation and coordination requires appropriate polarization of the epithelial lining. Epithelial cells are apical-basally polarized, such that the luminal (apical) face is exposed to air and airway surface
liquid, while the basolateral side forms attachments with the underlying basement membrane [99]. Apical-basal polarity is essential for airway tissue maturation and cilia formation [66, 69, 163]. Airway epithelial cells are also polarized in the planar direction via a mechanism involving reorganization of actin and microtubule-based structures that enables coordination of a sub-apical network of actin and microtubules within the cells and ultimately cilia alignment and coordination between individual MCCs within the epithelia [66, 70]. This planar polarization mechanism presumably functions to align cilia beat direction along the oral-lung axis [9, 70]. Generation of appropriately polarized epithelium is therefore necessary in order to achieve coordinated cilia beating and effective mucociliary clearance in an engineered tracheal construct. Current methods to generate airway epithelium in vitro however do not generate both apical-basal and planar polarized epithelium. Typically airway epithelial cells, isolated from primary donors, are grown on permeable transwell membrane cultures under air-liquid interface (ALI) culture conditions in which cells are supplied with nutrients from media in the basal compartment; this system promotes apical-basal polarization over an approximately four-week maturation period [163]. Although this ALI culture approach produces epithelium with mature cell types and MCCs, it lacks the instructive cues required to create planar polarized ciliated epithelium. This limitation results in cultures with disorganized cilia beating [95, 164, 165]. Strategies to generate both apical-basal and planar polarized epithelium are therefore required to engineer a functional airway epithelium [2, 299].

One strategy that has been previously used to induce morphological planar polarization of cells in engineered tissues is the use of topographic features, such as grooves, to guide and align cell organization. These features mimic similar-scale topographical cues present in the ECM of many tissues [14]. Specifically, the tracheal basement membrane is comprised of aligned
collagen fibres that run longitudinally along the proximal-distal axis [138, 245]. Surface grooves of different sizes, ranging from nano- to microscale, have been shown to induce different degrees of elongation in various cell types including epithelial cells [15, 264, 300-302]. For example, human corneal epithelial cells were found to align on grooved substrates when the pitch was larger than a threshold range of 0.8-1.6 μm [303]. Topographic cues therefore offer a potential strategy to induce planar alignment and cilia alignment in airway epithelial cells. Indeed we have previously reported on a strategy to expose the BEAS-2B epithelial cell line to grooved hydrogel topography that simultaneously enabled apical-basal and planar polarization of the cells [16]. In this work we observed that, while the BEAS-2B cells initially aligned to the gel grooves, whole cell alignment was lost after 24 h of ALI culture. In vivo planar cell polarity cues are often transient and present only during specific time windows of tissue development yet lead to structural polarization of cellular features at later time points [69, 70]. It is therefore possible that alignment of airway epithelium on grooves at initiation of ALI culture is sufficient to influence cilia coordination at later time points. Our previous work could not test this idea however because it was all performed using cell lines that do not generate MCCs.

Here, we set out to adapt our platform to characterize the effects of presenting topographical cues to primary airway epithelial cells, specifically with respect to cell organization during apical-basal polarization and to determine if planar cell alignment at early timepoints in ALI culture is sufficient to influence the organization of cilia that emerge at later time points in culture. We screened and identified the groove dimensions that produced primary human tracheal epithelial cell (HTEC) alignment in normal culture and found that HTECs grown on grooved collagen vitrigel membrane (CVM) aligned with the direction of the grooves. Alignment was maintained under ALI conditions until D14 but lost by D21. We also found that HTECs were able to terminally differentiate into MCCs on both flat and grooved CVM
membrane under ALI culture by day 21. While we attempted to quantify ciliary orientation and coordination on flat versus grooved substrates at 21 days of culture using super-resolution microscopy, this was not successful and future work will include optimization of this method to enable us to determine if the cell alignment over the first 14 days of ALI culture is sufficient to instruct ciliary alignment at day 21.

4.3 **Methods**

4.3.1 **Generation of microgrooves**

Microgrooves were produced as described previously [16]. Briefly, silicon wafers containing known microgroove topography (3.14 μm depth, 2 μm pitch) were silanized with (tridecafluoro-1,1,2,2,Tetrahydro Octyl 1)-trichlorosilane and then coated with a 1:10 mix of polydimethylsiloxane (PDMS) crosslinker:elastomer (Dow Corning Corporation, Midland, MI, USA) to create masters for replica molding. The film was vacuumed for 20 minutes and cured overnight at 60°C. After curing, the PDMS layer was peeled from the Si wafer. The resultant microgrooved PDMS masters were silanized before further replica molding.

For experiments where varying groove dimensions were required we generated masters with precisely-defined depth and geometry, from silicon masters created using a combination of electron beam lithography (EBL) and deep reactive ion etching (DRIE). Briefly, a Si wafer was spin-coated with 1 μm thick ma-N 2410 negative photoresist. Electron beam lithography (Vistec EBPG 5000+ Electron Beam Lithography System) was performed with regions containing 3, 5, or 10 μm-pitch lines. Unexposed photoresist was removed using MAD-525 developer. Etching was carried out using a deep reactive ion etcher (Oxford Instruments PlasmaPro Estrelas100 DRIE System). A Bosch etching process was used and repetitions were adjusted to obtain desired groove depths (1, 3, or 5 μm depth). Patterned Si wafers (1, 3, or 5 μm in depth, characterized by
scanning electron microscopy (SEM)) were washed with acetone, cleaned with oxygen plasma (TePla Technics 100-E Oxygen Plasma Asher), and silanized in a desiccator. Replica molding to obtain grooved PDMS masters was performed as described above.

Freestanding CVM inserts were generated from bovine dermis native collagen solution (5 mg/mL; KOU-IAC-50, AteloCell, Tokyo, Japan) as described by the manufacturer with some minor modifications. 1:1 (collagen solution: bronchial epithelial growth media (BEGM)) CVM solution was added on top of flat or microgrooved PDMS masters, incubated for 2 h at 37°C for gel formation, and allowed to desiccate in a humid chamber at 4°C for 2-3 days. Dried-out CVM films were re-hydrated by washing 3 times (10 minutes each) with phosphate buffered saline (PBS) (Lonza, Walkersville, MD, USA), and allowed to dry at 4°C for ~7 days. To create CVM films compatible with ALI culture, circular CVM inserts were cut out using a 6.0 mm biopsy punch (Fray Product Corporation, Buffalo, New York, USA) and carefully placed within 24-well transwell inserts (0.4 μm pore size) (Corning Incorporated, Tewksbury, MA, USA). CVM films were sterilized with 70% ethanol, rinsed with sterile PBS, and coated with 60 μg/ml PureCol (5005-100mL, Advanced BioMatrix, Carlsbad, CA, USA) diluted in 0.01 N hydrochloric acid (BDH7204-1, VWR, Mississauga, Ontario, Canada) prior to cell culture.

4.3.2 Microgroove geometry characterization

The topography of the PDMS replicas and CVM hydrogel films were visualized using SEM. All samples were gold coated (Edwards Coating System E306A, Tewksbury, MA, USA) and imaged using a Hitachi S-4500 (Hitachi High-Technologies Canada, Inc., Toronto, ON, Canada) at 1.5 kV. Transverse sections of microgrooved PDMS substrates were analyzed with ImageJ to measure groove dimensions.
4.3.3 Epithelial cell line culture

The human epithelial cell line BEAS-2B (bronchial epithelium) used in this study was from the American Type Culture Collection (ATCC) (Manassas, VA, USA). BEAS-2B were maintained in growth medium (Dulbecco’s modified Eagle Medium [DMEM]/F12 (Invitrogen, Grand Island, NY, USA)), 10% fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, Ontario, Canada), and 1 μg/ml penicillin and streptomycin (Sigma Aldrich, St. Louis, MO, USA).

4.3.4 Isolation and culture of HTECs

Normal human tracheal tissue was obtained as surgical waste from lung transplant operations with written informed consent from lung donors, and with the approval of the University Health Network Research Ethics Board (Study 08-0318-T). The tracheal tissue was healthy and was obtained from lungs deemed to be of suitable quality for lung transplants, but was not necessary for the transplant procedure. Basal cells were isolated following the procedure of Van de Laar et al. [166]. Briefly, tracheal tissue was digested with 0.1 mg/mL DNAse (DN25, Sigma Aldrich) and 1.4 mg/mL Pronase (P5147, Sigma Aldrich) in minimal essential media (11140050, Thermo Fisher, Burlington, ON, Canada) at 4°C for 24–48 hours. After digestion, tracheal epithelial cells were pelleted, rinsed with minimal essential medium, resuspended in BEGM (Lonza, Mississauga, Ontario, Canada) with 10% FBS and 10% dimethylsulfoxide, and stored in cryovials in a liquid nitrogen freezer. Primary HTECs were expanded in BEGM containing additional supplements as described by the manufacturer (Lonza), on plastic Petri dishes coated with 60 μg/ml PureCol (Advanced BioMatrix) diluted in 0.01 N hydrochloric acid. Cells were grown at 37°C in a 5% CO₂ incubator, and media was changed every other day. To subculture basal cells, cells were treated with 0.025% trypsin/EDTA, which was subsequently neutralized with an equal volume of BEGM (Lonza).
4.3.5 Cell seeding and ALI culture

For alignment experiments on PDMS microgrooves cells were seeded at densities to generate confluent monolayers immediately after seeding (50000 cells/cm$^2$). Cultures were maintained for 48 hours and prepared for immunocytochemistry.

For ALI studies, we seeded 150000 cells/cm$^2$ on grooved CVM inserts placed within transwells. Transwell filter inserts with flat CVM or no insert (0.4 μm pore-size) were seeded as controls. Cells were grown with BEGM present in the top and bottom compartments of the transwell filter until confluent. Once a confluent sheet was formed (approximately 24-36 h) media was removed from the apical compartment and the basal compartment was replaced with serum-free B-ALI differentiation media (193514; Lonza) to establish an ALI culture. Differentiation in ALI culture proceeded for various timepoints to 21 days after which the cells were prepared for immunocytochemistry.

4.3.6 Immunocytochemistry

Samples were fixed with 0.4% paraformaldehyde (28908, Pierce, Rockford, IL, USA) for 10 minutes, washed with PBS, then permeabilized with 0.1% Triton-100 (Sigma Aldrich) for 20 minutes at room temperature. Samples were washed with PBS and incubated in 5% FBS for 30 minutes at room temperature to block nonspecific binding. To detect cilia formation, samples were incubated overnight at 4°C with mouse anti-human primary antibody for acetylated α-tubulin (1:100; T7451, Sigma Aldrich). Samples were washed with PBS, and incubated with Alexa Fluor 546 goat anti-mouse secondary antibody (1:500; A11003, Life Technologies) at room temperature for 2 h. Nuclei were stained with Hoescht 33258 (2 μg/mL; 861405, Sigma), and F-actin with FITC-conjugated phalloidin (2 μg/mL; P5282, Sigma). Samples were then
mounted with Fluorescence Mounting Medium (Dako, Glostrup, Denmark) for imaging. Samples with no primary antibody but with a secondary antibody were run as negative controls.

For super-resolution microscopy, samples were fixed directly on filter with 100% methanol at -20°C for 10-15 minutes, washed with PBS and PBST (0.5% Tween20 diluted in PBS), then incubated in 5% FBS for 20 minutes at room temperature to block nonspecific binding. Samples were incubated overnight at 4°C in a humid chamber with the following primary antibodies: rabbit anti-human centriolin (1:50, sc-135020, Santa Cruz, Missisauga, Ontario, Canada) and mouse anti-human α-tubulin (1:500, T9026, Sigma-Aldrich). Samples were washed three times with PBST, and incubated with goat anti-rabbit conjugated with AlexaFluor-488 (1:500, A-11034, Thermo Fisher) and donkey anti-mouse conjugated with AlexaFluor-647 (1:500, A-31571, Thermo Fisher) secondary antibodies at room temperature for 1 h. Samples were washed three times with PBST, and incubated overnight at 4°C in a humid chamber with a 1:50 dilution of rabbit anti-POC1B antibody (PA5-24495, Invitrogen) directly labeled with AlexaFluor-555 using Apex Antibody Labeling Kit (A10470, Invitrogen). Samples were then stained for DNA using Hoescht 33258 (Sigma), washed three times with PBST and once with 50% glycerol in PBST. Samples were finally mounted with mounting medium (0.5% n-propyl gallate in glycerol).

4.3.7 Microscopy

Fluorescence microscopy was performed with a 20x/0.75 UplanSApo or 40x/1.3 Oil UplanFl objective, using an Olympus IX-81 inverted confocal microscope. Images represent either individual confocal sections or projections of sections that were chosen from Z-stacks (1-2 μm intervals), which were routinely acquired for all studied microgroove cultures. Image analysis, cell counts and cell shape measurements were done by evaluating Z-stacks, using the
FV10-ASW viewer (Olympus) and ImageJ software (NIH). Images were processed with the FV10-ASW viewer and ImageJ.

3D-SIM imaging was performed in collaboration with Quynh Nguyen and Vito Mennella (The Hospital for Sick Children, Toronto). Structured Illumination Microscopy (3D-SIM) was performed using a Zeiss ELYRA PS.1 3D Super-resolution Microscope and Zen software, using with Plan Apochromat 63X/1.40 Oil DIC M27 or alpha Plan 100X/1.46 Oil objective lens with an additional 1.6x optovar. The SIM images were acquired using An Andor iXon 885 EMCCD camera, with 101 nm/pixel z-stack intervals over a 5-10 µm thickness. Image stacks with typical heights of 4-10um were acquired with Z-distance of 0.101um. For each Z-slice, 15 grid excitation pattern images (5 phases, 3 rotation angles (-75°; -15°, +45°)) were taken. Raw images were then superimposed and computationally reconstructed to create images with sub-diffraction resolution using SIM module of ZEN Black Software (version 8.1). The reconstructed images were channel aligned by calibrating with super-resolution Tetraspeck™ fluorescent beads slide (Carl Zeiss Microscopy) and projected to single plane image based on maximal intensity and analyzed using Zen and Fiji/ImageJ software.

4.3.8 Quantification of cellular elongation and alignment

We quantified the elongation and alignment of cell plasma membranes by tracing fluorescent images by hand and using the fit ellipse and angle measurement tool of ImageJ. Within each image 20-30 random cells were traced and measured. Cells without complete borders or touching the edge of the image were excluded from the analysis. Elongation was defined as the ratio of the cellular major axis to the minor axis. Cells with an aspect ratio greater than 1.4 were defined as elongated while cells with elongation < 1.4 were deemed to be indistinguishable from perfect circles and considered noise. These data were excluded from the
analysis because non-elongated cells could not reliably be assigned an angular direction. Angular orientation of all detectable nuclei was detected with the analyze particles and angle measurement tools of ImageJ. Nuclei without complete borders or touching the edge of the image were excluded from the analysis. Alignment was defined as the percentage of cells or nuclei oriented within 15° of the groove direction. Angular histograms were created using the graphing software Rozetta by Jacek Pazera.

4.3.9 Statistical analysis

Unpaired, two-tailed Student’s t-tests or one-way ANOVA followed by Tukey’s (to compare among groups) post hoc tests were performed as appropriate. For all tests, p-values < 0.05 were considered significant. Prism 5 (GraphPad Software) was used for statistical tests and for the generation of graphs.

4.4 Results

4.4.1 Primary cells do not align on 3.14 μm deep grooves

Previous work in our lab demonstrated that BEAS-2B align on PDMS and gelatin grooves with depths of 600 nm and 3.14 μm [16]. We first set out to identify if 3.14 μm deep groove depth was also sufficient to align primary HTECs. Specifically we seeded BEAS-2B and primary HTECs at high enough densities to produce confluent monolayers on PDMS culture substrates containing microgrooves with 3.14 μm depth (5 μm pitch) (Fig. 20A-B, F-G). Using F-actin and nuclear staining, we assessed several readouts of alignment using image analysis software including cell elongation (i.e. aspect ratio), cell alignment and nuclear alignment. As expected we observed that BEAS-2B cells cultured on 3.14 μm deep grooves for 48 h had increased alignment of cells parallel to the direction of the groove axis (±15°), as indicated by the angular histograms (84.9% ± 8.0% vs. 31.1% ± 15.4%) (unpaired t test, p = 0.0058; Fig. 20D)
and increased nuclear alignment parallel to the groove direction (71.9% ± 7.1% vs. 38.2% ± 6.4% on flat, unpaired t test, p = 0.0037; Fig. 20E). These cells were also elongated compared to those cultured on flat PDMS (aspect ratio was 2.69 ± 0.11 versus aspect ratio 1.69 ± 0.35 on flat, unpaired t test, p = 0.0088; Fig. 20C). In contrast, primary HTECs did not exhibit a statistically significant difference in cell elongation (1.89 ± 0.34 vs. 1.83 ± 0.28) (unpaired t test, p = 0.6999; Fig. 20H) or nuclear alignment (42.5% ± 7.3% vs. 40.3% ± 13.8%) (unpaired t test, p = 0.6572; Fig. 20J) on 3.14 µm deep grooves compared to flat. While HTEC cell alignment increased slightly compared to flat substrates, the extent of this was less than observed in BEAS-2B cells (55.2% ± 12.8% vs. 39.2% ± 17.1%) (unpaired t test, p = 0.0294; Fig. 20I). These results suggested that while the 3.14 µm deep grooves produced a robust alignment response in BEAS-2B these groove dimensions were not sufficient to align primary cells.
Figure 20. **BEAS-2B and HTECs on 3.14 μm deep grooves.** Representative confocal images of BEAS-2B on (A) flat PDMS substrates or (B) PDMS substrates containing 3.14 μm deep microgrooves stained with. (C) BEAS-2B cell elongation on flat versus grooved substrates. (D) Corresponding rose plots and quantification of cell alignment of BEAS-2B on 3.14 μm deep grooves. (E) Corresponding rose plots and quantification of nuclear alignment of BEAS-2B on 3.14 μm deep grooves. Representative confocal images of HTECs on PDMS with (F) no topography or (G) 3.14 μm deep microgrooves. (H) HTEC cell elongation on flat versus grooved substrates. (I) Corresponding rose plots and quantification of cell alignment of HTECs on 3.14 μm deep grooves. (J) Corresponding rose plots and quantification of nuclear alignment of HTECs on 3.14 μm deep grooves. Stains are phalloidin (green) and Hoescht (blue). Error bars are standard error of the mean and * indicates p<0.05. (n = 3).

4.4.2 **Primary cells align on 5 μm deep grooves**

Since primary cells are much larger than BEAS-2B cells and exhibit a prominent cortical ring of F-actin that defines cell shape we hypothesized that these cells may require a stronger topographical cue to overcome. We therefore next set out to screen a range of groove dimensions to identify those that would induce robust alignment of confluent primary epithelial cells. To do this we fabricated silicon masters with grooves ranging from 1 to 10 μm in width, pitch and depth (see SI Table 1 for specific combinations) and assessed both HTEC and BEAS-2B alignment and elongation (Fig. 21). BEAS-2B cells oriented themselves in the direction of the grooves regardless of the feature sizes. Indeed, the angular histograms indicated that this cell line displayed a robust cell (Fig. 21I) and nuclear (Fig. 21J) alignment response at most groove geometries tested (SI Fig. 1). In contrast, HTEC displayed an increasing response to groove depth (Fig. 21K-N) for cell (Fig. 21O) and nuclear (Fig. 21P) alignment, but this response was statistically significant only at depths of 5 μm (Fig. 22). Specifically, for 5 μm x 5 μm x 5 μm microgrooved substrate the average cell elongation was significantly greater than flat culture (aspect ratio 2.29 ± 0.18 vs. 1.86 ± 0.27, one-way ANOVA + Tukey test, p < 0.0001; Fig. 22A). Furthermore, a significantly greater percentage of HTECs were elongated compared to primary cells cultured on flat substrates (90.4% ± 4.8% vs. 72.0% ± 10.0%) (one-way ANOVA + Tukey
test, p < 0.0001; Fig. 22B). In additional primary cell alignment was significantly higher than on flat substrates (61.6% ± 11.9% vs. 16.5% ± 9.3%) (one-way ANOVA + Tukey test, p < 0.0001; Fig. 22C), as was nuclear alignment (45.0% ± 14.5% vs. 26.1% ± 11.5% on flat) (one-way ANOVA + Tukey test, p < 0.0001; Fig. 22D). Since there were significant increases in elongation, cell and nuclear alignment we concluded that 5 µm x 5 µm x 5 µm grooves were sufficient to generate aligned primary epithelial cell monolayers.

Figure 21. Screening optimal groove dimensions for epithelial cell culture. (A) Scanning electron micrographs of (A) flat PDMS or microgroove substrates with depths of (B) 1 µm, (C) 3 µm, and (D) 5 µm. Groove dimensions are denoted as groove width (µm) x spacing (µm) x depth (µm). (E-H) Corresponding confocal images of BEAS-2B cultured for 48 h on varying groove depths. Cell (I) and nuclear outlines (J) were fitted with an ellipse and their orientations plotted as angular histograms (groove direction = 0°). (K-N) Representative confocal images of primary HTECs cultured for 48 h on varying groove depths with their corresponding angular histograms for cell (O) and nuclear (P) orientation. Stains are phalloidin (green) and Hoescht (blue).
4.4.3 Grooves induce HTEC morphological planar polarity in early ALI culture

We next set out to assess the impact of apical-basal polarization on the organization of primary HTEC grown on grooved substrates. To do this PDMS masters containing 5 µm x 5 µm x 5 µm microgrooves were used to generate grooved transwell inserts from gelatin or CVM. Initial pilot work indicated that the gelatin inserts used in our previous work with BEAS-2B were not compatible with primary HTEC culture (data not shown). CVM was therefore selected as unpublished work in our lab has suggested that this is an optimal substrate for HTEC growth. HTEC were grown on flat and grooved CVM membranes and cell alignment and elongation were assessed at D0, D14 and D21 of ALI culture.

We observed HTECs on grooved CVM at day 0, before initiation of ALI culture were more elongated compared to cells cultured on flat CVM (aspect ratio 2.16 ± 0.16 vs. 1.72 ± 0.20 on flat, unpaired t test, p = 0.04; Fig. 23G), remained elongated at ALI D14 (aspect ratio 2.55 ± 0.27 vs. 1.94 ± 0.14, unpaired t test, p = 0.0267), and lose this alignment by ALI D21 (aspect ratio 1.64 ± 0.11 vs. 1.66 ± 0.02, unpaired t test, p = 0.7739). Consistent with this we also observed that HTEC aligned with the direction of the grooves on grooved CVM membranes prior to initiation of ALI (ALI D0; Fig. 23D) while cells cultured on flat CVM did not align (Fig. 23A) (specifically 66.3% ± 7.6% compared to 10.7% ± 6.0% on flat, unpaired t test, p = 0.0006; Fig. 23H). Alignment on grooved CVM was maintained until D14 of ALI culture (69.3% ± 9.8% vs. 22.7% ± 8.5% on flat, unpaired t test, p = 0.0034; Fig. 23B and 23E) and lost by ALI D21 (17% ± 8.9% vs. 7.0% ± 0.2%, unpaired t test, p = 0.1223; Fig. 23C and 23F). Similarly, nuclear alignment was also statistically significant for HTECs cultured on grooved CVM at both ALI D0 (55% ± 3.6% vs. 13.7% ± 0.6%, unpaired t test, p < 0.0001; Fig. 23I) and ALI D14 (56.3% ± 4.9% vs. 20% ± 9.6%, unpaired t test, p = 0.0044) but not ALI D21 (26.3% ± 12.0% vs. 10% ± 1.0%, unpaired t test, p =0.0788). Together, these data suggested that grooves with the
appropriate dimensions were capable of producing HTEC alignment and morphological planar polarization (elongation) in confluent epithelial sheets over a significant time window during ALI induced apical-basal polarization.

Figure 22. Primary HTEC elongation and alignment on PDMS microgrooves. (A) Confocal images were analyzed to assess HTEC elongation (aspect ratio) on grooves of varying pitches and depths. (B) Proportion of HTECs with an elongated phenotype (defined as major axis/minor axis ≥ 1.4). Cell (C) and nuclear (D) alignment on flat versus different groove dimensions. Alignment was defined as the percentage of cells or nuclei oriented within 15° of the groove direction. Error bars are standard error of the mean and * indicates p<0.05. (n = 3).
Figure 23. Culture of primary HTECs on CVM microgrooves under ALI. Confocal images of HTECs cultured on flat CVM films (A) prior to ALI induction (Day 0), (B) after 14 and (C) 21 days ALI culture. HTECs grown on microgrooved CVM for (D-F) 0, 14 and 21 days ALI culture. (G) Cell elongation is enhanced on CVM microgrooves at Day 0 ALI and maintained until Day 14. Primary cells display cell (H) and nuclear (I) alignment in the direction of the grooves prior to induction of apical-basal polarization at Day 0, which is maintained to Day 14. Stains are phalloidin (green) and Hoescht (blue). Error bars are standard error of the mean and * indicates p<0.05. (n = 3).

4.4.4 Ciliation of polarized HTECs on CVM

We confirmed that HTECs were able to give rise to MCCs on CVM (Fig. 24). Ciliation was assessed by positive staining for the cilia-specific marker acetylated α-tubulin. HTECs were able to differentiate on both flat and grooved CVM by ALI D21, as indicated by the presence of groups of MCCs (Fig. 24B and 24C). Because our grooved substrate maintained alignment
throughout the early phase of ALI, during which the alignment of ciliary structures may be developing, we next wanted to assess the alignment and global coordination of cilia present in MCCs matured with ALI on grooved CVM substrates. In order to do this we assessed the angular orientation of ciliary basal bodies (BBs), a protein complex that is found at the base of ciliary axonemes. The angular orientation of a BB can be determined by staining for the BB and the basal foot, a projection from the BB complex that points in the same direction as the cilia beat direction and assessing with super-resolution microscopy [69, 70]. Super-resolution 3D-structured illumination microscopy (3D-SIM) visualization of poly-glutamylated tubulin (ciliary axoneme), γ-tubulin/POC1B (basal body) and centriolin (basal foot) staining allowed us to circumvent the traditional optical diffraction limit to visualize these two structures [65]. However, we found that scarce ciliation was observed by 3D-SIM in both transwell and CVM cultures (Fig. 24D-F). Only a few (<5) MCCs were detected within a single 3D-SIM field of view, effectively impeding our rotational polarity analysis of basal body orientation. Another issue that arose was that CVM substrates introduce optical aberrations into primary cultures. Specifically, CVM was a source of imaging background noise that obstructed the resolution of BB staining by γ-tubulin or POC1B staining (Fig. 25B). This challenge was compounded by cross-channel fluorescent leaking from directly-labeled primary antibodies for POC1B. Our results indicated that although primary cultures are populated by ciliated cells (as detected by laser scanning confocal), we are not currently successful with using super-resolution microscopy to quantify cilia orientation on flat versus grooved CVM substrates at ALI D21. We conclude that future work will be required to optimize culture conditions to produce airway epithelia densely populated with MCCs and robust imaging methods to determine rotational and tissue-level ciliary polarity.
Figure 24. Ciliation of polarized HTECs on CVM. Confocal image of cells stained with acetylated α-tubulin (red) on (A) transwell inserts, (B) flat CVM, and (C) 5 μm x 5 μm x 5 μm microgrooved CVM at ALI Day 21. 3D-SIM image of ciliated cells stained with centriolin (CNTRL; green), γ-tubulin (γ-Tub; red) and poly-glutamylated tubulin (poly-glutamylated Tub; white) on (D) transwell inserts, (B) flat CVM, and (C) microgrooved CVM at ALI D21. Scale bars: 10 µm.
Figure 25. CVM produces optical aberrations in 3D-SIM. 3D-SIM image of single ciliated cell cultured on (A) transwell insert or (B) CVM stained with centriolin (CNTRL; green) and γ-tubulin (γ-Tub; red) or POC1B (red). Scale bars: 10 µm.
4.5 **Discussion**

Here, we present an *in vitro* culture platform to understand alignment responses in multiciliated airway epithelium. We set out to customize our platform for characterizing the effects of substrate topography on epithelial cell organization during ciliogenesis to determine if planar polarization at early timepoints of ALI culture is sufficient to direct the organization of cilia that appear at later stages of culture. Specifically, we exposed primary HTECs to a variety of groove topographies and identified the groove dimensions that produced robust cell alignment in normal culture. We found that HTECs cultured on grooved CVM hydrogels displayed an alignment response under ALI conditions that was maintained in ALI until D14 but lost by D21. Furthermore, HTECs were able to differentiate into MCCs on both flat and grooved CVM substrates by day 21 of ALI culture. Although our topography strategy was able to present a planar polarization cue that was compatible with apical-basal polarization and ciliation, attempts to visualize and quantify ciliary orientation on flat versus grooved CVM at ALI D21 using super-resolution microscopy were not successful. Future work will be directed to optimize the conditions for primary cultures to produce epithelia with dense carpets of ciliated cells and to optimize the 3D-SIM method to enable us to determine if initial planar polarity is sufficient to direct ciliary alignment.

We found that alignment and morphological planar polarization of confluent HTECs induced by exposure to grooved topography occurs prior to and throughout a significant time window of apical-basal polarization. The loss of cell alignment in ALI is not due to cell-cell interactions or dense cell packing overriding topography cues as confluent sheets of HTECs remain aligned until at least ALI D14. It more likely reflects morphological changes of HTECs towards terminally-differentiated, columnar cell types. This is consistent with the observed decrease in cell elongation at ALI D21 (Fig. 23G). The establishment of a tissue-wide signal during
development remains poorly understood [70]. It is known that airway epithelium is molecularly planar polarized \textit{in vivo} and that this polarization precedes the emergence of cilia [69, 70]. This early polarization mechanism likely influences BB docking, as BBs have been shown to have a polarity bias towards the proximal (oral) direction immediately after docking [69]. Furthermore, cilia in multilicated epithelia refine their rotational and tissue-level polarity throughout development and this refinement is known to require molecular planar polarization [69, 87, 304]. The timing of our topography-induced morphological planar polarization is consistent with the timing of molecular and ciliary polarization in airway epithelium. These observations suggest that grooved topography could provide the initial guidance cue that orients cilia along a global tissue axis by influencing initial cilium orientation and later cilium refinement. Additional experiments will be necessary to determine the contribution of topographical cues to the coordination of molecular planar polarity.

Current \textit{in vitro} culture systems for human epithelial progenitors produce disorganized proximal airway epithelium. Topography is known to direct organization and migration of many cell types, including epithelial cells [16, 268, 303, 305]. We tested several hydrogel substrates for compatibility with primary epithelial cell culture prior to choosing CVM. We found that gelatin inserts were not suitable for HTEC culture. Gelatin is a denatured form of collagen derived by hydrolysis and the hydrolytic reduction of protein fibrils into smaller peptides could impair the ability of HTECs to bind and spread on gelatin substrates. We speculate that the presence of biological proteins in the CVM film are intact and form fibrils which could allow HTECs to better adhere to the substrate. One alternative explanation could be that that gelatin gels were not in the correct stiffness range. ECM materials that are too soft result in unstable, dynamic focal adhesions and low cell densities post-seeding [236], which may be the case for gelatin. CVM is
thin (~10 µm) and cells are able to sense deeply (10-20 µm) into underlying rigid substrates (e.g. polyester transwell membranes) that are not in direct cellular contact [306]. Another possibility could be that HTECs are extremely sensitive to toxic insults, such as the presence of glutaraldehyde required to crosslink gelatin.

We found that HTECs were able to terminally differentiate into MCCs on flat and grooved CVM substrates after 21 days of ALI culture (Fig. 24A-C). However, using super-resolution 3D-SIM, we were unable to find regions of epithelium that were densely populated by ciliated cells. A large population of representative MCCs is required within a single 3D-SIM image in order to perform the cilia rotational polarity analysis necessary to describe the orientation of cilia across a tissue. MCCs comprise 50-80% of all epithelial cells in the large airway epithelium [307-310] and it is unclear whether the low ciliation is an inherent biological response to in vitro culture. Primary culture methods will need to be optimized in the future for highly ciliated epithelium. Additionally, proper corrections for optical aberrations and antibody labeling are essential and will be necessary to remove background noise that complicates assessment of basal body orientation. Future work with optimized 3D-SIM measurements will enable us to determine whether alignment in submerged culture and early ALI can be translated to basal body alignment in later ALI culture.

4.6 Conclusions

We have adapted an in vitro platform for the primary culture of human airway epithelial cells and demonstrated that topographic cues can guide epithelial organization in transwell ALI culture. Our grooved hydrogel system was able to support cell growth and induced robust cell alignment in confluent epithelial monolayers. HTECs remained aligned for up to 2 weeks under ALI conditions, in contrast to cells grown on flat substrates. Our grooved culture system is
however limited as we were unable to determine the cilium rotational polarity due to low percentages of MCCs within the epithelium and nanometer-scale optical aberrations. We concluded that this approach required further optimization and future work to assess the impact of early morphological planar polarization on the organization of ciliated airway epithelium. This system enables the study of novel biological questions such as how grooved topography influences tissue organization and maturation, which can be used to inform the design of tissue-engineered airway replacements.
Chapter 5

Design of Biomimetic Substrates for Long-term Maintenance of Alveolar Epithelial Cells

5.1 Abstract

There is a need to establish in vitro lung alveolar epithelial culture models to better understand the fundamental biological mechanisms that drive lung diseases. While primary alveolar epithelial cells (AEC) are a useful option to study mature lung biology, they have limited utility in vitro. Cells that survive demonstrate limited proliferative capacity and loss of phenotype over the first 3-5 days in traditional culture conditions. To address this limitation, we generated a novel physiologically relevant cell culture system for enhanced viability and maintenance of phenotype. Here we describe a method utilizing e-beam lithography, reactive ion etching, and replica molding to generate poly-dimethylsiloxane substrates containing hemispherical cavities that mimic the architecture and size of mouse and human alveoli. Primary AECs grown on these cavity-containing substrates form a monolayer that conforms to the substrate enabling precise control over cell sheet architecture. AECs grown in cavity culture conditions remain viable and maintain their phenotype over one week. Specifically, cells grown on substrates consisting of 50 μm diameter cavities remained 96 ± 4% viable and maintained expression of surfactant protein C, a marker of type 2 AEC, over 7 days. While this report focuses on primary lung alveolar epithelial cells, our culture platform is potentially relevant and useful for growing primary cells from other tissues with similar cavity-like architecture and could be easily adapted to other biomimetic shapes or contours.
5.2 Introduction

There is a need to establish *in vitro* alveolar culture models to better understand the fundamental biological mechanisms that drive lung diseases. *In vivo*, the lung alveolus is an approximately 200 μm (in humans) or 50 μm (in mouse) spherical structure lined with alveolar epithelial cells (AEC) consisting of type 1 and type 2 AEC (AT1 and AT2) [22-25]. AT2 cells are cuboidal and often found in the mouth of the alveoli where neighbouring alveoli meet [129]. These cells produce and secrete pulmonary surfactant, which lowers the alveolar surface tension and contributes to host defense [36, 130, 131]. AT1 cells are flat or squamous and cover ~95% of the surface area of the lung [119, 311]. They play a primary role in gas exchange and regulation of fluid homoeostasis. An ideal model of the distal lung would contain both cell types in the appropriate proportions while in the right architecture.

Current AEC source options for building tissue-engineered lung models are i) cell lines, which are often derived from lung tumours and do not recapitulate healthy lung biology [154-156], ii) primary cells isolated from lung tissue: these often de-differentiate and only limited cell numbers can be generated [10, 11, 157, 312-314], and iii) stem cells, but these are often immature and require complex differentiation protocols [315-318]. Of these options, primary AEC cells enable the study of mature lung regenerative cell biology but generating models using this cell source is problematic because cells have limited proliferation after isolation [319] and extensive cell death (60-70%) occurs over the first few days in culture [157]. This therefore limits the number of cells available for assembling engineered lung models. Furthermore, the cells that do remain viable are not representative of the *in vivo* situation because the numbers of different types of AECs change over time in culture: specifically the percentage of AT2 cells decreases and cultures become enriched in AT1-like cells [11, 312]. It is generally thought that the AT2 cells lose their phenotype and differentiate to AT1 cells.
Traditionally, AECs have been grown on 2D plastic surfaces [320] or polycarbonate filter inserts [321] and, more recently, modeled in 3D spheroid systems [322]. In 2D culture, coatings such as Matrigel [323], a mixture of Matrigel and rat tail collagen [10, 323] and fibronectin [324] have been used to better maintain the surfactant levels within the culture, and hence the number of AT2 cells is thought to be better maintained. However, these studies did not directly assess the maintenance of cell viability using these coatings. Similar results have also been obtained in 2D cultures by supplementing the media with chemical factors such as keratinocyte growth factor (KGF) [319, 323] and rat serum [324]. In 3D culture, cells have been grown in bead suspensions [325, 326], in lung bud organoids embedded within Matrigel [327, 328], and cell suspensions embedded in type I collagen [295], Matrigel [329] and poly-ethylene glycol (PEG) [322], in which they form cystic structures with an average diameter ranging from 50 to 190 μm. While these systems improved the maintenance of cell viability, the ratio of different types of AEC was not maintained: specifically the AECs differentiated to AT1-like cells that express markers such as T1α and aquaporin-5 (AQP5) [322]. These models are also not easily scalable, and the dimensions of the 3D cysts are often heterogeneous or not within the relevant physiological range of the alveolar chamber in mouse or rat lung. Furthermore these 3D systems are challenging to analyze because they require sectioning or the use of staining and imaging methods that can penetrate within the 3D aggregate, which are generally low-throughput techniques.

We set out to develop a culture platform that captures the advantages of 3D culture for maintaining cell viability as reported [322], but that also enables precise control of 3D architecture and easy analysis of cellular phenotype. Here we describe a biomimetic strategy that enables prolonged (≥ 1 week) maintenance of primary AEC in culture. Specifically we generate
elastomeric substrates containing arrays of hemispherical cavities using electron beam lithography and deep reactive ion etching combined with soft-lithography. The dimensions of these cavities were designed to mimic the physiological dimensions of an adult mouse alveolar cavity (50 μm) or an adult human alveolar cavity (200 μm). We show that our substrate manufacturing method produces a highly reproducible architecture that can generate organized epithelial monolayer cultures in a physiologically relevant architecture. The cultures are easy to stain and analyze due to the accessibility of the cells in the hemispherical configuration and the predictability of the array. We can maintain cell viability when cells are grown in cavities of 50 μm (the size of mouse alveolar cavity) or 200 μm (human alveolar cavity). Furthermore, we found that the 50 μm cavities maintain stable expression levels for markers of AT1 and AT2 cells within the culture.

5.3 Methods

5.3.1 Fabrication of hemispherical architecture masters

Silicon substrates with an 800 nm thermal oxide (SiO\(_2\)) top layer were obtained from the Toronto Nano Fabrication Center (TNFC). A poly(methyl methacrylate) (PMMA) photoresist (MicroChem Corp, Westborough, MA, USA) was spin-coated onto the silicon substrate and soft-baked at 180°C for 3 min, resulting in deposition of a 200 nm thickness photoresist layer. A lithography mask in the form of a .GDS file was generated using Layout Editor® containing an array of filled circles. Dimensions of the circles were designed using a method described previously [330] to achieve the target cavity size. For example, if we aimed to etch hemispheres of 200 μm diameter in the silicon substrate eventually, the diameter of the filled circles should be 200/2.4 = 83 μm [330]. Patterns described in the GDS files were exposed and written onto the PMMA using an electron beam lithography (EBL) system (Vistec EBPG 5000+) at a 1000 μC/cm\(^2\) dose and a 15 nA current. After the exposure, the sample was developed in 1:3 methyl
isobutyl ketone:isopropyl alcohol (MIBK:IPA, Fisher Scientific, Canada) developer for 90 sec, rinsed in IPA for 30 sec and blow dried using nitrogen gas. During the development, the areas that were exposed to electrons were dissolved and circular holes were created in the PMMA layer. Next, using the PMMA as a mask, buffered oxide etcher (BOE) (Fisher Scientific, Mississauga, ON, Canada) was used to etch through the 800 nm SiO$_2$ layer. BOE etches SiO$_2$ at a 50 nm/min rate but cannot etch silicon or PMMA. After etching, the PMMA layer was removed from the sample using acetone (Fisher Scientific) and IPA. At this point in the process circular holes in the PMMA layer were successfully transferred onto the 800 nm SiO$_2$ layer on the wafer.

The sample was then loaded into a deep-reactive-ion-etching (DRIE) (Oxford Instruments PlasmaPro Estrelas100 DRIE System) tool for isotropic etching using a dry recipe with the following parameters: 10 mTorr chamber pressure, 2000 W ICP power, 300 sccm SF$_6$ gas flow, and 25°C temperature. Plasma was generated in the DRIE tool and the etching of silicon substrate proceeded isotropically [330]. Ensuring the correct diameter of the circular holes in the SiO$_2$ layer was important in ensuring that almost equal etch rates were achieved horizontally and vertically. We optimized the etch rate of silicon in this recipe. For example, the sample was etched for about 23 min in the plasma in order to create 200 μm diameter hemispheres. The 800 nm SiO$_2$ layer was thick and rigid enough to act as a hardmask during this prolonged etching. After DRIE etching, any residual SiO$_2$ was completely removed using BOE. This produced a silicon substrate with hemisphere cavities for molding culture substrates in the next step (see below).

5.3.2 Manufacture of cavity culture substrates

Silicon masters manufactured as described above were silanized with (tridecafluoro-1,1,2,2-Tetrahydro Octyl 1)-trichlorosilane to render them non-adherent and then coated with a
1:10 mix of polydimethylsiloxane (PDMS) crosslinker:elastomer (Dow Corning Corporation, Midland, MI, USA) to create masters for replica molding. Immediately after pouring the PDMS solution onto the silicon master, samples were placed in vacuum for 20 min to remove any air bubbles and then cured overnight at 60°C. After curing, the PDMS layer was peeled from the Si wafer to generate a surface containing hemispherical features. This PDMS master with inverse cavity geometry was then plasma-treated and silanized before further replica molding to generate PDMS substrates with the desired cavity geometry. PDMS substrates were then sterilized with 70% ethanol, coated with 50 μg/mL rat tail collagen 1 (354236, Corning, NY, USA) according to the manufacturer’s instructions, and rinsed with sterile phosphate buffered saline (PBS; Lonza, Mississauga, Ontario, Canada) prior to seeding cells for culture.

5.3.3 **Hemisphere geometry characterization**

The architecture of the PDMS substrates was visualized using scanning electron microscopy (SEM) and brightfield microscopy. Samples for SEM were sputter coated with a gold-palladium amalgam (Edwards Coating System E306A, Tewksbury, MA, USA) and imaged using a Hitachi S-4500 (Hitachi High-Technologies Canada, Inc., Toronto, ON) at 10 kV. Transverse sections to assess cavity geometry were obtained by sectioning the substrate with a scalpel and mounting at a 90° angle during SEM imaging. Cavity diameters were measured manually from brightfield images (see below for microscopy details) using ImageJ.

5.3.4 **Isolation of mouse alveolar epithelial cells**

C57BL/6 mice (≥ 8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Animal procedures were all approved by the University Health Network Animal Care Committee. Mice received care in compliance with the Principles of Laboratory Animal Care Committee.
Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care.

Cell suspensions were prepared from lungs isolated from at least 3 mice as described previously [331, 332] with some minor modifications. Briefly, mice were euthanized with an overdose of CO₂. The abdominal cavity was opened, and the diaphragm, chest plate, thymus and abdominal aorta were removed. With the use of a 27G needle, the lungs were perfused with 5 mL of PBS via the right ventricle to wash out excess blood. The trachea was isolated and cannulated with a 20-gauge iv catheter. 2 mL Dispase II solution (final concentration 2.0 U/mL; 04942078011, Roche Applied Science, Germany) was instilled through the cannula into the trachea. The lung-heart block was removed and transferred to a 50 mL tube containing 8 mL Dispase II solution. Subsequently, the lung-heart block was incubated in a 37°C shaking incubator for 30 min in 8 mL of Dispase II. The trachea and bronchi were removed, and the lungs were transferred to a new 50 mL tube containing 8 mL Dispase II solution, 1 mL Collagenase/Dispase (final concentration 1 mg/mL; 10269638001, Roche Applied Science) and 1 mL DNase I (0.1 mg/mL; D4527, >2000 U/mg , Sigma Aldrich, Mississauga, ON, Canada). The lungs were thoroughly minced and incubated for 15 min in the digestion solution. 5 mL of DMEM containing 10% FBS and 1% Penicillin/Streptomycin (“complete DMEM”; 12430-054, Invitrogen, Grand Island, NY, USA) were then added to the digestion solution. The minced lung tissue was passed through an 18G needle at least 5 times and the suspension was subsequently filtered through 100 µm and 40 µm cell strainers (352360, BD BioSciences, San Jose, CA, USA). The filtered cell suspension was centrifuged and depleted of red blood cells by incubation in 2 mL RBC lysis buffer (Sigma Aldrich). The cells were then centrifuged and resuspended in complete DMEM at 1 x 10⁶ cells/100 µL and were then incubated with MACS Mouse CD45 MicroBeads (130-052-301, Miltenyi Biotec, Germany) for 15 min at 4°C. Cells were then
washed before proceeding to magnetic separation using the AutoMACS cell separator system (Miltenyi Biotec) to deplete CD45\(^+\) cells according to the manufacturer’s instructions. The collected CD45\(^-\) cells were then positively selected for EpCAM (a marker specific for epithelial cells) by incubation with MACS Mouse CD326 (EpCAM) MicroBeads (130-105-958, Miltenyi Biotec) for 15 min at 4°C. After incubation and washing, the cells were separated magnetically by AutoMACS to collect the CD45\(^-\)EpCAM\(^+\) cells.

The collected cells were plated on coverslips and assessed by immunostaining for EpCAM, pro-surfactant protein C (SPC), AQP5, and vimentin (data not shown). 96.4% of the cells were positive for EpCAM, 63.7% of the cells were positive for SPC, and 50.8% of the cells were positive for AQP5. Vimentin was not detected, confirming that we had obtained an epithelial population.

5.3.5 Cell culture

The human epithelial cell line A549 (lung epithelium) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco’s modified Eagle Medium [DMEM]/F12 (Invitrogen) containing 10% fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, Ontario, Canada), and 1 \(\mu\)g/ml penicillin and streptomycin (Sigma Aldrich). For cell density experiments A549 cells were seeded onto flat plastic at densities ranging from 6 \(\times\) 10\(^4\) cells/cm\(^2\) to 1 \(\times\) 10\(^6\) cells/cm\(^2\). To seed A549 cells onto cavity substrates we seeded cells in highly concentrated (2 \(\times\) 10\(^7\) cells/mL) 1-2 \(\mu\)L droplets onto our cavity substrates. Cells were allowed to adhere for 4 h, after which the culture wells were supplemented with additional DMEM/F12.

For primary mouse alveolar cells, isolated CD45\(^-\)EpCAM\(^+\) cells were plated on flat PDMS substrates with a small airway growth media (SAGM) bullet kit (Lonza) and maintained
in a humidified 5% CO₂ incubator at 37°C. The media was changed every other day and cells were cultured until day 7 when cells were analyzed. Similarly to the cell line cavity seeding, cultures with confluent monolayers lining the hemispherical cavities were generated by seeded cells in highly concentrated (2 x 10⁷ cells/mL) 1-2 µL droplets onto our cavity substrates. Cells were allowed to adhere for 4 h, after which the culture wells were supplemented with additional SAGM.

5.3.6 Immunocytochemistry and microscopy

Samples were fixed with 4% paraformaldehyde (28908, Pierce, Rockford, IL, USA) for 10 min, washed with PBS, then permeabilized with 0.1% Triton-100 (Sigma Aldrich) for 20 min at room temperature. Samples were washed with PBS and incubated in 5% FBS (PAA Laboratories Inc.) for 30 min at room temperature to block nonspecific binding. Samples were incubated overnight at 4°C with the following primary antibodies: mouse anti-mouse EpCAM (1:100, MA5-12436, Pierce), rabbit anti-mouse pro-surfactant protein C (1:1000, AB3786, EMD Millipore, Etobicoke, ON, Canada), rabbit anti-mouse aquaporin-5 (1:200, ab78486, Abcam, Cambridge, MA, USA), and mouse anti-mouse vimentin (1:1000, ab8978, Abcam). Samples were washed with PBS, and incubated with Alexa Fluor 488 goat anti-rabbit (1:500, A11034, Thermo Fisher, Burlington, ON, Canada) and Alexa Fluor 546 goat anti-mouse (1:500, A11003, Thermo Fisher) secondary antibodies at room temperature for 2 h. Nuclei were stained with Hoechst 33258 (2 µg/mL; 861405, Sigma Aldrich Canada), and F-actin with FITC-conjugated phalloidin (2 µg/mL; P5282, Sigma Aldrich). Samples were then mounted with Fluorescence Mounting Medium (Dako, Glostrup, Denmark) for imaging. Samples with no primary antibody but with a secondary antibody were used as negative controls.
Fluorescence microscopy was performed with a 10x/0.3 UplanFI or 40x/0.8 LUMPlanFI dipping objective, using an Olympus IX-81 inverted confocal microscope. Images represent either individual confocal sections or projections of sections that were chosen from Z-stacks (1-2 µm intervals), which were routinely acquired for all studied cavity cultures. Image processing and analysis, cell counts and cell phenotype measurements were done by evaluating Z-stacks, using the FV10-ASW viewer (Olympus) and ImageJ software (NIH).

5.3.7 Quantification of nuclear area coverage, cell viability and phenotype

Nuclear coverage was used to assess maintenance of cell adhesion to the substrate. Cell coverage was used as a surrogate indicator of cell viability as only viable cells remained adherent to the substrate. To confirm this cell viability was also assessed. This also prevented inaccurate assessment due to the presence of cells in the process of dying that also remained adherent to the substrate. To assess nuclear coverage maximum intensity projections of confocal Z-stacks in the nuclear channel were thresholded using ImageJ. Total area of the nuclei was then divided by the area of the image to obtain nuclear area coverage. The nuclear area coverage at day 7 was then normalized to the day 1 area coverage for the same condition (i.e. flat or cavity culture) and this value was reported. Live and dead status of the cells was assessed by staining with calcein AM and ethidium homodimer-1 from a LIVE/DEAD kit (L3224, Molecular Probes, USA) and labelled all cells using Hoescht 33258 (2 µg/mL; P5282, Sigma Aldrich). Confocal Z-stack images were analyzed using ImageJ. The percentages of live and dead cells were determined by manual counting of the corresponding stain, and dividing by all nuclei present in the image. To assess cell phenotype we obtained confocal Z-stack images and assessed for SPC and AQP5 positivity using ImageJ. Specifically, the percentage of cells expressing SPC or AQP5 was determined by manual counting and dividing by all nuclei present in the image.
5.3.8 Statistical analysis

Unpaired, two-tailed Student’s t-tests or one-way ANOVA followed by Tukey’s (to compare among groups) or Dunnett’s (to compare each group to a control group) post hoc tests were performed as appropriate. For all tests, \( p \)-values < 0.05 were considered significant. Prism 5 (GraphPad Software) was used for statistical tests and for the generation of graphs.

5.4 Results and Discussion

5.4.1 Characterization of biomimetic substrate manufacturing strategy

We set out to generate a culture platform to maintain the cells in a biomimetic architecture that mimicked the geometry of the adult mouse and human lung alveolus. Specifically we designed culture substrates containing arrays of hemispherical cavities with diameters of 50 or 200 μm. To do this we generated silicon masters using DRIE and then generated culture substrates from PDMS using replica molding [333, 334] (Figure 26). Specifically we produced a 2D array of access holes in a 800 thick layer of SiO\(_2\) on a silicon substrate by first coating the wafer with PMMA photoresist. EBL was then used to generate an array of circular holes in the PMMA layer. The array pattern was then transferred to the SiO\(_2\) layer using buffered oxide etcher with the PMMA acting as an etch mask. Excess PMMA was then washed away and DRIE was used to isotropically etch hemispherical cavities in the silicon substrate with SiO\(_2\) acting as a hard mask. We optimized the diameter of the circular holes in the SiO\(_2\) layer to ensure equal etch rates in the horizontal and vertical directions. We also designed the substrates to contain dense arrays of cavities to maximize the culture area occupied by
cavities versus flat. In order to achieve this, we optimized the isotropic etching in DRIE to the diameter of the desired cavity diameters and the spacing of the circular holes in the SiO$_2$ layer.

We then used replica molding of the silicon wafer to generate PDMS masters from which we then further replica molded the actual PDMS cell culture substrates (Figure 26). In our initial design we used a close-packing organization of the cavities, however we found that we did not obtain accurate replica molding (SI Figure 3A-B) of the cavities due to limited infiltration of the PDMS into

**Figure 26. Biomimetic substrate manufacturing strategy.** Schematic of fabrication method using electron beam lithography and deep reactive ion etching. A silicon substrate with 800 nm thick layer of thermal oxide (SiO$_2$) was spin-coated and soft-baked to add a thin layer of PMMA photoresist. An array of circular holes was written onto the PMMA with electron beam lithography and developed to remove areas that were exposed to electrons. Using PMMA as a mask, buffered oxide etcher (BOE) was used to etch the circular holes into the SiO$_2$ layer. PMMA was then removed and the sample was then isotropically etched using a DRIE tool to produce hemispherical architecture in the silicon substrate. Residual SiO$_2$ was removed with BOE, and PDMS cell culture substrates were generated by traditional soft lithography and replica molding.
the inter-cavity wedges. We therefore re-designed the silicon master to contain arrays of cavities with a 5 µm spacing to enable better replication of the cavity features (SI Figure 3C-D). We also found that plasma cleaning the PDMS master before replica molding the PDMS culture substrate was critical to enable robust separation of the PDMS layers.

Using this fabrication approach we created dense assays of 50 and 200 µm cavities with uniform dimensions (Figure 27A and B). The DRIE approach successfully generated curved cavities with a hemispherical geometry (Figure 27C and D). We assessed variation in cavity dimensions both within an array of cavities, and between different substrate batches manufactured using replica molding from the same master, and found minimal differences in cavity diameter (Figure 27E and F): specifically within a 50 µm diameter array the standard deviation of the diameter was 1.33 µm and no significant difference was observed between mean cavity diameter between 3 independent substrate batches (one-way ANOVA + Tukey test, p = 0.263). Within a 200 µm diameter array the standard deviation of the diameter was 3.68 µm and there was no significant difference in mean cavity diameter between 3 independent substrate batches (one-way ANOVA + Tukey test, p = 0.2324). We therefore concluded that our manufacturing method was sufficient to create reproducible culture surfaces containing the desired biomimetic architecture.

5.4.2 **Optimization of seeding strategy to produce cavities with primary monolayers**

We next set out to establish a seeding protocol to generate a confluent monolayer of cells lining the cavity surface. To do this we first optimized seeding with the lung cell line A549 by seeding flat plastic substrates with different densities of cell suspension ranging from $6 \times 10^4$ cells/cm$^2$ to $1 \times 10^6$ cells/cm$^2$. We found that at seeding density of $1 \times 10^5$ cells/cm$^2$ produced cultures with confluent monolayers of A549 cells after 24 h. We used this optimized seeding
Figure 27. Characterization of substrate cavities. (A) DIC image of molded elastomeric substrate containing 50 μm diameter cavities. (B) DIC image of molded elastomeric substrate containing 200 μm diameter cavities. (C) SEM image of 50 μm diameter cavity cross-section. (D) SEM image of 200 μm diameter cavity cross-section. (E) Boxplot of cavity diameters for different batches of substrates for 50 μm cavity substrates. (F) Boxplot of cavity diameters for different batches of substrates for 200 μm cavity substrates. In E and F the central bar indicates the population median and the outer lines of the box indicate the first and third quartile respectively (n= 30 per independent substrate batch).

density to seed our cavity substrates (SI Figure 4A and 4D). We found that this seeding density deposited a uniform number of cells in each cavity (SI Figure 4H): the standard deviation from the mean was 14% and 13.5% for 50 and 200 μm cavities, respectively. Furthermore, the number
of A549 (human) cells occupying one 200 μm cavity (the approximate size of a human alveolus) was 126 ± 17, which is similar to the number of reported epithelial cells in an adult human alveolus (117 cells) [119]. Within the cavity the cells self-organized into a monolayer (SI Figure 4C and 4F) in the 200 μm cavities but completely filled the 50 μm cavities. We speculate that this was due to the large size of the human cells relative to the 50 μm cavities. Taken together, our data showed a robust seeding method resulting in cultures with confluent monolayers of cells lining the cavity surface.

We next seeded different densities of primary mouse alveolar cells onto our substrates. Obtaining large numbers of these cells is challenging, therefore we seeded the cells in very concentrated (4 x 10^6, 20 x 10^6, or 40 x 10^6 cells/mL) droplets over a small surface area of the substrate. We found that seeding with droplet densities of 20 x 10^6 cells/mL produced confluent cultures of primary cells after 24 hours (Figure 28A and E). Primary cells formed a monolayer lining the curved cavities at both sizes (Figure 28C and G). The number of cells per cavity was 22 ± 5 and 383 ± 112 cells for 50 and 200 μm diameters, respectively. The number of primary cells occupying one 50 μm cavity is similar to the total number of epithelial cells reported for a mouse alveolus (26 cells) [335]. We assessed variation in cell seeding both within an array of cavities, and between different biological replicates, and found the variation was consistent (Figure 28D and H): specifically within a 50 μm diameter array the standard deviation of cells per cavity was 5 cells and no significant difference was observed between mean number of cells per cavity between 3 independent biological replicates (one-way ANOVA + Tukey test, p = 0.5892). Within a 200 μm diameter array the standard deviation of cells per cavity was 112 cells and there was also no significant difference in mean number of cells per cavity between 3 independent biological replicates (one-way ANOVA + Tukey test, p = 0.8206). Since the mean
number of cells per cavity and the variation in cell number between cavities was consistent between biological replicates we concluded that our droplet seeding protocol for primary cells was a useful approach to generate confluent and reproducible monolayers within the cavity substrates.

Figure 28. Optimization of primary cell seeding to produce monolayers within the cavities. (A) DIC image of primary mouse AECs cultured in 50 µm diameter cavities. (B) Corresponding confocal microscopy image of (A) with nuclei stained with Hoescht (blue). (C) Corresponding confocal side profile of (A). Cavities are outlined in white. (D) Boxplot of number of cells per cavity for cultures from different biological replicates for 50 µm cavity substrates (n = 15 per biological replicate). (E) DIC image of primary mouse AECs cultured in 200 µm diameter cavities. (F) Corresponding confocal microscopy image of (D) with nuclei stained with Hoescht (blue). (G) Corresponding confocal side profile of (D). Cavity is outlined in white. (H) Boxplot of number of cells per cavity for cultures from different biological replicates for 200 µm cavity substrates (n = 10 per biological replicate).
5.4.3 Cell viability is maintained in cavity culture

We next wanted to assess the ability of the substrates to maintain cell viability compared to standard 2D cultures. To do this we assessed both the number of cells at day 1 versus 7 using a nuclear stain and the viability of the cells at day 7 using a live-dead stain. Using image analysis software we quantified the number of adherent cells by measuring the area of the substrate covered by nuclei. We observed primary mouse AECs lift off flat PDMS substrates over time in culture (Figure 29A) and that the area covered by nuclei at day 7 relative to day 1 was 0.59 ± 0.18 (Figure 29A and 29D), which is statistically lower than 1 (the expected ratio of cells if they remain completely attached). In contrast, no reduction in nuclear area coverage was observed for AECs cultured in 50 µm cavities: the area covered by nuclei at day 7 relative to day 1 was 1.11 ± 0.25, which was not statistically significantly different from 1 (Figure 29B and 29D) suggesting that there was no cell loss on 50 µm cavity substrates. Area coverage at day 7 by AECs cultured in 200 µm cavities was 0.74 ± 0.20, which was also not statistically significantly different from 1 (Figure 29C and 29D). Furthermore, adherent AECs all showed a positive viable signal with more viable AECs in 50 µm cavities compared to flat culture (one-way ANOVA + Tukey test, p < 0.0001, Figure 29E-G). Specifically, in 50 µm and 200 µm cavities adherent cell survival was high with 96 ± 4% and 89 ± 12% of adherent cells staining positive for the live marker after 7 days of culture respectively. In contrast the viability of cells grown on flat substrates was low with only 2 ± 1% cells staining positive for the live marker after 7 days culture. Many cells stained positive for both live and dead dyes suggesting these cells were in the process of dying and hence were still adherent to the substrate. These results suggest that the 50 µm cavities preserve the adhesion and viability of primary mouse AECs up to 7 days, in contrast to flat culture. The 200 µm cavities on the other hand showed a slightly reduced nuclear coverage and viability compared to 50 µm cavities at day 7, but neither were statistically significant from
Figure 29. Assessment of cell viability maintenance on flat versus cavity substrates. (A) Confocal image of nuclei stained with Hoescht for primary mouse AECs cultured on flat substrates at day 1 and day 7. (B) Confocal image of nuclei stained with Hoescht for primary mouse AECs cultured in 50 µm diameter cavities substrates at day 1 and day 7. (C) Confocal image of nuclei stained with Hoescht for primary mouse AECs cultured in 200 µm diameter cavities substrates at day 1 and day 7. (D) Graph comparing substrate coverage by nuclei at day 7 normalized to day 1 for flat and cavity cultures. Errors are standard error of the mean. * indicates p<0.05. (n= 4). (E–G) LIVE/DEAD stain of cells on flat or cavity cultures. The mean percentage of adherent cells ± st. dev. that were viable is indicated at the bottom of each image.
Figure 30. Assessment of AT2 phenotype on flat versus cavity substrates. (A) Confocal image of cells stained with Hoescht for nuclei (blue) and SPC (green) on flat substrates at day 1 and 7. (B) Confocal image of cells stained with Hoescht for nuclei (blue) and SPC (green) on substrates containing 50 µm diameter cavities at day 1 and 7. Corresponding confocal side profiles underneath. (C) Confocal image of cells stained with Hoescht for nuclei (blue) and SPC (green) on substrates containing 200 µm diameter cavities at day 1 and 7. Corresponding confocal side profiles underneath. (D) Quantification of SPC positive cells as a percentage of total cells in the culture as a function of time for flat and cavity cultures. Error bars are standard error of the mean and * indicates p<0.05. (n= 4).

values at day 1. We speculate that perhaps the benefit of the 200 µm cavity is more limited than in the 50 µm cavity case because the cavity is too large for mouse cells, which occupy a 50 µm alveolus in vivo.

We therefore concluded that our biomimetic culture model, specifically with 50 µm cavity
dimensions improves the maintenance of primary mouse AEC in culture.

5.4.4 Cavity culture maintains SPC and AQP5 expression over 7 days

We next wanted to assess the phenotype of the AECs in cavity culture compared to standard 2D cultures. To do this we evaluated the expression of SPC, which is a standard AT2 epithelial cell marker and AQP5, a standard AT1 epithelial cell marker, using immunocytochemistry and quantified the number of cells positive for SPC and AQP5. We observed that, as expected, the percentage of SPC-positive cells decreased over time in flat PDMS culture (Figure 30A and 30D). Specifically, a starting population of 63.7% ± 26.8% SPC-positive cells at day 0 decreased to 24.5% ± 16.7% SPC-positive cells by day 7. Consistent with changes in the SPC-positive population, the percentage of AQP5-positive cells increased over time in flat PDMS culture (Figure 31A and 31D). Specifically, a starting population of 50.8% ± 17.4% AQP5-positive cells at day 0 increased to 76.9% ± 18.3% AQP5-positive cells by day 7. In contrast, AECs cultured in 50 μm cavities maintained their levels of SPC-positive cells for up to 7 days (63% ± 22.5%, Figure 30B and 30D) and maintained stable levels of AQP5-positive cells for up to 7 days (61.0% ± 25.4%, Figure 31B and 31D). Preservation of the SPC-positive population was not observed for AECs cultured in 200 μm cavities however (30% ± 17.6%, Figure 30C and 30D). The AQP5-positive cell population however remained stable and was not statistically significantly different from the day 0 population in 200 μm cavities (46.4% ± 18.8%, Figure 31C and 31D). Again we speculate that the less effective maintenance of AT1 and AT2 type cells in the 200 μm cavity culture arises because 50 μm cavities better mimic the mouse alveolus compared to 200 μm cavities or flat culture.
Figure 31. Assessment of AT1 phenotype on flat versus cavity substrates. (A) Confocal image of cells stained with Hoescht for nuclei (blue) and AQP5 (green) on flat substrates at day 1 and 7. (B) Confocal image of cells stained with Hoescht for nuclei (blue) and AQP5 (green) on substrates containing 50 µm diameter cavities at day 1 and 7. Corresponding confocal side profiles underneath. (C) Confocal image of cells stained with Hoescht for nuclei (blue) and AQP5 (green) on substrates containing 200 µm diameter cavities at day 1 and 7. Corresponding confocal side profiles underneath. (D) Quantification of AQP5 positive cells as a percentage of total cells in the culture as a function of time for flat and cavity cultures. Error bars are standard error of the mean and * indicates p<0.05. (n= 4).

Based on these results we conclude that our biomimetic culture model containing 50 µm cavities maintains a stable balance of AT1 and AT2 cells in culture. A number of mechanisms could explain the benefit of cavity culture on SPC maintenance, including sequestering of signalling molecules within the
confined space of the cavity [234], or mechanical signals due to the stresses on the cellular monolayer during conformation to the topography of the cavity [21, 281]. These effects could explain how cavity culture prevents differentiation of AT2 to AT1-like cells and inhibits the expression of AT1 markers such as AQP5. Future work will focus on understanding which of these cellular mechanisms play a role in the effects we observe on AECs in cavity cultures.

In this report, we have described a biomimetic culture model that mimics the architecture of the lung alveolus. Our system is “2.5D” as cells are not embedded within a hydrogel but do experience 3D architecture and bending of the cellular monolayer. In contrast to cyst cultures, cells in our system adhere to a solid polymer substrate (PDMS) that does not enable nutrient delivery to the basal surface. While this is a limitation of the current study because this type of polarized nutrient delivery can be important for some biological questions, we envision our system could be easily adapted to allow for this by molding hydrogel cavities as opposed to PDMS ones, using a method similar to one we have described previously [16]. A hydrogel culture system could also facilitate co-culture experiments where pulmonary fibroblasts or endothelial cells could be presented in the hydrogel surrounding the epithelial layer. A key advantage of our system is the 3D-like architecture within a 2D plane, which is particularly advantageous for both achieving highly reproducible and controllable cellular architecture and accessibility for data analysis. Unlike previous methods in which cells are grown as a heterogeneous population of self-organizing cysts, our methods enables precise definition of the cavity size and hence the number of cells in the local tissue unit. Processing in our system for image analysis is also much easier than in typical 3D cultures as all cells in the cavity are accessible to staining reagents unlike in aggregates or cysts where limited penetration of stains through hydrogels or multiple cell layers can be a challenge. Our system also offers the
advantage of being potentially scalable as large sheets containing cavities for mass culture are feasible. Scale up however, would require large starting populations of AECs, since cells typically do not proliferate *in vitro*. It would therefore be interesting to use these methods to assess the behaviour of stem or progenitor AECs in the future, since this would create the possibility of greater cell numbers. Indeed, a combination of lung biomimetic architecture and large scale production of mature AECs would enable the development of a manufacturing process to generate model systems at the scale useable for evaluating or screening potential therapeutics. Furthermore, while we have demonstrated the relevance of our system for growing primary lung cells, it is a potentially relevant and useful platform for growing primary cells from other tissue that are constructed with a similar cavity-like architecture, such as intestinal crypts and breast mammary glands.

**5.5 Conclusions**

We have developed a highly reproducible fabrication method and cell culture platform for growing cells in a curved architecture. Primary mouse AECs cultured on cavity substrates form epithelial cell monolayer conforming to the culture substrate architecture. Cells remain viable and maintain their expression levels of SPC and AQP5, specific markers for AT2 and AT1 cells respectively, in contrast to cells grown on flat substrates. Our alveolar culture system will enable the exploration of numerous novel biological questions in the future such as how can architecture dimensions be systematically varied to engineer desirable cell phenotypes. We speculate that this model will provide a valuable tool for those studying the biology of the lung epithelium, as well as screening potential therapeutics for diseases affecting the distal lung epithelium.
Chapter 6

Discussion, Conclusion and Future Directions
Much of the \textit{in vitro} work to model airway epithelium has been conducted in traditional 2D culture systems that do not replicate the biological structures cells experience \textit{in vivo}. The airway and lungs exhibit specific architecture linked to cell utility but microstructural organization has not been employed in engineering strategies to generate epithelial tissue. The overall objective of this thesis was to explore the utility of physical attributes to manipulate airway and lung epithelium. We used three engineered scaffolding approaches to investigate the effect of different biophysical cues (stiffness, topography, and architecture) on cell properties and behaviour with a view to generate highly functional \textit{in vitro} epithelial models. We found that architectural cues can have pronounced effects on cell behaviour when appropriately micropatterned to capture aspects of native biological structures. These results advance primary culture models by producing more physiologically-relevant phenotypes such as epithelial planar polarization and distal lung progenitor maintenance. The findings in this thesis have highlighted advancements in synthetic and fabrication techniques, unlocking future opportunities to rationally design biomaterials for engineered lung epithelium which we discuss below.

6.1 \textbf{Biomaterials with UV-tunable properties enable precise spatial control of cell alignment}

In \textit{Chapter 3} we described a tissue-engineered (TE) scaffolding approach to UV pattern cells with a planar polarity cue that was also compatible with apical-basal polarization. We initially set out to pattern anisotropic stiffness into a hydrogel but were not successful in this regard. Instead we found that our device spatially patterns regions with differences in adhesion with resolution as low as 13 µm. The patterns were able to form through the depth of the hydrogels (fidelity maintained at least up to 50 µm) and we demonstrated that this patterned gel is free of surface topography and local variations in substrate stiffness.
Although this hydrogel could not be used to culture epithelial cells, certain mesenchymal cell types (human mesenchymal stem cells (hMSCs) and valvular interstitial cells (VICs)) were able to adhere and spread at both sparse and confluent seeding densities. Our results demonstrate the ability of cells to spread on a material can be tailored by the simple localization of the peptides that are incorporated into the hydrogel. These cell types were able to morphologically align to poly(ethylene glycol)-norbornene (PEG-NB) hydrogels with spatially-patterned CRGDS, in contrast to cells cultured on PEG-NB with no CRGDS adhesion pattern. We found differences in morphology and proliferation between different cell types which could allow for future experiments with patterned adhesion to produce elongated tissue types such as bone, ligament, or skeletal/cardiac muscle. The ability to control where mesenchymal cell types adhere to a synthetic material would be particularly important for applications to induce migration in wound-healing situations such as bone defects or heart valve repair [220, 221]. Other work has shown that micropatterned bone marrow-derived MSCs exhibit changes in their differentiation outcome when their aspect ratios are altered [336]. This culture platform will enable the exploration of questions such as how can adhesion be systematically varied to engineer cell shape to achieve desirable cell lineage commitment in a heterogeneous tissue.

For most cell types this biomaterial did not support culture at high or confluent densities. The inability of PEG-NB to support epithelial progenitor adhesion or hMSC proliferation suggests that this material is not optimized for culture of challenging cell types and is better suited as a tool for investigating biological questions rather than a platform for new medical products. However due to limitations regarding the inability to support epithelial cell types we chose not to move forward with this approach as we were focused on designing epithelial tissues.
Historically, covalent chemistries have dominated the biomaterials field [337]. Materials produced from these covalent chemistry technologies generally possess inert and uniform properties and display limited capacity towards generating complex presentation of biophysical and biochemical cues. It is known that spatially controlled extracellular matrix (ECM) signals play a role in different processes in vivo [167, 338, 339]. Advances in biomaterial chemistry have enhanced our capacity for engineering precisely-tuned materials to generate the cell-ECM interactions and organization necessary to form tissues with complex structures [337, 340]. Specifically, the photopatterning of scaffolds by light-mediated reactions have become instrumental towards the spatial control of biochemical signals on hydrogel surfaces. In this technique, specific regions are exposed to light to initiate the desired reactions (e.g. thiol-ene crosslinking [341] or photocleavage [342]). Single photon [343] or multiphoton [344, 345] irradiation methods have been developed with patterning resolution limits of 50 µm and 5 µm, respectively. Our strategy complements these techniques by improving the resolution of spatial control (down to 13 µm in the xy plane) without the need for complex confocal patterning setups. This simple photomask patterning technique allows for single cells to be exposed to multiple adhesion cues and interfaces within the same surface (i.e. non-isotropic changes in peptide presentation), enabling the study of the effect of differential adhesion on cell behaviour.

Although the utility of biomaterials has been widely explored to direct fibroblast alignment [343], neural extension outgrowth [346] and bone regeneration [347], relatively little work has been done investigating the use of biomaterials to support the epithelium of the large airways. PEG-based polymers present an advantageous platform to incorporate specific biochemical cues [216, 224] such as RGD, an amino acid sequence found in fibronectin, vitronectin, laminin and collagen that is known to induce cell adhesion [348]. In Chapter 3, we found that our photopatterned culture system was limited as it was unable to support the adhesion
and culture of primary human tracheal epithelial cells (HTECs) despite the presence of CRGDS adhesion sites. It is possible that PEG-NB hydrogels are too mechanically soft to support HTEC culture as epithelial cells are capable of growing on stiffer substrates in vitro. Primary airway epithelial cells possess a prominent, rigid actomyosin cortex which suggests the need for robust mechanical properties in addition to adhesive cues [257, 349]. In fact, stiffer substrates may be preferred as primary cells adhere on several materials with mPa stiffness including polydimethylsiloxane (PDMS), the polyester membranes of transwell inserts, silk fibroin [204] and collagen vitrigel membrane (CVM) [200]. ECM stiffnesses that are too soft result in round morphologies, unstable focal adhesions and low cell densities post-seeding.

We further tested several hydrogel substrates including gelatin, PEG-diacrylate, silk fibroin and CVM prior to selecting CVM as our cell culture substrate for our grooved scaffolding approach in Chapter 4. Our pilot studies indicated that gelatin and PEG-diacrylate did not support the ability of HTECs to bind and spread (data not shown). Experiments with silk fibroin and CVM suggested that HTECs could form epithelia on these naturally-derived biomaterials. Both substrates permitted differentiation into multiciliated cells (MCCs), but ciliation was delayed on silk fibroin (Waddell lab unpublished data). We speculate that silk fibroin and CVM retain their native structures to better recapitulate fibrous ECM and allow better adhesion by HTECs, in contrast to PEG which is biologically inactive and gelatin which is a denatured form of collagen [183]. It is likely that HTECs have different adhesion and mechanical requirements than the cell types currently supported by our PEG-NB system. Indeed, VICs cultured in dynamic 3D PEG-NB gels showed greater elongation and α-smooth muscle actin activation in response to RGDS addition (0 to 1.5 mM), and the magnitude of these responses was greater than to dynamic changes in matrix modulus (1 to 6 kPa) [350].
We sought to test the impact of local stiffness variations on epithelial organization in a culture system compatible with apical-basal polarization. Direct measurement of tracheal matrix mechanical properties has not been performed but the makeup of aligned collagen fibres [138, 245] implies that significant local variances in mechanical stiffness exist [135, 246]. We initially hypothesized that anisotropic substrate stiffness would have a significant effect on epithelial alignment. Cells are able to respond not only to the magnitude of substrate stiffness but also to the directional presentation (anisotropy) of that stiffness. Our hypothesis could not be tested however as PEG-NB patterned with 50 µm photomasks did not differ its mechanical properties across the surface. Indeed, other studies have tried patterning stiffness using photomasking techniques with similar results. The Burdick group have reported spatial patterning of substrate mechanics using methacrylated and norbornene-modified hyaluronic acid [250, 251]. Using transparency photomasks and atomic force microscopy (AFM) with 1 µm diameter SiO₂ bead tips, these investigators reported significant changes in mechanics varying from 2.5 kPa to 25 kPa within the same gel surface. It was reported that stiffness changes were achieved with a resolution of 50 µm and that patterning with 25 µm photomasks was not observed. These results are consistent with our findings that differential UV dosing could tune mechanical properties at the macro scale (i.e. bulk stiffness changes) but not at the local scale (i.e. stiffness does not vary in regions that are ≤50 µm apart).

More recently, Yang and colleagues were able to produce patterned stiffness (2-10 kPa) at the micrometer scale [351]. These experimenters were able to generate and map patterns of 2 µm x 2 µm squares on PEG di-photodegradable acrylate (PEGdiPDA) hydrogel surfaces using chrome photomasks and AFM. This improvement in resolution was achieved by exploiting the photodegradation of the nitrobenzyl ether crosslinker in PEGdiPDA. Photolabile mechanisms
may present an advantageous approach for the purpose of patterning mechanical properties. In contrast, photocrosslinking chemistries rely on thiol-radical mechanisms to form the covalent bonds of polymerization, which could propagate for an indeterminate distance before terminating, leading to a loss of resolution [220]. Mechanically-tunable in vitro hydrogel platforms with subcellular resolution could allow for novel experiments to test the impact of stiffness variations at the scale of a single mature focal adhesion (1-5 µm²) [19, 352]. Although we were not able to test the effect of differential stiffness cues on cell polarity, it would still be important in the future to probe how spatial changes in substrate stiffness can regulate cell organization, particularly in airway epithelium. This could be accomplished by adopting polymers with the aforementioned properties and chrome photomasking approaches. Testing this hypothesis will advance our fundamental understanding of the relationship between biophysical cues and epithelial alignment, but will first require optimization of other chemistries to support the culture and differentiation of primary cells.

Advancing our capacity to model native tissues will require not only heterogeneous presentation of mechanical properties but also complex biomolecule presentation over a wide range of length scales. We have adapted a PEG-based hydrogel to robustly pattern adhesion at the subcellular scale (~13 µm), opening several novel areas of investigation. The incorporation of adhesive peptides into PEG-NB is easily achieved by the addition of a cysteine residue, which can participate in the thiol-ene crosslink reaction [224]. This presents the opportunity for sequential patterning of multiple peptides within the same gel. Specifically, a portion of norbornene moieties can be crosslinked with dithiol crosslinker molecules in an initial gel-forming step. The remaining norbornene groups would be available for subsequent crosslinking. Gels would be incubated with a solution of photoinitiator and mono-thiolated peptide, then
exposed to UV light through a photomask, which permits thiol-ene reactions at the locations where light is exposed. This technique would yield a PEG-NB hydrogel with spatially patterned adhesion of more than one adhesive biomolecule. Indeed Wade et al. have utilized a norbornene-functionalized hyaluronic acid nanofibrous hydrogel to spatially pattern 3 peptides independently within one scaffold to modulate the adhesion and morphology of NIH 3T3 fibroblasts and HUVECs [343]. Advances to engineer phototunable synthetic PEG hydrogels to mimic the complex biochemical properties of native ECM would provide an alternative to natural biopolymers like hyaluronic acid, gelatin, and alginate, which inherently contain uncontrolled bioactive sites (e.g. RGD, matrix metalloproteinase (MMP)-sensitive sequences) that could confound interpretations of experimental results [183].

The lessons learned in the development of our hydrogel patterning strategy opens another area of potential investigation. Specifically, spatiotemporal control afforded by light enables the design of hydrogels with chemistries that allow controlled degradation. Culture systems could be dynamically softened \textit{in situ} to understand matrix remodeling by cells. Applications include providing a temporary structure for cells to secrete their own ECM (e.g. biodegradable sutures, biodegradable films) [353, 354] or to study cell responses to changes in elasticity or topography [169, 355]. Other applications for photodegradation would be to stimulate the release of molecules (e.g. drugs, growth factors) at desired rates [356, 357]. The controlled release of molecules could be harnessed to treat a variety of diseases to either stimulate or impede tissue growth including myocardial infarction [358], rheumatoid arthritis [359], and cancer [360].

Traditionally, culture scaffolds were initially designed to permit cell survival and proliferation [168, 361]. Advances in contemporary biomaterials have shifted their focus on surface functionality but many examples discuss bulk modifications. Technical improvements
are necessary to precisely present matrix properties in an appropriate manner so that these signals can be integrated to instruct cell behaviour. Recent developments in the biomaterials field include the ability to control hydrogel properties at multiple length scales (from molecular to macroscopic), the introduction of dynamic behaviour, and additive functionalities to capture the complexity of native tissues. In Chapter 3 we have described structurally simple biomaterial options, with increasing functional sophistication in their ability to tune and manipulate complex physical and biological properties. These advances in biomaterial science capture the multifunctional aspects of native biological structures, and will enable investigators to ask novel biological questions to provide insights into the manner by which matrix interactions influence cell behaviour [222, 337]. Applications toward tissue engineering and regenerative medicine will require further advances in material biocompatibility with airway epithelialization.

6.2 Micro-grooved topography aligns primary airway epithelium during apical-basal polarization

As an alternative to asymmetric stiffness in Chapter 4 we pursued a grooved scaffolding approach to create epithelial planar polarity. We wanted to explore whether substrate topography could provide a polarizing signal to align ciliated airway epithelium. We hypothesized that grooved gels would induce a morphological cell alignment response that could be translated into alignment of basal bodies (BBs) along the corresponding axis. Several grooved topographies were screened to align confluent primary HTECs. Our device aligned primary airway epithelial cells in both normal and air-liquid interface (ALI) culture, indicating that our culture method could induce planar polarization that was compatible with apical-basal polarization. Primary cells were able to give rise to MCCs on both flat and grooved hydrogel substrates by ALI D21. We found that planar cell alignment was induced by hydrogel CVM grooves at only early timepoints in ALI culture (up to D14) and lost afterwards. Future work is required to determine
the effect of morphological planar polarization in early ALI timepoints on the alignment of cilia that emerge at later ALI timepoints. This approach was limited as the biomaterials that support epithelial ALI culture are few and the factors that promote adhesion are poorly understood. Consequently the materials compatible with airway epithelial cells do not allow for simultaneous modification of substrate stiffness with topography, or allow for the encapsulation of a second cell type such as fibroblasts.

We tested several hydrogel substrates and found that both 5% silk fibroin hydrogels and CVM were biocompatible with HTECs. Ciliation on flat silk biomaterial was delayed compared to transwell culture (ALI D45 versus D31), and cilia were completely absent in silk gels imprinted with grooved topography. We speculate that there is some chemical or physical factor in silk fibroin hydrogels that inhibits the terminal differentiation of HTECs into ciliated epithelial cells. CVM allows ciliogenesis on comparable levels to transwell culture in both flat and grooved conditions. Biomaterials screening for optimal HTEC culture is currently under active investigation in the Waddell lab.

We sought to test the impact of micro-grooved architecture on epithelial organization in a gel culture system compatible with apical-basal polarization. We hypothesized that grooves would direct the intra- and intercellular organization of motile cilia in differentiating airway epithelium. We initially attempted to bypass lengthy ALI culture (which requires up to 45 days of culture time) by visualizing primary cilia of BEAS-2B and HTECs in submerged culture on flat and grooved PDMS substrates. Determination of primary cell alignment was not possible because primary cilia are ultrastructurally different than motile cilia. Specifically, primary cilia have multiple basal foot projections in that project radially around the BB, making the determination of cilia rotational polarity not possible (unlike motile cilia, which have one basal foot per cilium).
Consequently we generated ciliated epithelium with ALI culture on CVM hydrogel substrates (flat and grooved). Our hypothesis could not be tested however as further technical optimization is required to produce the 3D-SIM images necessary for BB orientation analysis. Primary epithelial cells exhibit significant strain-to-strain variability in terms of their differentiation capacity and screening is required to select strains with high ciliation potential. Future work will be necessary to generate epithelia with sufficiently dense regions of cilia required for super-resolution microscopy. Specifically, we will continue to focus on comparing ciliary BB polarity between flat and grooved culture conditions, using 3D-SIM with optimized imaging parameters.

A number of approaches are available to fully test our hypothesis in future work. Optimized 3D-SIM measurements will enable us to determine whether alignment of primary cells in submerged culture and early ALI can be translated to BB alignment in later ALI culture. If BB are planar polarized by grooves in fully differentiated epithelium that suggests topography creates a proximal-distal tissue axis, along which cells are able to rearrange their cytoskeletal dynamics and translate polarity into cilia alignment. A potential area of investigation following this outcome would be to characterize the kinetics and mechanism of ciliary alignment throughout ALI culture at earlier timepoints. Transmission electron microscopy (TEM) is a well-established method for determining the polarity of the cilia ultrastructure [70, 71] and provides an alternative approach if technical challenges with 3D-SIM cannot be overcome. If the cilia polarity cannot be determined by 3D-SIM or TEM, determination of cilia beat direction by fluorescent bead tracking could provide a metric for epithelial function [86, 362]. High speed imaging of bead movement on top of ciliated epithelium allows for the determination of cilia orientation and beating frequency.
6.3 **Biomimetic architecture maintains alveolar epithelial cell utility**

Underscoring the functional importance of architectural organization, current 2D culture systems that lack instructive biophysical cues produce not only disorganized airway epithelium but also dysfunctional distal lung cells. In *Chapter 5* we described a TE scaffolding approach to use biomimetic architecture to preserve alveolar epithelial cells (AECs). We hypothesized that alveolar-mimetic architecture would prolong the viability and phenotype of AECs compared to culture on flat substrates. Substrates were fabricated with densely-packed arrays of alveolar-mimetic hemispherical cavities and we demonstrated that our manufacturing method generated highly reproducible epithelial monolayers in physiologically-relevant curved architecture. Primary mouse AECs remained viable in cavities relevant to the sizes of mouse (50 µm) and human (200 µm) alveoli. Furthermore we found that mouse AECs are able to maintain stable expression levels of type 1 (AT1) and type 2 (AT2) markers within 50 µm cavities. This system presents a useful platform that enables precise control of 3D architecture that could be easily adapted for primary culture of cells from other tissues with curved architecture. Our current experiments however do not show the utility of this device with respect to providing a valuable tool for basic science of the lung epithelium or for screening therapeutically for lung diseases. We plan to perform additional studies polarizing cells in molded hydrogel substrates to strengthen the impact of the presented model in the revised journal article. In the long term, future work will be focused on elucidating the mechanism by which cavity culture confers benefit to AECs.

The major limitation for our cavity culture system is a loss of viability beyond 7 days of culture. No observable differences were detected between AECs cultured on flat and cavity surfaces as most cells stained positive for the dead marker at D14 in both conditions. This negative result implies that architecture alone does not provide the cues necessary to support
very long term culture (>1 week) of primary alveolar epithelium. It is likely that, in addition to architectural cues, the maintenance of AEC viability requires signaling from soluble factors or interactions with pulmonary fibroblasts or endothelial cells. This is consistent with findings from organoid culture systems whereby alveolospheres are unable to be generated without stromal support cells [363]. A technical limitation of our system is the access to large sources of mature cells. Obtaining primary AECs is technically challenging and time-consuming which restricts scale up to generate model systems that would be suitable for drug screening.

Another area of future work would be to define the underlying mechanism of cavity culture. We speculate that the architectural cues could be influencing cell-cell signaling between AECs, by increasing cell density and crosstalk. Studies culturing hepatocytes [364] and mESCs [234] have demonstrated that paracrine signaling between cell colonies is limited to <400 µm. These principles could be applicable to signaling pathways in a wide range of cell types. Our biomimetic culture system could be concentrating signaling molecules within the confined space of the cavity and altering endogenous signaling gradients, or enhancing paracrine signals between cavities. The first hypothesis could be tested by culturing cells on the inverted (convex) geometry; if the effect is cell-intrinsic (i.e. not sequestering signaling molecules within the small cavity space) then the same phenotype would be observed. The substrates for testing larger cavities would however require technical optimization as gravitational forces would lead to cells rolling off the curved substrates into the inter-bump wedges. The second possibility could be tested by cavity culture in substrates with varying inter-cavity spacing. Cavities that are spaced effectively far apart can be considered independent and cell phenotypes could be assessed and compared to the densely-packed configuration.
Alternatively, the geometric organization of cells within a tissue could be exposing different regions to different amounts of mechanical stress [275]. Intercellular adhesion and cortical tension integrate to produce a tissue surface tension signal [276] that regulates the local behaviour of cells and their neighbours [277]. For instance, geometry can spatially pattern epithelial-to-mesenchymal transitions (EMT) at regions of high mechanical stress in micropatterned mammary epithelial sheets [279]. Geometric cues have been shown to influence a variety of behaviours including proliferation [20], stem cell characteristics [278], and integrin-mediated cancer stem cell signaling [281]. The cellular response to geometry is likely a universal phenomenon as it has been shown to guide cell behaviours in many cell types including A549 lung cancer cell lines [281]. This possibility could be tested by treatment of cells in cavity culture with cytoskeletal (e.g. blebbistatin or cytochalasin D) or ROCK inhibitors and assessing whether the benefits of cavity culture are disrupted.

An ideal model of the distal lung would contain both AT1 and AT2 cells in the appropriate proportions within the correct architecture. The *in vivo* niche is complex and several local microenvironmental parameters govern the alveolar niche including ECM, cell-cell interactions and soluble factors. Our platform can be modified to isolate the contribution of local signaling molecules within and nearby the confined space of the cavity by screening various cavity sizes or cavity spacing. Creating cavities that are too small or too large will allow us to characterize the functional consequences of niche-size-dependant signaling control. Similarly, varying the spacing between cavities would allow experimenters to identify the minimum distance by which signaling between cavities can be considered independent of one another. In this way our system is useful for *defining* the boundaries of the governing parameters in the alveolar niche. This platform could be utilized for the systematic prediction, detection and manipulation of
endogenous signaling gradients or mechanical strain \textit{in vitro} to regulate cell viability and phenotype.

6.4 \textbf{Overall Research Contributions}

Current models of airway epithelium are limited by culture methods that do not adequately reproduce the \textit{in vivo} situation, leading to disorganized or dysfunctional cell behaviour. Specifically, methods to produce ciliated airway epithelium do not incorporate any tissue-level instructive cue for cilia beating directionality, leading to ineffective mucociliary clearance. In the case of distal lung epithelium, available sources of mature primary cells have limited viability after isolation and the cells that do survive in culture change their phenotype over time. New biomaterial design has been driven by an increased understanding of native tissue architecture and the role it plays in actively interfacing with biologically complex environments to direct tissue function. The respiratory system presented opportunities for utilizing region-specific architectural cues to manipulate the organization of respiratory epithelium. We designed fabrication techniques and synthetic approaches that afford defined properties at the length scale that is biologically relevant to trachea and lung epithelial cells. Specifically, our engineered scaffolds enable complex cell-material interactions to generate planar polarized airway epithelium and AEC progenitor cell maintenance. These cell culture platforms with complex architecture presentation contribute to the toolbox of synthetic scaffolding approaches for rational biomaterial design.
Chapter 7

Supplemental Figures and Tables
<table>
<thead>
<tr>
<th>Estimated dimensions (width x spacing x height (μm))</th>
<th>Measured dimensions (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>width</td>
</tr>
<tr>
<td>3x3x1</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>3x3x3</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>3x3x5</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>5x5x1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>5x5x3</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>5x5x5</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>10x10x1</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>10x10x3</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>10x10x5</td>
<td>9.7 ± 0.1</td>
</tr>
</tbody>
</table>

**SI Table 1. Characterization of microgrooved PDMS.** Feature dimensions were measured from SEM images. ($n = 3$).
SI Figure 1. BEAS-2B alignment on PDMS microgrooves. (A) Confocal images were analyzed to assess BEAS-2B cell elongation on grooves of varying pitches and depths. (B) Proportion of BEAS-2B with an elongated phenotype (defined as major axis/minor axis ≥ 1.4). Cell (C) and nuclear (D) alignment on flat versus different groove dimensions. Alignment was defined as the percentage of cells or nuclei oriented within 15° of the groove direction. Error bars are standard error of the mean and * indicates p<0.05. (n = 3).
SI Figure 2. At higher groove depths (10 μm), monolayer formation is compromised. Representative confocal images of phalloidin (green) and Hoescht (blue) staining for BEAS-2B or primary HTECs cultured for 48 h on 10 μm deep PDMS grooves with (A,D) 3, (B,C) 5, and (C,F) 10 μm pitches.
SI Figure 3. Optimization of cavity substrate manufacturing process. (A) SEM image of 50 µm diameter cavities in close-packed configuration. (B) SEM image of individual 50 µm diameter cavity in close-packed configuration, depicting shallow curvature. (C) SEM images of 50 µm diameter cavities in grid-packing configuration. (D) SEM image of individual 50 µm diameter cavity in grid configuration.
SI Figure 4. Optimization of cell seeding to achieve reproducible monolayer cultures within the cavities. (A) DIC image of A549 cells cultured in 50 µm diameter cavities. (B) Corresponding confocal microscopy image of (A). Nuclei stained with Hoescht (blue) and actin stained with phalloidin (green). (C) Corresponding confocal side profile of (A). (D) DIC image of A549 cells cultured in 200 µm diameter cavities. (E) Corresponding confocal microscopy image of (D). Nuclei stained with Hoescht (blue) and actin stained with phalloidin (green). (F) Corresponding confocal side profile of (D). (G) Graph showing the number of cells per cavity for 50 µm and 200 µm diameter cavities (n= 10). Error bars are standard error of the mean.
Chapter 8

References


