Generation of Planar Cell Polarity (PCP)

in vitro for Epithelium Tissue Engineering

by

Ana Cristina Paz Mejia

A thesis submitted in conformity with the requirements for the degree of
Master of Science

Department of Chemical Engineering and Applied Chemistry

Institute of Biomaterials and Biomedical Engineering

University of Toronto

© Copyright 2011 by Ana Cristina Paz Mejia
Generation of Planar Cell Polarity (PCP)

in vitro for Epithelium Tissue Engineering

Ana Cristina Paz Mejia

Master of Science

Department of Chemical Engineering and Applied Chemistry
Institute of Biomaterial and Biomedical Engineering

University of Toronto

2011

Abstract

Engineering epithelium with correct structure is essential for generating functional tissue. During tissue development, cells organize in defined patterns through cellular signalling. Artificial generation of the signalling that organizes cells within the tissue offers a novel approach for engineering tissues with appropriate structure. Planar cell polarity (PCP) is a cellular signalling pathway involved in the organization of epithelial cells. Our goal is to study the effect that co-culturing genetically distinct populations of epithelial cells, with variable levels of one of the core PCP proteins, has in epithelial cell sheet organization. MDCK cells transduced with a tagged PCP core protein (GFP-Vangl2) and wild type MDCK cells were co-cultured side-by-side. The effect of tight junction and cilia formation, and localization of the GFP-Vangl2 protein were evaluated. The results suggest that tight junction and cilia formation are not affected. On the other hand, the GFP-Vangl2 protein seems to be affected at some level.
Acknowledgment

I would like to thank everybody who has helped me to go through this learning process and personal growth. To my family, my boyfriend and friends for being such as strong support; this achievement is much mine as it is theirs. To my supervisor Alison for her help and support during this process. Working with her was a great experience. To all the members of the McGuigan lab for all their help and for making the work in the lab such a special and unforgettable experience. To Professor Helen McNeill and the members of her lab for all their technical assistant and helpful feedbacks. Finally, thanks to all the members of my committee for their time and help.
# Table of Contents

Acknowledgment .................................................................................................................. iii

Chapter 1. Thesis Aim and Hypothesis ....................................................................................... 1

1.1 Thesis Overview ................................................................................................................. 1

1.2 Hypothesis .......................................................................................................................... 3

1.3 Aims .................................................................................................................................... 3

Chapter 2. Background ............................................................................................................. 4

2.1 Importance of engineering epithelium ................................................................................. 4

2.2 Epithelial Tissue and Polarization ....................................................................................... 4

2.2.1 Apical Basal Polarization ............................................................................................... 5

2.2.2 Planar Cell Polarity (PCP) .............................................................................................. 5

2.3 *In vitro* systems in epithelial tissue studies ........................................................................ 8

2.4 Examples of current advances in epithelium tissue-engineering ......................................... 10

2.5 Tools from tissue engineering to organize epithelium ......................................................... 15

2.5.1 Cell Micropatterning ....................................................................................................... 15

2.6 Conclusion .......................................................................................................................... 17

2.7 Reference ........................................................................................................................... 17

Chapter 3** Developing methods of micropatterning epithelial cells in mono and co-culture on filters insert culture system ............................................................................. 21

3.1 Introduction ........................................................................................................................ 21

3.2 Method ............................................................................................................................... 23

3.2.1 Cell Culture ................................................................................................................... 23

3.2.2 Master and PDMS stamp fabrication .............................................................................. 23

3.2.3 Parafilm insert fabrication ............................................................................................... 23

3.2.4 Filter insert patterning strategies .................................................................................... 24

3.2.5 Filter insert culture conditions ......................................................................................... 26

3.2.6 Assessing patterned epithelial sheets ............................................................................. 27

3.2.7 Assessing epithelial cell polarization markers ................................................................. 27
Chapter 4. Definition of organization readouts on modified MDCK_GFP-Vangl2 cells

4.1 Introduction ......................................................................................................................... 41

4.2 Methods ................................................................................................................................. 42
  4.2.1 MDCK culture conditions ................................................................................................. 42
  4.2.2 Virus production ............................................................................................................... 42
  4.2.3 MDCK transduction with GFP-Vangl2 construct and eGFP construct ......................... 43
  4.2.4 Assessment GFP-Vangl2 protein localization on modified MDCK cells ..................... 43
  4.2.5 Assessment of epithelial cell polarization markers of MDCK cells ................................ 43

4.3 Results ................................................................................................................................ 44
  4.3.1 Generation of modified MDCK_GFP-Vangl2 cells ......................................................... 44
  4.3.2 Assessment of GFP-Vangl2 protein localization on modified MDCK_GFP-Vangl2 cells 45
  4.3.3 Assessment of epithelial cell polarization markers on MDCK_GFP-Vangl2 cells ...... 48

4.4 Discussion ............................................................................................................................. 51

4.5 Conclusion ............................................................................................................................. 54

4.6 Reference ............................................................................................................................... 54

Chapter 5. Co-culturing patterned sheets of MDCK_GFP-Vangl2 cells and wild type MDCK cells

5.1 Introduction ............................................................................................................................. 56

5.2 Method .................................................................................................................................. 57
  5.2.1 Co-culture experimental design ...................................................................................... 57
  5.2.2 Assessment of the effect of co-culture condition on cell sheet organization ............... 58
5.3 Results .......................................................................................................................................................... 59

5.3.1 Assessment of GFP-Vangl2 protein localization in MDCK_GFP-Vangl2 cells when Co-cultured with wild type MDCK ........................................................................................................................................... 59

5.3.2 Assessment of epithelial cell polarization markers on MDCK_GFP-Vangl2 and wild type MDCK cells ........................................................................................................................................................................... 62

5.4 Discussion .................................................................................................................................................... 66

5.5 Conclusions ................................................................................................................................................ 69

5.6 Reference .................................................................................................................................................... 69

Chapter 6. Conclusions, Challenges and Future Works .............................................................................. 70

6.1 Conclusions ................................................................................................................................................. 70

6.2 Challenges .................................................................................................................................................. 72

6.3 Future Work ............................................................................................................................................... 72

6.4 Work Significance .................................................................................................................................... 73

Appendix ......................................................................................................................................................... 74
Tables

Table 5.1 Dynamics of MDCK_GFP_Vangl2 cell in Monoculture .............................................68

Table 5.2 Dynamics of MDCK_GFP_Vangl2 and wild type MDCK cell in Co-culture ...............68
Figures

Figure 2.1 PCP core proteins interaction .................................................................6

Figure 2.2 Domineering non-autonomy phenomenon in Drosophila wing...................7

Figure 3.1 Schematic of mono-pattern and co-pattern methods..................................26

Figure 3.2 Micro-patterning of epithelial cells on filter insert membranes using agarose method at day 1..................................................................................................................28

Figure 3.3 Micropatterning of epithelial cells on filter insert membranes using agarose method at day 15....................................................................................................................29

Figure 3.4 Co-patterning of GFP expressing and non-GFP epithelial cells on filter insert membrane at Day 0 using parafilm and fibronectin method. .................................................................31

Figure 3.5 Co-patterning of GFP expressing and non-GFP epithelial cells on filter insert membrane at Day 15 using parafilm and fibronectin method........................................................................31

Figure 3.6 ZO-1 expression on monopatterned, co-patterned and un-patterned MDCK cells on filter inserts membrane over time. ........................................................................................33

Figure 3.7 Primary cilia formation on monopatterned, co-patterned and un-patterned MDCK cells on filter insert membrane over time.................................................................34

Figure 4.1 Generation of a homogeneous population of transduced MDCK cells........45

Figure 4.2 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cell on agarose microwells with different confluences. ..........................................................................................46

Figure 4.3 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cell on agarose microwells on glass. ..........................................................................................................................46

Figure 4.4 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells culture over time...............................................................................................................................48

Figure 4.5 Assessment of Zo-1 in un-patterned MDCK_GFP-Vangl2 cells and wild type cells on filter insert membranes over time. ...........................................................................50
Figure 4.6 Assessment of primary cilia formation on un-patterned MDCK_GFP-Vangl2 cells and wild type MDCK on filter insert membranes over time. ................................................................. 51

Figure 5.1 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells co-cultured with wild type MDCK over time. ........................................................................................................ 60

Figure 5.2 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells co-cultured with wild type MDCK on early time point........................................................................................................ 61

Figure 5.3 Close up of interface between MDCK_GFP-Vangl2 and MDCK wild type population on co-culture at early time points........................................................................................................ 62

Figure 5.4 Assessment of ZO-1 in co-cultured MDCK_GFP-Vangl2 with wild type MDCK cells on filter insert membranes over time........................................................................................................ 64

Figure 5.5 Assessment of primary cilia formation on co-cultured MDCK_GFP-Vangl2 with wild type MDCK cells on filter insert membranes over time.................................................................................. 65
Appendix

Figure 2.1 Example of PCP defects in vertebrate and Drosophila..........................74

Figure 3.1 Co-patterning of GFP expressing and non-GFP epithelial cells on filter
insert membrane at Day 7 using fibronectin method. ................................................74

Figure 4.1 Unsorted MDCK-GFP-Vangl2 cells..........................................................75

Figure 4.2 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells on day 0......75

Figure 4.3 Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells on patterned
filter insert membrane culture over time......................................................................76

Figure 4.4 Assessment of ZO-1 patterned MDCK_GFP-Vangl2 cells on filter insert
membranes over time.................................................................................................76

Figure 4.5 Assessment of primary cilia formation on patterned MDCK_GFP-Vangl2
cells on filter insert membranes over time...................................................................77

Figure 5.1 Domineering Non-autonomous phenomenon in Drosophila wing cells........77

Figure 5.2 Generation of a homogeneous population of lower transduced MDCK cells.....78

Figure 5.3 Co-culture of MDCK wild type cells with low transduced MDCK with
GFP-Vangl2 cells over time. ........................................................................................78

Figure 5.4 Primary cilia formation on wild type MDCK cell on day 5............................79
Chapter 1. Thesis Aim and Hypothesis

The epithelium is one of four major tissues in the body and it lines most major organs (skin, lung, kidney, intestines, etc). In general the epithelium consists of an organized single or multi layer of epithelial cells resting over a basement membrane. Many diseases such as cancer and polycystic kidney disease (PKD) are caused by damage of the epithelium structure, which leads to tissue malfunction. Engineering functional epithelium and understanding the factors that control epithelial maturation/organization is therefore an important factor in generating whole artificial organ replacements. Furthermore, engineered epithelium could allow us to model normal and diseased epithelium in vitro. This could be an invaluable tool to understand the differences between diseased and normal epithelium and study the mechanisms that lead to theses differences. Therefore, engineered epithelium is not only valuable for the field of tissue engineering but also for fields such as developmental biology and drug discovery.

1.1 Thesis Overview

Engineering epithelium with correct organization is essential to create functional epithelial tissue and consequently to engineer fully functional organs such as trachea, lungs and kidneys. Currently engineered epithelium does not exhibit appropriate organization and hence does not completely perform all its functions. For instance, due to the lack of organized specific structures, such beating cilia in the respiratory epithelium, current engineered epithelium does not fully perform as expected. Therefore, it is important first to understand the mechanisms that create this specific organization in the epithelium, and second create better strategies that allow us somehow recreate these mechanisms to engineer fully organized epithelium. During assembly of a tissue in a developing embryo, cells organize in defined patterns due to local and global signals (mechanical and chemical). We want to understand how local and global cues can be combined to produce specific tissue organization for engineering epithelial tissues with appropriate organization and hence better functionality. Creating tools that allow us to understand how global and local signals interact, will help us design strategies to engineer tissues with better organization and consequently with better functionality. We have focused on chemical signalling cues between cells and how they influence cell sheet organization. Specifically we focused on a particular signalling pathway, the planar cell polarity signalling pathway (PCP signalling), that influences epithelium organization during embryo development. PCP signalling is a good example where a local signal (interaction of PCP core proteins within the cell) influences the global organization of the epithelium. In PCP signalling the interaction of local and global signalling cues can be observed
clearly in the domineering non-autonomy phenomenon, in which a modification in the PCP signalling in a group of cells within the epithelium (local signal) affects the organization of neighboring non-mutated cells (at a global scale). The modification in the PCP signalling in the cells consists in overexpression or knockdown of one of the PCP core proteins, while the effect in the organization on the neighboring cells consist in the disarrangement of cellular structure, such as cilia, in the plane of the epithelium. This in vivo phenomenon of domineering non-autonomy where local modification within the cell have global effects on cell organization over the tissue, inspired us to ask if it is possible to affect the organization of an epithelial cell sheet in vitro by geometrically patterning genetically distinct epithelial cell populations, with variable levels of one of the PCP core proteins. To begin to answer this question in this study, first we needed to develop patterning techniques that allowed us to create monocultures and side-by-side co-culture of epithelial cells on filter insert culture system. These techniques are described in chapter 3. It is important that theses techniques are developed on filter insert culture system because this system is essential to fully maturate or apical-basal polarize epithelial cells in vitro. Secondly we needed a pure population of modified epithelial cells with clear markers of cell sheet organization. To do this an epithelial cell population with a modification (overexpression) in one of the PCP core proteins was generated and an assessment of readout of epithelium structure organization was done. Specifically, we want to understand the behavior (localization and dynamics over time) of the exogenous PCP protein on the modified epithelial cells culture on filter insert membranes, to later use this knowledge as a reference/control for the co-culture experiments. Also, we wanted to determinate if the overexpression of the PCP protein affected the apical-basal polarization of the cells in any way to assess if our standard polarization markers in wild type cells still could provide a reliable readout of epithelium organization in the co-culture experiments where one of the cell populations had elevated levels of PCP protein. All this is described in chapter 4. Using the developed co-patterning technique and our purified cell populations we co-culture side-by-side genetically distinct epithelial cell populations, with variable levels of one of the PCP core protein and characterized how the organization of the sheet was affected. To quantify cell sheet organization we assessed the localization of a PCP core protein, formation of cellular structures (specifically cilia), and expression of markers for apical-basal polarization (specifically ZO-1). These experiments are described in chapter 5. Finally, in chapter 6 over-all conclusions are discussed and areas of future work for further understanding of some of the results are suggested.
1.2 Hypothesis

We hypothesize that by co-culturing genetically distinct populations of epithelial cells, with variable levels of one of the core PCP proteins we can affect the localization of a PCP core protein, formation of cellular structures such as primary cilia or and expression of markers for apical-basal polarization such as ZO-1 on an epithelial cell sheet in vitro.

1.3 Aims

AIM 1 To develop methods of micropatterning epithelial cells in mono and co-culture on filters insert culture system.

Aim 1.1 To establish a micro-patterning protocol for monocultures of epithelial cells on filters insert membrane.

Aim 1.2 To establish a micropatterning protocol for co-culture epithelial cells on filters insert membrane.

Aim 1.3 To characterize wild type epithelial cells in micropatterns monoculture and co-culture to demonstrate patterning does not affect apical-basal polarization

AIM 2 To define the structural organization of MDCK cells overexpressing one of the PCP core proteins (Vangl2)

Aim 2.1 To generate a homogeneous population of MDCK transduced with a GFP-Vangl2 construct

Aim 2.2 To characterize the localization and expression of exogenous GFP-Vangl2 protein in pure populations of MDCK-GFP-Vangl2 cells over time cultured on filter insert membrane.

Aim 2.3 To characterize apical-basal polarization on MDCK-GFP-Vangl2 cells by assessing ZO-1 expression and primary cilia formation over time.

AIM 3 To characterize the effect of co-culturing MDCK_GFP-Vangl2 cells and wild type MDCK cells on the structural organization of a polarized MDCK epithelial sheet.

Aim 3.1 To characterize the localization and expression of GFP-Vangl2 protein in the GFP-Vangl2-MDCK population over time under co-culture conditions.

Aim 3.2 To characterize apical-basal polarization in a co-cultured epithelial cells sheet by assessing ZO-1 expression and primary cilia formation over time.
Chapter 2. Background

2.1 Importance of engineering epithelium

The epithelium is one of four major tissues in the body and it lines most major organs (skin, lung, kidney, intestines, etc). In general the epithelium consists of an organized single or multi layer of epithelial cells resting over a basement membrane. This tissue is constantly exposed to environmental damages due to its function as a protective barrier. Therefore, epithelium substitutes are broadly needed in different organs such as skin and respiratory track to repair different damages caused by traumas such as burns. Furthermore, many diseases such as cancer, polycystic kidney disease (PKD), smoke inhalation injury and Kartagener's syndrome or immobile cilia syndrome are caused by damage of the epithelium structure, which leads to tissue malfunction. Engineering functional epithelium and understanding the factors that control epithelial maturation/organization is therefore an important factor in generating whole artificial organ replacements. Furthermore, engineered epithelium could allow us to model normal and diseased epithelium in vitro. This could be an invaluable tool to understand the differences between diseased and normal epithelium and study the mechanism that leads to these differences. Therefore, engineered epithelium is not only valuable for the field of tissue engineering but also for fields such as developmental biology and drug discovery.

2.2 Epithelial Tissue and Polarization

Epithelial tissue is one of the four tissue types found in the body and makes up part of the majority of organs (lung, skin, kidney, liver, etc). It consists of an organized single or multi layer of epithelial cells resting over a basement membrane. Its main functions are to: (i) form a protection barrier between the organism and the environment, and (ii) regulate the permeability, transportation, and secretions of ions or molecules between body compartments. Polarization of different proteins within epithelial cells is one of the most important properties of epithelial tissue organization, ensuring its correct function. Epithelial tissue has two axes of polarization: (i) a polarization along the apical-basal axis, (ii) a polarization perpendicular to the apical-basal axis, which is known as Planar Cell Polarity (PCP). Both of these polarizations contribute to the functional organization of epithelial tissue.
2.2.1 Apical Basal Polarization

Apical-basal polarity is established and maintained by two key components of epithelial tissue architecture: tight cell-cell adhesions and cell-base membrane interactions. The tight cell-cell adhesions establish a physical barrier between the apical and basolateral domains of the cell plasma membrane, which creates a functional and structural distinction between the two plasma membrane domains. The formation of the tight cell-cell adhesions is established through specialized lateral local junctions; tight junctions and adherence junctions. The cell-basement membrane interactions have been reported to play an important role in the establishment of the apical-basal polarity, also provides structural support to the tissue and gives appropriate cues for differentiation. Apical-basal polarity permits the vectorial transport of molecules and ions through body compartments and provides protection against pathogens. Also, it has been reported that this polarization can act as a tumour suppressor.

During apical-basal polarization there is an extensive cell architecture remodelling. These structural changes allow us to assess when a cell or cell sheet is polarized. Some of this structural changes are: (i) formation and localization of tight junction in the apical-lateral side of the plasma membrane, this can be assessed by the expression of tight junction protein ZO-1; (ii) protusion of primary cilia on the apical plasma membrane surface; (iii) apical and basolateral proteins located in their respective domains; (iv) localization of the Golgi complex to the apical region above the nucleus; and (iii) reorganization of cytoskeleton, such as actin accumulation to the apical part of the cytoplasm.

2.2.2 Planar Cell Polarity (PCP)

In the literature, PCP is defined as the organization of cells or subcellular structures in the plane of the epithelium, perpendicular to the apical-basal axis. This type of polarity is established through a specific signalling pathway that results in the asymmetrical localization within a cell of a group of PCP signalling core proteins along the axis perpendicular to the apical-basal axis. This set of core proteins was first identified in Drosophila melanogaster and were found to be evolutionary conserved on vertebrates. The PCP core proteins include the membrane proteins Frizzled (Fz), Flamingo (Flmi), Strabismus or Van Gogh (Stbm or Vang or Vangl) and cytoplasmatic proteins Dishevelled (Dhs or Dvl), Diego (Dgo), and Prickle (Pk), which associate with the membrane during PCP signalling. Through studies in which some of the PCP core proteins are overexpressed or silenced it has been established that asymmetrical localization of the PCP core proteins in the cell is due to antagonistic relationships between some of these proteins; however, the complete mechanism is still not fully understood. The most accepted model for the asymmetric localization of the PCP core proteins consists of the formation of two protein complexes in the cell; one on the
proximal side of the cells (Vang, Pk, Fmi) and another on the distal side of the cell (Fz, Dsh, Dgo, Fmi)\(^{11,14}\) (Figure 2.1) It is proposed that the asymmetry between the protein complexes is achieved through i) mutual inhibition of the proteins inside the cells, ii) stabilization of the complexes through communication at the cells boundaries, or iii) a combination of both mechanisms\(^ {13}\). Observation of asymmetric distribution of the core proteins comes mainly from studies in *Drosophila*. In vertebrates, this asymmetry has been reported in the sensory hair cells and in cells involved in convergent extension movement during gastrulation and neurulation\(^ {13}\). The mechanism of how the asymmetrical localization of the PCP core proteins is translated into cells or subcellular structure organization is not still clear\(^ {15}\).

![Figure 2.1 PCP core proteins interaction. (a) Apical view of asymmetrical formation of the two PCP core protein complexes on two cells that are planar polarized. Vangl and Pk assemble the proximal complex and Dhs, Dgo and Fz assemble the distal complex. Fmi is not asymmetrical localized and it seems to mediate interaction between neighbouring cells and helps to stabilize the protein complexes. Even though, this model is appealing still more studies have to be done to completely clarify protein interaction. This model comes from studies on *Drosophila* wing. (b) Lateral view of asymmetrical localization of the two PCP core protein complexes on two cells that are planar polarized.](image)

In studies in which patches of cells with mutations in some of the PCP core proteins are generated within the tissue, a very interesting phenomenon called domineering non-autonomy is exhibited. Domineering non-autonomy refers to the loss of planar polarity in the wild type cells adjacent to the mutant cells. This phenomenon is observed in all *Drosophila* tissues, but is mostly described as occurring in the wing\(^ {12}\) and the inner ear epithelium of vertebrates\(^ {15}\). The most interesting observation is that the localization of the wild type cells that lost correct polarity depends on the type of PCP mutation in the neighbouring mutant cells. For example, in Fz mutants’,
only wild type cells that are localized distal to the mutant patch lose their planar polarization \(^{16,17}\) (Figure 2.2). In contrast, in Vangl mutants', only wild type cells that are localized proximal to the mutant patch lose their cell polarization\(^{18,19}\). This phenomenon exposes how PCP signalling allows communication of spatial information from one cell to the next\(^{18}\) and gives a model to study how a local change within a group of cells is translated into a more global signal.

**Figure 2.2. Domineering non-autonomy phenomenon in Drosophila wing.** Normally hair cells in the Drosophila wing arise and grow toward the distal side of the cell. Cells with FZ mutation (glow red cells) affect the planar polarization of wild type cells located distally to the mutant cells. The lost of planar polarity is observed by the disorganization of the hair cells.

In addition to the core proteins, there is a second group of proteins involved in PCP signalling that was also discovered in *Drosophila*. This second group includes two protocadherins Fat (Ft) and Dachsous (Ds) that interact across the cell boundaries, and a transmembrane protein Four-jointed (Fj)\(^{18}\). This group of proteins seems to provide a global directional cue in PCP signalling over the whole tissue\(^{14}\). An early model of the role of this second group of global PCP proteins signalling suggests that this group acts upstream of the core PCP proteins and direct their asymmetry distribution\(^{12,14}\). However, new evidence challenges this model, and, currently it is not clear if this group acts upstream or parallel to the core proteins and how the information is propagate from one system to the other\(^{12,14}\). The role of this second group of proteins in vertebrate has not been determined significantly\(^{13}\).

The final PCP signalling readout is the activation of different cellular responses such as regulation of cytoskeleton organization, cell adhesion and migration, and movement and orientation of the mitotic spindle. In *Drosophila* and vertebrates tissue-specific effectors have been identified. For instance, in *Drosophila* wing and abdomen PCP signalling results in the formation of a hair on a specific side of the cell as a result of cytoskeleton arrangement and in the eye PCP signalling produces planar organization of the ommatidia as a result of directed cell rotation\(^{20, 21}\)In
vertebrates, organization of the hair on the back of the mouse and in the inner ear the parallel arrangement of sensory hair cells (Appendix-Figure 2.1). More specific, in the vertebrate inner ear it has been shown that mutation in Vangl protein cause the disarrangement of sensory hair cells (stetocilia and kinocilia) which show a link between PCP signalling and the cell organelle cilia. Cilia are long study cellular structures found in many vertebrate cells types. There are two types of cilia: (i) non motile or primary cilia: this type of cilia is found as a individual structure on the apical surface of the majority vertebrate cell and serves as a sensory structure, (ii) motile cilia: this type of cilia is found as cluster of 100-300 cilia on the surface of specialized cells such as ciliated cells from the respiratory epithelium. This cilia have a motor protein which allow them to beat in one direction. The study of the link between cilia and PCP signalling have graped the attention of many research groups due to the possible involvement of PCP signalling in ciliopathies, which is a group of diseases that are caused by defects in cilia formation and function. Some examples of ciliopathies are polycystic kidney disease (PKD) and Bardet-Biedl syndrome (BBS). In 2005 Ross and colleagues showed that mice with mutations in some genes identified for BBS show PCP phenotypes such as disturbed stereociliary bundles in the inner ear and neural tubule defects. However, so far there is no clear evidence that shows that mutations in any of the PCP core protein can cause ciliogenesis defects. Therefore, currently the exact connection between cilia and PCP signalling is still to be elucidated.

2.3 In vitro systems in epithelial tissue studies

In vitro culture systems for epithelial tissue are used as models to investigate physiological and morphological properties, and diseases mechanism of the tissue. Currently, the most common in vitro systems used to study different aspect of epithelial tissue are: (i) filter insert culture system in which cells are culture as a 2D or 3D (multilayer) and (ii) Biological gels in which cells can also be seeded as a 2D (monolayer over the gel) or 3D (cells embedded in the gel).

Filter insert culture system is broadly used due to its closer resemblance to the in vivo situation as opposed to standard cell culture systems. It has been shown that in this system epithelial cells apical-basal polarization better than on plastic or glass substrates. For instance, MDCK cells grown on plastic and glass substrates are usually 3-5 µm tall as opposed to 10-15 µm in filter insert culture system. Also, this system enhance the grow and differentiation of primary epithelial cell. It is mostly used to study physiological and architectural properties of the epithelium, such as vectorial transport, permeability, and reorganization of protein and organelles during the differentiation of epithelial cell in vitro. The filter insert culture system consists of a porous membrane hold by a plastic insert placed in a plastic well; this creates a structure with
Comparison of different studies shows batch changes which affect the reproducibility of the experiment and therefore it is difficult to limit the effect.

The limitation of this gel system is that due to the nature of the gels the composition from batch-to-batch changes which affect the reproducibility of the experiment and therefore it is difficult to compare different studies.

Biological gels are also a broadly used in vitro to induce cysts differentiation in epithelial cells to study tissue architecture, physiology and disease mechanisms (mainly in cancer) 7,31,32. In particular, the 3D gel system is largely used to recapitulate and study factors that lead to morphogenesis of tubular ducts and alveoli or acinar structure in the mammary tissue, which is essential to understand breast cancer33,34. Also, it has been shown the 3D gel system allows better maintenance of morphological and functional characteristics of mammary primary cell comparing with tissue culture dish, in which these characteristics are completely lost 33,35. There are two gels type mainly used 7,35: (i) Gels purified from extracellular matrix such as collagen (type I, III, IV), laminin and fibronectin; (ii) Reconstituted basement membrane gels, which are commonly derived from tumours due to the abundant amount of basement membrane found in tumour compare with normal tissue. Matrigel™ is the reconstituted basement membrane most used due to it capacity to induce apical-basal polarization. Matrigel™ consist of laminin, type IV collagen, entactin, heparan sulfate proteoglycans, and fibronectin. Theses gels are used as a thin coat layer over the dish in which cell can grow as a monolayer. Also, due to their thermoresponsive properties, they are liquid at 4℃ and gel at 37℃, cell can be embedded on the gel to create a 3D structure. The 3D structure can be cultured attaching to the bottom of the dish or floating free in the medium. Furthermore, the gels can be later digested with enzymes to release the cells for additional analysis. One of the limitation of this gel system is that due to the nature of the gels the composition from batch-to-batch changes which affect the reproducibility of the experiment and therefore it is difficult to compare different studies 36. Also, changes to the physical and biological properties of the gels are
limited. Hence, there is an emerging trend to create new biomaterial base on hydrogels that provide the same biological clues as natural gels, and at the same time their properties can be modulated.

Both of these in vitro systems give us epithelium tissue models that closely resemble the in vivo situation and can be viable surrogates to animal models. Furthermore, it is feasible to use the principles of theses systems to create devices for tissues engineering in which tissue can be grown and mature in vitro to later on being implanted into the patient. For instance, most of the approaches in engineering complete corneal substitutes used the filter insert culture system as a structural platform to construct the three layer corneal tissue. Although, the material of the insert membrane is not ideal for use in humans, these experiments show a prove of concept that the system can be used as starting point to design a device for engineering corneal tissue in vitro.

2.4 Examples of current advances in epithelium tissue-engineering

Most of the engineered epithelium reported in the literature is generated with the intent to be used as part of an engineered organ such as the trachea or skin. In other cases the engineered epithelium is used as a temporary or permanent substitute to repair damaged epithelium in an organ. For example, engineered epithelial cells layers are used as substitute to repair the corneal epithelium layer in the eye’s cornea and the epithermal layer of the skin. Researchers in the field of tissue engineering have worked over the last decades to find strategies to engineer epithelium for different organs. Some of the main organs in which researches have focus are skin, trachea, lung and cornea. Therefore, we will discuss some of the work that has been done in engineering epithelium for these organs.

ENGINEERED SKIN SUBSTITUTES

The skin is the largest organ in the body and the first barrier of protection that the body has against the environment. It is composed of two tissue layer: (i) A epithelial tissue layer called epidermis which is compose mainly stratified layer of keratinocytes, melanocytes (for pigmentation) and appendages such as hair, nail, sweat and sebaceous glands, (ii) a connective collagen-rich layer called dermis. Engineered skin substitutes are a great alternative for treatment of acute or chronic skin wounds caused by surgical procedures, burns or chronic diseases such venous leg ulcers, in which large portion of skin is compromised. There are two types of engineered skin substitutes: partial engineered skin substitutes which consist of engineered epidermal or dermal tissue, and composite engineered substitutes which consist of both skin layers, the epidermis and dermis. The epithelial or epidermal substitutes generally are composed of just autologous keratinocyte from different sources that are grown in vitro, or a combination of autologous
keratinocytes with biomaterial such as hyaluronic acid membranes and gel-like fibrin, which improve the mechanical stability of the graft\textsuperscript{39}. Examples of current commercially available epidermal substitutes are Epicel, EpiDex \textsuperscript{38}. Composite skin substitutes are made up of both skin layers, the epidermal and dermal layers, which basically consist of keratinocyte cells growing on a dermal substitute. This dermis substitutes are generally autologous dermal fibroblast or allogeneic neonatal foreskin fibroblast grown in biomaterials such as collagen, silk, fibrin glue and polyglycolic acid \textsuperscript{38,40}. Some examples of composite skin substitutes are Apligraft, and OrCel \textsuperscript{41}.

Although all these engineered substitutes have had certain level of success in the clinical setting for wound healing, they still not completely resemble the function and structure of native skin. For instance, these substitutes lack skin appendage such as hair, sebaceous and sweat glands. These appendages are important in obtaining fully functional and aesthetic appearance skin. Currently, researchers are investigating the usage of stem cell in skin substitutes. It is believed that adult stem cells found in the skin, such as stem cells residing in the bulge region of hair follicles, could be a good cell source for skin substitutes due to their multiple differentiation capacity and easy access\textsuperscript{38}. Also, the better understanding of the biology of wound repair would allow to improve the design of the future engineered skin substitutes \textsuperscript{40}. Currently, engineered epithelium in skin lack of the organized multi cellular structure of native epidermis. Therefore, is still necessary to create engineering strategies that allow the generation of epidermis that contains an organized structure with all cell types and appendages find in the native epidermis. In general, the ultimate goal for skin is to create an engineered substitute that provides an effective scar-free wound healing process, which will allow to produce a final tissue that resembles native tissue structure\textsuperscript{42}.

**ENGINEERED EPITHELIUM IN THE RESPIRATORY TRACT**

The respiratory tract is a tree of tubular structures that are composed mainly of epithelial, connective and muscle tissue \textsuperscript{43}. Two different zones composed the respiratory system: a) conditioning zone, b) respiratory zone. The conditioning zone consists of the nasal cavities, pharynx, larynx, trachea, bronchi, and large and terminal bronchioles. The respiratory zone, also known as lung, consists of respiratory bronchioles, and alveolar ducts and sacs \textsuperscript{44}. Epithelium lines the interior of both of these zones, but its structure and function is different in each zone. In the conditioning zone the epithelium is mainly pseudo-stratified columnar \textsuperscript{45}. Its main function is to act as a physical and immune barrier against potential dangerous foreign particles \textsuperscript{46}. In the respiratory zone the epithelium becomes thinner and goes from simple cuboidal epithelium in the respiratory bronchiole to simple squamous in the alveolar ducts and sacs\textsuperscript{44}. In this zone is where the exchange of gases happens; therefore, the lining epithelium is essential for the correct function of the respiratory tract.
The respiratory tract can be damaged due to respiratory diseases, congenital anomalies, cancer and traumas, which is why clinicians and researchers are in the continue quest of finding tissue substitutes and regenerative medicine therapies. Engineering functional respiratory epithelium is important to the more general task of engineering fully functional respiratory tract's substitutes, such as trachea, bronchi, and respiratory ducts and sacs or lungs.

**Trachea**

For many decades clinicians and researchers have seen the need of engineering a trachea substitute. Extensive tracheal resections are needed in patients with large tumours or benign stenosis. When more than 50% of tracheal need to be resected the treatments available are not completely effective. For years researchers in the field of tissue engineering have been focusing on creating a functional engineered trachea. One of the main problems that many group encounter when engineering the trachea is the difficulty of generating a functional lining epithelium due to its complex structure. The respiratory epithelium is composed of different types of epithelial cell such as goblet, ciliated, and basal cells. Therefore the isolation and *in vitro* culture of respiratory epithelial cells is not simple. Different group have investigated approaches to create a lining epithelium for engineered trachea.

In general the strategy that many group have used to engineered respiratory epithelium *in vitro* is isolating epithelial cell populations from different sources such as nasal epithelium, embryonic stem cells, and bone marrow stem cells. Then, these cells are grown and differentiated using air-liquid culture condition in filter insert culture system, or grown in biomaterial such Pluronic F127 and decellularized cadaveric tracheas. Finally, this engineered epithelium is applied as a lining layer to a tubular engineered structure to form a composite engineered trachea *in vitro*. On 2008 one of this strategies was used successfully in the replacement of a part of the trachea in a human patience. Although, the patient had a positive outcome, it took 3 month to develop the engineered graft, which is not suitable for urgent cases. Therefore, currently they are working in improving the procedure to develop a short time protocol.

The major problem of some of these approaches is that they do not test the functionality of the engineered epithelium such as mucous production and mucociliary clearances *in vitro* or *in vivo*. Other consideration that needs to take into account in the future is the unidirectional beating of ciliated cells, which appear to be regulated by the planar cell polarity pathway. There is not doubt that more studies needs to be done to find the optimal cell source, isolation and culture protocol, and *in vitro* system to generate a functional lining respiratory epithelium layer for tracheal replacement.
Lung

The lung is composed of a simple layer of different specialized epithelial cells such as Clara cells, type I and type II alveolar cells. Currently, the only way to replace lung tissue in patient with terminal lung disease is through lung transplantation. Therefore, researcher and clinicians are interested in finding engineering strategies to create lung tissue replacement. Comparing with other tissues very few work have been done in lung tissue engineering. The majority of the work consists in showing the capacity of different biomaterial to sustain and promote proliferation of alveolar cell in vitro. For instance, Sugihara and colleagues reported 1993 for the first time the reconstruction of alveolar-like structure in vitro by growing rat alveolar epithelial type II cells in a three-dimensional collagen gel. Cells form luminal structure with specific polarization and produce surfactant protein. Lin and colleagues demonstrated in 2006 the potential of using Poly-DL-lactic acid (PDLLA) as a scaffold for engineering lung. They successfully grew a murine lung epithelial cell line (MLE-12) on 2D and 3D PDLLA scaffold. However, an organized structure was not found in either of 2D or 3D scaffold. Cortiella and colleagues showed the potential of polyglycolic acid (PGA) and Pluronic F127 to promote the proliferation in vivo and in vitro of isolated adult lung progenitor cells from mammalian tissue. The cells seed on the material express clara cell protein and surfactant protein C and generate in vitro and in vivo pulmonary-like structures. Following the same approach that Micchiarini group used to engineer trachea a group from Yale University reported in 2010 the repopulation of a decellularized rat lung with pulmonary epithelial cells and endothelial cells. The organization of the cells and general microstructure of the engineered lung was very close to the native lung. Moreover, they showed the capacity of the engineered lung to produce gas exchange for short periods of time when implanted into rats. However, the authors state that multiple issued need to be address to obtain long term functionality.

There is still a lot of work ahead in the field of lung tissue engineering. A suitable source for pulmonary epithelium that gives rise to different pulmonary epithelial cell types, such as stem cell, is essential to allow approaches such as the decellularized lung to be used in clinical settings. Also, to be able to create lung tissue with native lung microstructure it is important to better understand in more detail the lung structure to be able to create strategies capable of give environmental clues to the cells to allow them anatomically organized, like the decellularized matrix seems to be doing.

ENGINEERED CORNEA

The cornea is composed of three tissue layers, an external stratified epithelium, a middle stromal layer and an internal endothelium layer. The specific arrangement of the stroma extracellular matrix together with the size, density and structure of all corneal cells provides the
cornea with optical transparency and refraction power, which are the most important properties of the native cornea. Research in tissue engineering cornea have been focused in develop single tissue cornea layer substitutes such as epithelial and stromal layer, and complete cornea substitutes compose of more that one layer. In this introduction we will be focusing in cornea epithelial layer substitutes and in some new approaches for complete cornea substitutes.

As mention above the epithelial layer is located in the cornea surface, and due to this position it is constantly expose to environmental damages. Therefore, the epithelium is often renew by stem cells that are located in the transitional area between the cornea and the bulbar conjunctiva, called limbal area. When this source of stem cell is damaged due to a trauma such as ocular burns or diseases such as Stevens-Johnson syndrome the cornea gets opaque causing a severe vision lost. Current treatment for cornea opacification is transplantation of autologous limbal epithelial stem cells from the healthy patient eye. However, this procedure requires a large limbal graft and is not suitable with patient with bilateral lesion. When there is a bilateral damage the current treatment is allograft transplantation, which require the use of immunosuppression drugs and finding suitable donors. Therefore, in the last years clinician and researcher have developed alternative treatment using tissue engineering approach. The general approach to engineered epithelium corneal layer in vitro is to expand epithelium cell from the healthy eye or mucosa cells, and growing them in natural scaffold such as amniotic membranes and fibrin gels or in special temperature responsive cell-culture dishes developed by a group in Japan (cell sheet technology). Finally, this engineered epithelium corneal layer is transplanted to the injured eye. These methods have been successfully used in treatment of unilateral and bilateral cornea lesion in animals and humans.

Regarding the development of complete substitutes, researchers are also investigating different tissue engineering approaches. Currently, the only treatment for irreversible complete corneal damage is allograft corneal transplantation, which is not ideal treatment due to risks of tissue rejection, complication with immunesupresor drugs, and low availability of donor tissue. The common approach for engineering a three layer corneal construct in vitro consists in isolate and expand human or animal corneal cell in vitro. Then a filter insert filter is used as a support for 3D construct creation. First, stromal cell mix with different material such as collagen gel, collagen/chondroitin sulphate and fibrin/agarose or not material is seeded on the filter insert and then a layer of epithelial and endothelial cells are applied on the top a bottom of the stromal layer, respectively. In general the studies claim that their corneal construct had similar structure to the native cornea and that however these construct are still not suitable for transplantation they can be used for biomedical and pharmaceutical research. Currently, due to the recent success of the
usage of decellularized matrices in engineering other tissues 54 two groups are investing the usage of decellularized porcine corneas matrix repopulated with corneal cells63,70.

In general strategies of engineering epithelium for epithelial corneal layer have been successful. However, it is clear that there is still a lot of work ahead to find approaches that can engineer complete corneal substitutes for implantation. First, suitable cells sources for the three layers will need to be study. Also, it is known that the specific arrangement of the stromal extracellular matrix together with the size density and structure of all corneal cells provides the cornea with optical transparency and refraction power 63,70. Therefore, future designs need to have into account this specific organization to be able to engineer transplantable functional corneas.

Most of these strategies to engineer epithelial tissue from different organs do not include design factors that lead to generate epithelium with correct/native like structure. This results in tissues that are partially functional. To be able to introduce design factors that allow to generate an epithelium with better structure it is important introduce knowledge that biologist have about epithelial cell biology and tools that they use to general this knowledge in future strategies to engineer epithelium.

2.5 Tools from tissue engineering to organize epithelium

2.5.1 Cell Micropatterning

Cell micropatterning comprises a group of methods that allow to spatial and geometrically control cell placement and shape in a substrate. These techniques are applied in different fields such as cell biology and tissue engineering 73. In cell biology is used to study cellular migration 74, mechanical stress influence in cell behaviour75, proliferation 76, and differentiation 77. In tissue engineering theses techniques have been broadly used due to their capacity to control and reproduce the natural cell environment in vitro78,79. For instance, with this techniques different cell populations can be static 79,80 or dynamically 81 co-culture side by side on a substrate. This creates a more tissue-like structure and natural environment in vitro, which allows to engineer tissue models to use for different purpose such as drug screenings. For epithelium tissue engineering and epithelial cell biology cell micropatteming is not commonly used, but it has a great potential to answer different biological question and generating epithelium tissue with better organized structure. A reason for this could be the lack of developing these techniques in filter insert membranes, which is the suitable substrate or culture system for growing and differentiating epithelial cells. One of the few example in where cell micropatteting is used in epithelium studies has elucidate the importance of mechanical stress in the epithelial-mesenchymal transition 75.
A number of micropatterning techniques exist to spatially organize cells into distinct microsheets on solid substrates. Currently the simplest and most classic methods for micropatterning cells are microfluidic patterning, microcontact printing, and the use of microstencils.

1) **Microcontact printing** utilizes an elastomeric stamp or mould, usually made of poly(dimethylsiloxane) (PDMS), prepared by casting the liquid prepolymer of an elastomer against a master, fabricated using photolithography, which has a patterned relief structure. This patterned stamp is then coated with a desired adhesion protein and brought into contact with a cell-repellent substrate. When the stamp is removed, a pattern of the adhesive protein remains on the substrate. Cells preferentially adhere to substrate regions containing the adhesive protein. The most common proteins used are fibronectin, laminin, collagen and gelatin. To generate monopattern using this method, the no printed area is treated with a material that resist protein adsorption such as bovine serum albumina or pluronic F27 to stop cells from growing out from the printed protein. To generate patterned co-cultures using this method, the first cell type is seeded in serum free medium and allowed to adhere to the microstamped proteins. Then a second cell type is seeded in serum-containing medium and adheres to the locations where the first cell type is not present.

2) **Stencil-based techniques** involve generating thin poly(dimethylsiloxane) (PDMS) or cell-repellent hydrogel membranes containing holes of a desired pattern. These membranes are formed by molding PDMS or the hydrogel around an elastomeric stamp, usually also made of PDMS, prepared by casting the liquid prepolymer of an elastomer against a master, fabricated using photolithography. This step requires access to a clean room. The PDMS membrane or hydrogel layer containing hole regions is generated on a desired cell adhesive substrate, such as glass of tissue culture polystyrene (TCPS). Cells cannot adhere in regions where PDMS or hydrogel is present but can adhere in the “hole regions” where the underlying cell adhesive substrate is exposed.

3) **The Microfluidic technique** uses a PDMS membrane with patterned microchannels network molded into its surface. The PDMS is place over a solid surface to close the microchannels of the PDMS membrane. Then the microchannels are injected with cells or with a particular solution to create the patterns in exposure regions. This technique is used to mono-pattern or co-pattern side by side multiple cell types.

The first two of these three methods are the most adaptable technique to use for patterning epithelial cell on filter insert culture systems to generate complete structure that resemble the
native epithelium structure. Additional, in general all these methods are powerful tools to understand the influence of different factors such as physical, mechanical, chemical in the cell and tissue integrity.

2.6 Conclusion

The epithelium has very specific structural properties that allow it to perform all its functions in the body. This structure is not easy to mimic or generate in vitro. Biologists have found tools such as filter insert membranes that allow resembling part of the epithelium structure in vitro, the apical-basal polarization. However, other features of the epithelium structure such as the planar organization have not being generated in in vitro system. Engineering tools such as micropatterning techniques that could allow to create a more organized epithelium structure have not been introduced in strategies to engineer epithelium. This could be due to the lack the type of substrates that normally are used in micropatterning techniques, which are no suitable to induce correct structure in epithelial cells. Therefore, is important to be able to integrate knowledge from basic science and engineering to create new approaches for engineering more mature/functional epithelium.

2.7 Reference


Chapter 3**

Developing methods of micropatterning epithelial cells in mono and co-culture on filters insert culture system

**Two manuscripts based on this work have been submitted to Lab on a Chip (manuscript in press) and Journal of Epithelial biology and Pharmacology (manuscript in review). This work was completed in collaboration with S. Javaherian. The contributions of A. Paz to this work were developing the agarose microwell pattern method and developing the fibronectin co-culture method.

3.1 Introduction

Apical-basal polarization of the epithelial cells is a key requirement for developing functional and mature epithelium \(^1\). Currently, the most widely used 2D system to generate polarized epithelium *in vitro* involves the filter insert culture system. This culture system provides a better system to study physiological and morphological properties of the epithelium due to its closer resemblance to the *in vivo* situation \(^2\), as opposed to standard cell culture systems. Generally, this *in vitro* culture system uses either pure populations of epithelial cells or unorganized mixtures of primary cell populations harvested from epithelial tissues. Also, epithelial cells are cultured together with different cell types grown on the lower chamber, such as stromal cells. This co-culture condition has shown to improve the proliferation and differentiation of epithelial cells \(^3\). Some of the disadvantages of this system are: (i) it is challenging to generate a uniform epithelial sheet throughout the surface of the insert membrane which makes it difficult to characterize the effect of a sample treatment on epithelial sheet organization, (ii) the system only allows to create a co-culture condition limited to study the role of diffusible factors, and completely leaves out the role of direct cell-cell interactions.

In order to set up our experiment we needed to spatially control the epithelial cell population on the filter insert membrane to first allows us to better characterize the organization of a population epithelial sheet, and second to allow us to co-culture side by side two different epithelial populations to study the role of direct cell-cell interactions in epithelial cells sheet organization. Currently, the simplest and most common methods for micropattening cells or spatially control cell seeding are designed for solid substrates such as glass or plastic \(^4\); however, these techniques have not been translated for use with epithelial cells cultured using the filter insert culture system. Therefore, we set out to adapt solid substrate patterning methods \(^5\text{--}8\) to enable micropatterning of one and two types of epithelial cell population on filter insert
membranes. Specifically we aimed to develop a method that allowed us to (i) generate monocultures of epithelial microsheets with controlled dimensions and shape and (ii) spatially control two epithelial cell populations in a side by side co-culture while maintaining the culture conditions required for epithelial cell apical-basal polarization and maturation.

The most common methods for micropattening cells reported in the literature that provided a starting point for our method development are microfluidic patterning, microcontact printing, and the use of microstencils. None of these patterning strategies has been used previously to generate patterned monocultures or co-culture of epithelial cell sheets for subsequent apical-basal polarization and maturation of epithelial cells in the filter insert culture system. Furthermore, in the case of mono-patterning cells in glass or plastic substrates with most of the existing methods the spatial patterning of the cell can only be maintained between two to seven days, which is not ideal for epithelial cell applications where an extended culture period is required to induce apical-basal cell polarization; and in the case of patterned co-cultures produced using these strategies it is not clear how well it can be maintained over time.

Of the existing microprinting methods microcontact printing, and the use of microstencils appeared to be the most adaptable for use on filter insert membranes and provided a starting point for developing our monopattening and co-patterning strategies.

In this chapter we describe:

1) A method we adapted from hydrogel micropatterning to generate patterned monoculture epithelial sheets and demonstrate that within these patterned “microsheets” epithelial cells apically-basally polarize in the same way as unpatterned sheets.

2) A set of strategies for micropatterning co-cultures of epithelial cells on filters insert membranes. Specifically, we have adapted the extracellular matrix protein stamping technique to generate patterned co-cultured epithelial sheets and we have also developed a novel patterning method using Parafilm inserts that do not require a clean room or complex equipment, making it more adaptable for epithelial biology labs without access to photolithography tools. Using Parafilm inserts we show that we can pattern co-culture epithelial sheets into stripe patterns. Our patterning methods provide a set of tools for patterning epithelial cell populations while maintaining the culture conditions necessary to generate polarized epithelium.
3.2 Method

3.2.1 Cell Culture

We used two different epithelial cell lines, the ARPE-19 human retinal epithelial cell line (ATTC, USA) and the MDCK dog kidney epithelial cell line (ATTC, USA) at passages between P10-20. ARPE-19 cells were grown in Dulbecco's modified Eagle's medium/nutrient F-12 (DMEM/F-12, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Canada) and 1% Penicillin/Streptomycin (VWR, Canada) and maintained in an incubator at 5% CO₂. MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM, Biowhittaker) supplemented with 10% FBS and 1% Penicillin/Streptomycin and maintained in an incubator at 5% CO₂.

3.2.2 Master and PDMS stamp fabrication

We created an SU-8 master containing specific topographic features as described previously. Briefly, we designed a photomask with the desired geometric patterns (200 µm-widestripes) using AutoCad and printed the pattern in high-resolution black ink on a transparent sheet. We exposed negative photoresist (MicroChem Corp, USA), spin-coated on to a clean 3 x 5 glass slide, to UV light through our mask to transfer the geometric patterns from the photomask to the photoresist. We then washed away unexposed photoresist with a developer solution (MicroChem Corp, USA) to generate a master with the desired topographic features. The master was silanized under vacuum for 3 hours using 1-Trichlorosilane (UCT, USA). We generated a PDMS stamp by replica molding as described previously [37]. Briefly, we poured a degassed 10:1 (w/w) mixture of PDMS polymer:curing agent ((Dow Corning Corp, USA) over our master and cured at 60 ºC for 3 hours. The hardened PDMS stamp was then peeled off from the master.

3.2.3 Parafilm insert fabrication

To generate parafilm inserts to fit into filters inserts for a 6-well plate we generated a stripe pattern in the parafilm inserts using surgical blades (No 10, Feather, Japan). The cut parafilm pieces were removed using tweezers leaving behind holes of rectangular shape in the parafilm inserts. To bind the parafilm inserts onto the filter insert membrane we pressed the parafilm insert firmly onto the filter insert surface to ensure full contact and a good seal with the substrate material. We then added 1 mL of phosphate buffer saline (PBS, VWR, Canada) into each well and degassed the films for 5 minutes at a pressure of 30 psig. This step was critical to ensure liquid infiltration into the small stripes and subsequent cell patterning. We UV sterilized the films in PBS for 20 minutes and then removed the PBS from the wells before cell culture. We then added FBS to the filters and incubated at 37 ºC for 30 minutes prior to seeding of the first cell type.
3.2.4 Filter insert patterning strategies

Figure 3.1 outlines our strategies for mono or co-patterning epithelial cells population on filter insert membranes using an agarose layer (A), and fibronectin microprinting (B) or parafilm inserts (C). All methods used filter inserts with 0.4 µm pores PET membrane (Corning, USA).

To monopattern cells using agarose microwells (Figure 3.1A) we adapted a previously reported method for micropatterning cells on glass slides by micromolding agarose around a PDMS stamp to generate an agarose layer containing holes replicating the shapes of the PDMS posts of the stamp. We generated a patterned agarose layer using a 0.5 cm x 0.5 cm PDMS stamp with 200 µm square pattern arrays. We cleaned the PDMS stamp with 70% ethanol for at least 30 min prior to use. We placed the PDMS stamp against the filter insert’s membrane with the features side down and perfused a hot solution of three parts of 1% agarose (Sigma Aldrich, USA) with two parts anhydrous ethanol under the PDMS stamp. Leaving the stamp in place, we gelled the agarose by incubating in the freezer (-20 ºC) for 3 min and then at room temperature for 5 min. We removed the PDMS stamp with forceps by pulling straight up to generate the patterned agarose layer on the filter insert membrane. Before the cells were seeded the micropatterned filter insert membrane was exposed to UV light for 30 min and coated with 25 µg/ml of fibronectin for 1 hour at room temperature and then rinsed twice with cell culture medium. To seed cells we added 500 µl of a 2×10⁶ cell/ml solution in serum medium over the agarose patterns and incubated at 37 ºC, 5% CO₂ for an hour. Around the agarose patterns we used a PDMS frame (the PDMS frame was cleaned by submerging it in 70% ethanol for 30 min and dried with air) to contain the cells over the pattern. After one hour, we removed the PDMS frame and any unattached cells by gently washing two to three times with serum medium. We added 1.5 ml of serum-containing medium to the top compartment and 2.6 ml of serum-containing medium in the lower compartment of the filter insert culture system. On the next day we switched the medium in the top compartment to serum-free medium and continued the culture for up to 2 weeks periodically supplying the cells with fresh medium in both compartments.

To co-pattern two epithelial cell populations we developed two different strategies. Our first strategy (Figure 3.1B) is an adaptation of cell patterning techniques carried out by controlled deposition of ECM proteins originally developed for use on glass or plastic substrates. This strategy makes use of a PDMS stamp generated by soft-lithography by replica molding the stamp over topographic features generated using photolithography. To generate patterned co-cultures on the filters insert membrane using this fibronectin microprinting method, first a 0.5 cm x 0.5 cm PDMS stamp with 200 µm-wide stripes pattern arrays was sterilized by submerging the stamp in 70% ethanol solution for at least 30 min and dried with air. Then, the stamp was placed in a sterile...
Petri dish with the pattern side up, covered with 20 µl of 100 µg/ml fibronectin solution (Sigma Aldrich, Canada), and incubated for one hour at room temperature. Excess of not absorbed fibronectin onto the stamp was then carefully removed by blotting the stamp on a kimwipe and rinsing it once with double distilled water. The stamp was then blotted again on a kimwipe until there was no visible liquid left on the stamp. Immediately after drying, the inked stamp was placed for 10 min on the filter insert membrane, and a 6 g weight was placed over the stamp to ensure conformal contact. After 10 minutes the weight was removed, a PDMS frame was placed around stamp (this frame was later used to contain the cell seeding solution), and the stamp was removed with tweezers. A 0.05% (W/V) BSA (Sigma Aldrich, Canada) solution was then added over the deposited fibronectin pattern and incubated for one hour at 37°C. Finally, the BSA solution was removed and the membrane was rinsed with serum-free medium.

To seed the first cell type (GFP MDCK or GFP ARPE-19) we prepared a cell suspension of 2 × 10^6 cells/mL in serum-free medium and placed 500 µL of the suspension over the fibronectin pattern. The sample was incubated at 37 °C, 5% CO₂ for 1.5 hours for ARPE-19 and 2.5 hours for MDCK cells. Next, un-adhered cells were removed by rinsing the filter insert membrane with serum-free medium. We added 1 ml of serum-free medium to the bottom compartment of the filter insert culture system to better visualize the generated patterns on the filter insert membrane by microscopy. To generate patterned co-cultures the sample was incubated with FBS for 20 min at 37 °C to deactivate the BSA blocking before adding the second cell type. We then prepared a cell suspension (of the second cell type) of 3 × 10^6 cells/mL in serum medium and added 500 µL of it to the sample and incubated at 37 °C, 5% CO₂ for 2 hours for ARPE-19 and 3 hours for MDCK cells. Un-adhered cells were then removed by rinsing the filter with serum medium. Finally, we added 1.5 ml of serum medium to the top compartment and 2.6 ml of serum medium to the bottom compartment. On the next day we switched the medium in the top compartment to serum-free medium and continued the culture for up to 2 weeks periodically supplying the cells with fresh medium in both compartments.

Figure 3.1C shows our alternative more simple strategy for co-patterning that uses parafilm inserts. While this parafilm strategy is limited to only striped geometric patterns and is not suitable for applications where features smaller than 100 µm are desired 6, this method is significantly easier to optimize than the microprinting technique, does not require use of a photolithographic master, and the geometry is adequate to answer a wide range of questions requiring spatially organized epithelium. To generate patterned co-cultures using the parafilm insert method we placed a parafilm insert containing 300 µm stripe arrays and firmly bonded the parafilm insert to the filter insert membrane. To seed the first cell type (GFP MDCK or GFP ARPE-19) we added
500 µL of a cell suspension of 2 × 10⁶ cells/ml making sure that the entire surface of the insert was covered by the cell suspension. We incubated the samples at 37 °C, 5% CO₂ for 3 hours. Next, we removed the cell suspension and gently washed away any un-adhered cells with our cell culture medium. To generate the co-cultures, we removed the parafilm inserts 3 hours after seeding of the first cell type for MDCK and 12 hours for ARPE-19 and incubated the patterned cells in fetal bovine serum for 20 minutes, at 37 °C. We next removed the FBS and seeded 500 µl of a second cell type (MDCK or ARPE-19) at a seeding density of 3×10⁶ cells/ml. We incubated the patterns for 1 hour to allow the additional cells to adhere to the surface free from the first cell type and then thoroughly washed the substrates with growth medium to remove any un-adhered cells. Finally, we added 1.5 ml of serum medium to the top compartment and 2.6 ml of serum medium to the bottom compartment. On the next day we switched the medium in the top compartment to serum-free medium and continued the culture for up to 2 weeks periodically supplying the cells with fresh medium in both compartments.

**Figure 3.1 Schematic of mono-pattern and co-pattern methods.** (A) Schematic of agarose mono-patterning method. (B) Schematic of fibronectin co-culture method. (C) Schematic of parafilm co-culture.

### 3.2.5 Filter insert culture conditions

For the three strategies we cultured the patterned mono and co-cultures overnight, for one week or for two weeks. In the case of the one and two week cultures, to generate a full-polarized epithelial sheet we cultured the cells with serum medium in the top and bottom compartments of the filter insert culture system for 1 day and then replaced the medium in the top compartment with serum-free medium for the further days of culture. To generate un-patterned control samples for comparison we seeded 2 × 10⁶ cells/mL on to filter insert and incubated overnight with serum
medium in the top and bottom compartments of the filter insert culture system. After one day we replaced the medium in the top compartment with serum-free medium and cultured the cells for one or two weeks.

3.2.6 Assessing patterned epithelial sheets

To assess the formation of mono and co-patterned sheets of MDCK or ARPE-19, the sheets were fixed on the filters in 4% paraformaldehyde, washed in phosphate buffered saline, permeabilized using 0.1% triton-X100. For the monopatterns we stained MDCK cells with monoclonal antibodies against β-catenin (Invitrogen, 1:200) and FITC-conjugated anti-mouse (1:250, Sigma Aldrich) secondary antibodies. We stained ARPE-19 with Rhodamine phalloidin (Invitrogen, 1:100). To characterize the distribution of the two cell types on the filter inserts over time we stained co-cultures with DAPI (Invitrogen, Canada) and observed the GFP signal from the cells expressing GFP. Images were taken on an Olympus IX81 microscope and a Nikon D-eclipse-C1 confocal microscope.

3.2.7 Assessing epithelial cell polarization markers

To determine if the patterned cells undergo the same expected apical-basal polarization as is seen in unpatterned cultures we assessed the typical apical polarization marker ZO-1. We also assessed the presence of cilia using an antibody against acetylated α-tubulin (Sigma Aldrich, USA). We fixed cells (patterned and unpatterened) on the filters in 4% paraformaldehyde, washed in phosphate buffered saline, permeabilized using 0.1% triton-X100, and stained with DAPI (Invitrogen), and monoclonal antibodies against ZO-1 (Invitrogen, 1:100), and acetylated α-tubulin (Sigma Aldrich, 1:500) for fluorescent microscopy. We used Cy3-conjugated anti-mouse (1:250, Millipore, USA) and FITC-conjugated anti-mouse (1:250, Sigma Aldrich) secondary antibodies for imaging. Images were taken on a Nikon D-eclipse-C1 confocal microscope.

3.3 Results

3.3.1 Monopatterning cells using agarose microwells

Using the agarose patterning strategy outlined in Figure 3.1A we generated microepithelial sheets with controlled geometry from two different epithelial cell lines, MDCK canine kidney epithelial cells and ARPE-19 human retinal epithelial cells. Figure 3.2 shows arrays of MDCK and ARPE-19 epithelial cell microsheets patterned using the adapted agarose method (A-D) at day 1 after cell seeding. We next wanted to determine how long we could maintain our micropatterned sheets before cells started to outgrow the patterns. Ideally, we want to culture the epithelial cells for 15 days to allow maturation; therefore we assessed patterns after 15 days in culture. Figure 3.3 shows microsheets of MDCK (A-B) or ARPE-19 (C-D) epithelial cell microsheets
patterned using the adapted agarose method after 15 days of culture. MDCK and ARPE-19 cells remained contained within the pattern for up to 15 days in culture, the average timeline necessary for apical-basal polarization of the epithelial cells on filter insert culture system. However, an edge effect was observed on MDCK cells, in which cells started forming multilayer structures at the pattern edges (this can be see in the ZO-1 stained samples at 7 and 15 days shown in Figure 3.4). We speculate this was due to continued growth of the MDCK cells, and a lack of contact growth inhibition at the pattern edges. We stopped the experiment on day 15 because this was our experimental timeline; however, at this time point the patterns remained in a good condition and there were no signs of pattern degradation. We therefore anticipate the patterns could be maintained for even longer time periods, if required. When using the agarose patterning on glass or tissue culture plastic the patterns start degrading in about 7 days; this was not the case when we adapted this method for use with the filter insert culture system.

Figure 3.2. Micro-patterning of epithelial cells on filter insert membranes using agarose method at day 1. (A) Overview of MDCK cells on 200μm square array patterns stained for β-catenin. (B) MDCK cells on 200μm square pattern stained for β-catenin. (C) Overview of ARPE-19 cells on 200μm square array patterns stained for Phalloidin. (D) ARPE-19 cells on 200μm square pattern stained for Phalloidin. Scale bars, 200 μm A and C; 50μm B and D.
3.3.2 Co-culturing cells using fibronectin microprinting and parafilm insert methods

Here we present two methods for co-patterning epithelial cell populations on filter insert membranes. Firstly we generated a method to co-culture two population of cells by adapting a combination of two previously developed methods for microprinting ECM protein on rigid substrates for generating cell patterns and a co-culture method using microstencils. This method allows generation of any arbitrary size and shape pattern. We also devised a method to co-culture cells into simple stripe patterns using parafilm inserts, which although simplistic is adequate for a number of applications and can be performed without access to specialized equipment. Figure 3.4 shows co-patterned MDCK/GFP MDCK and ARPE-19/GFP ARPE-19 epithelial cell sheets, zero days after patterning, generated using our adapted fibronectin microprinting and our parafilm insert methods. As expected, both methods resulted in patterns of alternating stripes of GFP and non-GFP cells. For the fibronectin microprinting method the stripes were about 200 µm in width for the GFP cells and 500 µm for non-GFP cells. For the parafilm insert method the GFP cells stripes were about 250 µm and non-GFP cell stripes about 400 µm. As the

Figure 3.3 Micropatterning of epithelial cells on filter insert membranes using agarose method at day 15. (A) Overview of MDCK cells on 200µm square array patterns stained for β-catenin. (B) MDCK cells on 200µm square pattern stained for β-catenin. (C) Overview of ARPE-19 cells on 200µm square array patterns stained for Phalloidin. (D) ARPE-19 cells on 200µm square pattern stained for Phalloidin. Scale bars, 200 µm A and C; 50µm B and D.
paraflm inserts are generated manually the accuracy and precision of the stripe sizes are not as high as that of fibronectin microprinting method.

Selecting the correct cell density for seeding and the correct timing for the incubation period was critical for achieving robust patterning in each of the methods and with each cell type. For example, in the fibronectin microprinting method for MDCK cells, an initial seeding density of $2 \times 10^6$ cells/mL with an incubation period of 2.5 hours before addition of the second cell type produced good patterning of the cells, and for ARPE-19 cells an initial seeding density of $2 \times 10^6$ cells/mL with an incubation period of 1.5 hours produced good patterning. Also, the incubation of the filter with FBS for 20-30 min before the addition of the second cell type was crucial to promote the attachment of the additional cells on the filter insert membranes. We found that although the fibronectin microprinting method allowed arbitrary pattern generation, stamping on the filter insert membrane was more difficult than on glass or tissue culture substrates. It was, therefore, very important to remove the excess fibronectin by blotting the stamp on kimwipe paper, instead of drying the stamp with air or nitrogen gas. Also, the stamping step had to be done quickly so the inked stamp did not completely dry. After the stamp was placed on the filter it was important not to attempt to move the stamp until after the stamping period was complete. All the procedures were done with the filter insert placed in the well plate holder.

For the paraflm insert method the critical step in ensuring the successful generation of patterns was bonding of the paraflm insert to the filter membrane. Here, it is crucial to ensure the strongest possible bonding by applying pressure without disturbing the patterns in the paraflm insert. We found that the degassing step provided a good indication of whether the bonding between paraflm and the membrane was sufficiently strong. In the case of inadequate bonding the paraflm insert detached from the membrane under vacuum during the degassing step. Figure 3.5 shows maintenance of patterns over 15 days periods of culture with serum free medium in the top compartment. Using both techniques MDCK cell sheets remained clearly patterned after 15 days in culture suggesting limited cell mobility within the polarized epithelial sheet (Figure 3.5 A, B). However, with the fibronectin microprinting method the patterns appear to change over time (Figure 3.5 B) more than in samples patterned using the paraflm insert method (Figure 3.5 A). ARPE-19 cells moved more significantly over the 15-day period and significant pattern distortion was observed when using both patterning techniques (Figure 3.5 C, D). This is most pronounced in the fibronectin microprinting method (Figure 3.5 D), where even by day 7 (Appendix-Figure 3.1) the GFP expressing cells mix with the wild type cells. ARPE-19 cell pattern fidelity was lower than in MDCK cells with the paraflm insert method as well, where by day 15 the pattern, while still distinguishable, was not sharp and the two cell populations had mixed.
Figure 3.4. Co-patterning of GFP expressing and non-GFP epithelial cells on filter insert membrane at Day 0 using parafilm and fibronectin method. (A) Co-culture of GFP MDCK and wild type MDCK cells generated by parafilm insert method. (B) Co-culture of GFP MDCK and wild type MDCK cells generated by fibronectin microprinting method. (C) Co-culture of GFP ARPE-19 and wild type ARPE-19 cells generated by parafilm insert method. (D) Co-culture of GFP ARPE-19 and wild type ARPE-19 cells generated by fibronectin microprinting method. Scale bars 200 μm. Blue-Nucleus, Green-GFP

Figure 3.5. Co-patterning of GFP expressing and non-GFP epithelial cells on filter insert membrane at Day 15 using parafilm and fibronectin method. (A) Co-culture of GFP MDCK and wild type MDCK cells generated by parafilm insert method. (B) Co-culture of GFP MDCK and wild type MDCK cells generated by fibronectin microprinting method. (C) Co-culture of GFP ARPE-19 and wild type ARPE-19 cells generated by parafilm insert
3.3.3 Assessing apical-basal polarization on the filter insert

To ensure micropatterning the cells did not alter the apical-basal polarization of the epithelial cells on the filter insert culture system we stained patterned and un-patterned MDCK epithelial sheets for ZO-1, a typical marker of apical-basal polarization ¹⁴, and acetylated α-tubulin, a marker of cilia, a typical indicator of mature epithelium ¹⁵,¹⁶. **Figure 3.6** compares the ZO-1 expression in patterned microsheets (A-C), co-cultured sheets (D-F) and un-patterned (G-I) MDCK cells cultured for 1, 7 and 15 days on filter insert membranes. In the three conditions ZO-1 localized to the cell membrane in a sub-apical location from day 1 as expected ¹⁶. No difference was observed in the level of ZO-1 protein and its localization between the patterned microsheets, co-cultured and un-patterned MDCK cells (**Figure 3.6** J-R).

**Figure 3.7** compares the primary cilia distribution in patterned microsheets (A,B), co-cultured sheets (C,D) and un-patterned (F,G) MDCK cells cultured for 7 and 15 days on filter insert membranes. As expected, primary cilia were only present in a sub-set of the MDCK cells ¹⁷. We manually quantified cilia numbers in cells under the three conditions at day 15 in a 50 µm × 50 µm square area. Approximately, 25 cilia in co-cultured, 20 cilia in unpatterned filters and 22 cilia in patterned microsheets were present in the same area, suggesting that patterning the epithelial cells into microsheets did not adversely affect epithelium maturation. Furthermore, under the three conditions samples showed the presence of cilia by day 7 suggesting the rate of epithelial polarization was also not significantly affected by our patterning and co-culture technique.
Figure 3.6. ZO-1 expression on monopatterned, co-patterned and un-patterned MDCK cells on filter inserts membrane over time. Images A to I show projected stacks of ZO-1 over time. (A,B,C) ZO-1 staining on monopatterned MDCK cells at day 1, 7 and 15 respectively. (D,E,F) ZO-1 staining on co-cultured GFP-MDCK and wild type MDCK cells generated by parafilm insert method at day 1,7,15 respectively. (G,H,I) ZO-1 staining on un-patterned MDCK cells at day 1, 7 and 15 respectively. Scale bars 50 μm. Red-ZO-1, Blue-nucleus, Green-GFP. Images J to R show the z position of ZO-1 over time. (J,K,L) ZO-1 position on monopatterned MDCK cells on day 1,7 and 15 respectively (M,N,O) ZO-1 position on co-cultured cells on day 1,7 and 15 respectively. (P,Q,R) ZO-1 position on un-patterned cells on day 1,7 and 15 respectively. Scales bars 5 μm. Red ZO-1 protein, Blue-nucleus.
Figure 3.7. Primary cilia formation on monopatterned, co-patterned and un-patterned MDCK cells on filter insert membrane over time. (A,B) Primary cilia on monopatterned MDCK cells at day 7 and 15 respectively. (C,D) Primary cilia on co-cultured GFP MDCK and wild type MDCK cells generated by parafilm insert method at day 7,15 respectively. (E,F) Primary cilia on un-patterned MDCK cells at day 7 and 15 respectively. Scales bars A,B,E and F 50 μm and C and D 100 μm. Red- primary cilia marked by acetylated α-tubulin

3.4 Discussion

Cell patterning is a strategy to spatially control the location of one and multiple cell populations on a cell culture substrate. Generating distinct monopattered microsheets allows many sheets to be exposed to the same experimental conditions in parallel. This allows for better testing of the reproducibility of different experimental treatments on cell sheet behaviour. Additionally, monopatterned epithelial cells in microsheet could also be a useful tool to better characterize the organization of a population epithelial sheet because it allows generation of a more uniform sheet.

We therefore set out to adapt solid substrate patterning methods to enable micropatterning of epithelial cells into microsheets with controlled dimensions and shape, while maintaining the filter insert culture conditions required for complete epithelial cell apical-basal polarization and maturation. A recent report described a filter insert culture system patterning method that involved infiltration of a PDMS pattern into the filter insert’s membrane. However, this does not allow one cell type to be micropatterned over the extended time periods required for maturation of epithelial cells; therefore the usefulness of this method for patterning epithelial cells undergoing apical-basal polarization was unclear. We therefore decided to adapt an agarose patterning method that allows the generation of microsheets with arbitrary shape and dimensions originally on glass or culture plastic substrates.

A key issue we needed to address was maintaining the microsheet patterns for an extended culture period (10-20 days) to allow adequate maturation of the epithelial sheets on the filter insert culture system. Typically, micropatterning agarose methods contain cell patterns for a few days (4-9 days) depending on the cell type. We found however that on filter insert membrane the agarose layer was capable of containing cell growth within the patterned microsheet regions for up to 15 days for both ARPE-19 and MDCK epithelial cells. While we only observed patterns for up to 15 days, we anticipate cell growth and maturation could have been maintained even longer if required, since there was no indication of pattern degradation by day 15. We speculate that agarose allows better cell containment on the filter insert membrane because during moulding of agarose some gel infiltrates the pores of the membrane creating a tight interlocking bond between the gel layer and the membrane substrate. This bond prevents detachment of the agarose layer from the
substrate (as is seen when this technique is used on glass or culture plastic) and, hence, allows longer pattern containment.

Another surprising advantage of patterning the epithelial cells into microsheets was improved uniformity across the microsheets. When cells were cultured on large filter inserts (24 mm diameter) we noticed large variations in cell density and cell shape in different regions of the filter insert membrane. Patterning the cells significantly reduced this variation within individual microsheets making image analysis more simple and allowing easier analysis of the epithelium structure. This could have advantages in applications such as studying the effects of mechanical stress associated with the shape of colony on a behaviour of mature apically-basally polarized epithelial cells. Finally, micropatterned cells can be used for the study of paracrine signalling in mature epithelial cells.

In order to set up our experiment we needed to spatially control epithelial cell populations on the filter insert membrane to allow us to co-culture side by side two different epithelial populations to study the role of direct cell-cell interactions in epithelial cells sheet organization. Therefore, we also set out to adapt solid substrate patterning methods to enable co-culture micropatterning of epithelial cells into patterns with controlled dimensions, while maintaining the filter insert culture conditions required for epithelial cell complete apical-basal polarization and maturation. We adapted two methods, originally developed for cell patterning on glass or culture plastic substrates: a fibronectin microprinting method, which allowed us to produce co-patterned epithelial sheets with arbitrary features dependent on the design of the stamp, but requires the use of photolithographically produced stamps; and a parafilm insert method that allows the generation of stripe patterns only but is more easily adoptable by any lab since it does not require photolithography.

Our first method was based on previously reported strategies and involved fibronectin microprinting. While fibronectin microprinting allows the generation of any arbitrary pattern, this method required significant optimization to ensure reproducible patterning. We found that the success of using this method to generate patterns was sensitive to seeding cell density and timing of each step: Seeding at too low a cell density or incubation for too short a time results in incomplete coverage of the fibronectin stamped area (results not shown). Seeding at too high a cell density or incubating for too long a time resulted in cell clump formation and overgrowth of the cells beyond the patterned fibronectin stamped areas (results not shown). Both these scenarios lead to poor quality of cell patterning. For the particular cell types we used we found that 2x10⁶ cells/mL and 2.5 hours for MDCK and 1.5 hours for ARPE-19 incubation time produced robust patterning. Therefore, cell density and seeding times will likely have to be optimized for different epithelial cell
types. Also, we found that the concentration of fibronectin solution was critical. Using less than 100 µg/mL fibronectin solutions resulted in poor fibronectin printing. We speculate that this is due to poor confocal contact of the stamp with the porous membrane and therefore the fibronectin transfer or stamping is not as effective as on rigid surface. Alternatively the fibronectin may not absorb as effectively on the filter material compared to glass and tissue culture polystyrene. Using a higher concentration of fibronectin counteracts the effect of poor fibronectin transfer. The FBS incubation before the second cell type seeding was also very important to promote faster attachment of the second cell type. This avoided the migration of the first cell type out of the patterns.

In contrast to the fibronectin method that allows arbitrary pattern generation we also describe a very simple parafilm insert patterning method. While this method can only generate stripe co-patterns, it is extremely easy and accessible to epithelial biology labs without access to a clean room or soft-lithography stamps. We found the parafilm insert method easier to implement but as with the fibronectin microprinting method it was important to optimize cell seeding densities and incubation times to ensure robust pattern generation. In the case of MDCK cells, if cells were seeded at too high a cell density or allowed to grow for too long then removal of the Parafilm insert without disrupting the epithelial sheets became very challenging. This was not a problem with ARPE-19 cells, which do not apically-basally polarize as quickly as the MDCK cells on the filters. Depending on the epithelial cell type being patterned it is therefore important to establish when the insert can be removed without disruption of the cell sheet on the membrane. Other important parameters that influenced the quality of the co-pattern were (i) adequate bonding of the parafilm insert to the membrane’s surface, (ii) the FBS wash step prior to seeding of the second cell type, and (iii) the cell density of the second cell type. We suggest using the degassing step in the insert preparation as a test for the strength of the bonding between parafilm and the membrane surface. If the parafilm detaches at this step even in some places, the probability of the successful patterning is very low. We also found the FBS wash step following the removal of the parafilm insert very important for promoting fidelity of the desired pattern. We speculate that FBS masks any cell-repellent parafilm residue left behind, promoting faster attachment of second cell type to the areas free of the first cell type. In the absence of the FBS wash the first cell type started migrating out of the pattern before the second cell type had a chance to adhere, disturbing the imposed pattern (results not shown). Finally, with the parafilm insert method the success of the patterning depended more on the seeding density of the second cell type as opposed to that of the first cell type. Here, if the density of the second cell type was too low the first cell type migrated out of the pattern into the areas unoccupied by the second cell type, disrupting the pattern.
As with the monoculture methods, a key issue we needed to characterize for both our co-patterning methods was pattern maintenance over an extended culture period (10-20 days) to allow adequate maturation of the epithelial sheets on the filter insert membranes. Pattern integrity over time varied depending on the motility of the epithelial cells and their tendency to move within the sheet. After 15 days in culture the co-culture patterns in MDCK cell sheets remained sharp suggesting little cellular re-arrangement within the sheet occurs. Patterns in ARPE-19 cell sheets on the other hand became disrupted over the 15 days maturation period due to cell migration within the sheet. This increased migration behaviour may reflect the fact that ARPE-19 cells do not polarize as quickly as MDCK epithelial cells do over the 14-day culture period 20. One potential strategy to decrease cell migration within the patterned sheets is to increase the adhesiveness of the filter substrate 21, for example by stamping with higher concentrations of fibronectin, to limit cell mobility on the filter surfaces. Assessing the ability of specific cell types to move within the sheet over time is therefore an important factor when designing an experiment that requires robust pattern maintenance. Therefore for our future experiments we selected MDCK cells, which displayed limited motion within the cell sheet.

Another key issue we wanted to assess for all methods was whether our mono and co-patterning methods disrupted normal epithelial cell polarization and maturation. All our patterning methods allow an even nutrient supply from the basal compartment of the filter insert to the epithelial layer; therefore we predicted that our techniques should have no effect on sheet polarization and maturation. To assess polarization we focused on MDCK cells which polarize over 7-15 days on filter insert culture system 22 during which ZO-1, a widely used marker of apical polarization, relocates to the apical subdomain of plasma membrane, and primary cilia form on the apical surface of the cells 17. As expected ZO-1 was expressed at the same sub-apical level and localized to the plasma membrane of the cells in monopattered, co-cultured and un-patterned MDCK cells at all time points analyzed. The other marker of epithelium maturation analyzed was the presence of primary cilia. Primary cilia were observed in on monopattered, co-cultured and un-patterned MDCK sheets at day 7 and day 15 suggesting that our patterning methods did not alter the capability of the epithelium in the microsheet to mature. We did not observe any difference in the timing of primary cilia formation and growth between patterned and unpatterned epithelial sheets. In all cases, we could see a faint signal for the basal bodies one day from the start of the culture (results not shown). By day 7 we observed presence of primary cilia in both unpatterned and patterned microsheets in a subset of the cell population. In both samples approximately the same number of cilia per mm² were observed. By day 15 the subset of the cell population with primary cilia remained unchanged in both culture conditions with primary cilia becoming longer than at day 7.
Beyond our experimental question our methods could be useful for addressing a number of biological questions. For instance, the influence of different epithelial cell populations on tissue structure and function in vitro is not well characterized, in part due to the lack of available methods for spatially controlling multiple cell populations while maintaining the culture conditions necessary to generate polarized and mature epithelium. The fibronectin microprinting method is most useful when the epithelium sheet shape or pattern size is important factors for the experimental design. For example, using this technique we could assess if the invasion of cancer cells into normal epithelial cell sheets depends on the size or the shape of the cancer cell sheet. We envision the parafilm patterning technique is most useful when the cell sheets size or patterns size are not important factors for the experimental design. Also, this method is appropriate when the use of protein such as fibronectin can affect the experimental outcome. For example using this technique we could assess the effect of a drug on pattern disruption rate and hence cell mobility within the epithelial sheet. Both co-culture systems are therefore powerful tools for understanding epithelial cell biology in vitro and for optimizing the design of tissue-engineered epithelium.

3.5 Conclusion

We have developed a set of micropatterning techniques to spatially control the organization of epithelial cells into microsheets and co-culture on filter inserts under the culture conditions necessary to induce epithelial cell apical-basal polarization. Micropatterning epithelial cells into microsheets offers a number of advantages such as uniformity within each microsheet, multiple sheet analysis on one filter insert. The co-culture methods offer different advantages depending on the requirements of the system necessary for cell patterning. Our fibronectin microprinting method allows co-patterning of distinct populations of epithelial cells in any arbitrary pattern. Our Parafilm patterning method allows co-patterning distinct populations of epithelial cells in stripes but is extremely straightforward and could be adopted by any laboratory without the need of specialized microfabrication equipment and access to a clean room. Theses developed micropatterning methods, specifically the agarose microwell method and parafilm co-culture method will be used to set out the experiment that will allows us to begin to understand how to generate planar organization in an epithelial cell sheet in vitro. More generally our methods will be useful for probing the importance of cell organization on epithelium function for applications in tissue engineering and generating relevant in vitro models of diseased epithelium to better understand disease mechanisms.
3.6 Reference

Chapter 4.
Definition of organization readouts on modified
MDCK_GFP-Vangl2 cells

4.1 Introduction

In chapter 3 we developed a system that allowed us to spatially organize one or two populations of epithelial cells on filter insert membranes. The next step in our study was to generate a population of epithelial cells with modified levels of one of the PCP core proteins. Additionally, it was necessary to define a readout of epithelium organization in the modified cells population to later be able to characterize and study the effect on the cell sheet organization when this modified cell population is co-cultured with a wild type cell population.

Epithelial cell lines are used broadly for studying in vitro morphological and physiological properties of the epithelium. For this study we decided to work with Madin-Darby Canine Kidney (MDCK) cells line due to the following reasons: (i) it is one of the most commonly used cell lines for in vitro studies in epithelium tissue and therefore they are very well characterized\(^1\), (ii) can apical-basal polarize and develop primary cilia in vitro\(^2\), (iii) come from kidney epithelium and kidney epithelium disease such as ADPKD has been linked with PCP defect\(^3\), (iv) grow in monolayer, which makes them suitable for easy characterization, (v) as we observed on chapter 3 they do not migrate or move as much as other epithelial cell lines.

As mentioned in chapter 2 in Drosophila a group of 6 proteins (Vang,Dgo,Pk,Fz,Dvl,Fmi) have been identified as the core protein necessary to generate planar organization in the epithelium. However, only a subset of them (in Drosophila Vang, Fz,Dvl,Fmi; in mouse Vangl2,Vangl1, Fz3,Fz6,Dvl2,Celsr1) have been identified in a few mammalian tissues, such as the inner ear\(^3-5\). For the generation of the modified MDCK population with one of the PCP core protein we decided to overexpress a mouse Vangl2 protein for the following reasons: (i) it is one of the main core proteins found in mammals and it has not been shown to be involved in any other wnt signalling pathway other than PCP signalling, (ii) it has been shown that Vang and Vangl2 overexpression and knockdown creates domineering non-autonomy phenomenon in Drosophila wing actin hairs\(^6,7\) and in vertebrates inner ear stereociliary bundles\(^8\) respectively, (iii) There are clear PCP phenotypes in mice when Vangl2 protein is mutated such as neural tubule closure defects\(^9\) and bundle orientation defects in the inner ear\(^8\) (iv) although Vangl 1 and Vangl2 share 70% similarity, the function of Vangl1 is still not clear\(^9\).
Therefore, for this part of the study we designed a set of experiments that allowed us to:

1) Understand the behaviour (localization and dynamics over time) of the mouse exogenous Vangl2 protein on the modified MDCK cells culture on filter insert membranes, to later use this knowledge as a reference/control for the co-culture experiments.

2) Determine if the overexpression of Vangl2 protein affected the apical-basal polarization of the cells in any way to assess if our standard polarization markers in wild type cells still could provide a reliable readout of epithelium organization in the co-culture experiments where one of the cell populations had elevated levels of Vangl2.

4.2 Methods

4.2.1 MDCK culture conditions
We used MDCK dog kidney epithelial cell line (ATTC, USA) at passages between P10-20. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Biowhitaker) supplemented with 10% FBS and 1% Penicillin/Streptomycin and maintained in an incubator at 5% CO₂.

4.2.2 Virus production
On the lentiviral vector production 293T human embryonic kidney cells (ATCC, USA) were used as packaging cells. The lentiviral packaging genome PAX2, lentiviral envelope genome MD2G and the optimized transfection protocol were kindly provided by Dr Jason Moffatt. The day before the transfection 3.5 x 10⁶ of 293T cells were grown on a 100 x 20 mm tissue culture dish using high glucose Dulbecco’s Modified Eagle (DMEM) culture medium (Lonza, USA) supplemented with 10% fetal bovine serum (FBS) and 0.1% antibiotic (100 U/ml penicillin / 100 ng/ml streptomycin) (Lonza, USA). Cells were grown overnight at 37°C in a 5% CO₂ humidified chamber. On the day of the transfection 36ul of FuGENE6 transfection reagent (Roche, Germany) was diluted on 540ul of OPTI-MEM medium (GIBCO, USA) and left at room temperature for 5 min. On a separate tube the mix of three transfection plasmids (5.4ug PAX2, 0.6ug MD2G and 6ug of GFP-Vangl2) was made. Then, the plasmid mixture was added to the diluted FuGEN6 and incubated for 30 min at room temperature. The transfection mix was added to the 293T packaging cells and incubated for 18 hours at 37°C in a 5% CO₂ humidified chamber. After 18 hours the medium from the 293T packing cells was removed and replaced for DMEM supplemented with 1.28 % BSA, and 1% antibiotic. For the next two days the medium was collected and concentrated using centrifugal filter concentrators (Millipore, Ireland). The titer of the produced virus was measured using a lentiviral titer kit (MellGen Laboratory Inc, Canada).
4.2.3 MDCK transduction with GFP-Vangl2 construct and eGFP construct

GFP-Vangl2 construct was introduced to MDCK cells via lentiviral transduction. On a 24 well tissue culture plate 200µl of lentiviral vector carrying the GFP-Vangl2 construct or 100 µl of lentiviral vector carrying the eGFP construct, 3µl of 1X Polybrene (Sigma,USA) and 40,000 MDCK cells were mixed and left for 18 hours at 37°C in a 5% CO₂ humidified chamber. Then, the medium was changed for DMEM supplemented with 10% FBS, and 1% antibiotics. In the case of MDCK transduced with GFP-Vangl2 construct the cells were passaged until at least 10 x 10⁶ cells were obtained. Then, MDCK_GFP-Vangl2 cells were sorted to get a homogeneous transduced population using flow cytometry. This homogeneous population of MDCK_GFP-Vangl2 cells was used for all experiments.

4.2.4 Assessment GFP-Vangl2 protein localization on modified MDCK cells

To assess the localization of GFP-Vangl2 protein on modified MDCK the cells were seeded on patterned and un-patterned insert filter membranes to allows us to better understand localization of GFP-Vangl2 protein over time. The method used was the agarose micropattern method described on Chapter 3. Patterned and un-patterned cells cultured for different days were fixed with 4% paraformaldehyde, washed in phosphate buffered saline, permeabilized using 0.1% triton-X100. MDCK_GFP-Vangl2 cells were stained with monoclonal antibodies against GFP (Invitrogen, 1:1000), FITC-conjugated anti-sheep (1:250, Sigma Aldrich) secondary antibodies and stained with DAPI (Invitrogen). Images were taken on an Olympus IX81 microscope and a Nikon D-eclipse-C1 confocal microscope.

For the live image microscope experiment we culture 3 x10⁴ MDCK_GFP Vangl2 cells in a 96 tissue culture well plate. We have observed the same behaviour of the GFP-Vangl2 protein on filter insert membrane as in tissue culture plates or glass slides (Appendix-Figure 4.1). Therefore, we used tissue culture plate instead insert filter due to technical issues using insert filter on the live image microscope. The cell were grown for 16 hours and then stained with 50 µg/ml of Hoechst for 30 min at 37°C. Then, cells were wash with medium for one hour at 37°C. Finally, the plate was placed on the microscope and pictures were taken every hour for 16 hours for FITC channel using a 20X lens.

4.2.5 Assessment of epithelial cell polarization markers of MDCK cells

To determine if the introduction of the exogenous GFP-Vangl2 protein had any effect on the dynamics of apical-basal polarization in modified MDCK cells we assessed the typical apical polarization marker ZO-1 and the presence of primary cilia using an antibody against acetylated α-tubulin (Sigma Aldrich, USA) on un-patterned and patterned cells. Cells (un-patterned and patterned) were fixed in 4% paraformaldehyde, washed in phosphate buffered saline, and
permeabilized using 0.1% triton-X100. Cells were stained with DAPI (Invitrogen), monoclonal antibodies against ZO-1 (Invitrogen, 1:100), or acetylated α-tubulin (Sigma Aldrich, 1:500). The following secondary antibodies were used for imaging: Cy3-conjugated anti-mouse (1:250, Millipore, USA) and FITC-conjugated anti-mouse (1:250, Sigma Aldrich). Images were taken on a Nikon D-eclipse-C1 confocal microscope.

4.3 Results

4.3.1 Generation of modified MDCK_GFP-Vangl2 cells

In order to create a population of MDCK cells with Vangl2 protein overexpression, MDCK cells were transduced with a tagged GFP-Vangl2 construct using a lentiviral vector. The transduced construct was designed with tagged GFP protein to allow us to assess the localization of the exogenous mouse Vangl2 protein in the epithelial sheet. When cells were seeded we noticed a high heterogeneity in the amount and localization of protein throughout the cell sheet (Appendix-Figure 4.1). Our initial conclusion was that this was due the broad distribution of transduction levels, which could be observed by the different levels of GFP fluorescence shown by flow cytometry analysis (Figure 4.1A). Furthermore, the transduction efficiency was between 50% to 80% (data not show), so after the transduction there was a mixed population of transduced and non-transduced cells. To conduct experiments with a more homogeneous cell population therefore, the transduced MDCK cells were further sorted by using activated cell sorting (FACS) (Figure 4.1B) to eliminate the possibility that differences in the amount and localization of the GFP-Vangl2 protein were due to the broad distribution of transduction levels and presence of the non-transduced cells. We decided to sort a population with high levels of transduction because it was easier to observe the localization of the Vangl2 protein in these high transduced cells than on the low transduced ones. Furthermore, we did not observed any evident effect in the cell morphology due to the high levels of infection. However, we avoided to sort cells with the highest transduction levels (the tail of the transduction distribution (Figure 4.1A) because these cells had a level of overexpression that made them completely green. The final sorted homogeneous population of cells was expanded and used for all the experiments.
4.3.2 Assessment of GFP-Vangl2 protein localization on modified MDCK_GFP-Vangl2 cells

Even though cells had similar level of infection, differences in the amount and localization of the GFP-Vangl2 protein throughout the epithelial sheet could still be observed. Therefore, we decided to pattern the cells on 200μm x 200μm square agarose microwells to better visualize and understand the localization of the GFP-Vangl2 protein. First, sorted MDCK_GFP-Vangl2 cells at 2,5x10^5 cell/ml were seeded over the patterns and cultured the cells for five days. On the first day, the cells were cultured with serum medium on top and lower compartment and for the next four days with serum-free medium in the top compartment and serum medium in the lower compartment to promote apical-basal polarization. The patterns were randomly filled with different numbers of cells, resulting in patterns with variable levels of cell confluency in individual patterns (Figure 4.2). This difference in confluence between the patterns allowed us to observe the influence of the level of cell confluency on the localization of the protein GFP-Vangl2 protein within the cell. In patterns with medium and low confluency GFP-Vangl2 protein is localized in the cell cytoplasm, more precisely around the nucleus (Figure 4.2A and 2B). In contrast, on patterns with high cell confluency GFP-Vangl2 protein localized mostly to the cell membrane (Figure 4.2C). This suggests that cells need to be in tight contact with neighbour cells to localize the GFP-Vangl2 protein on the cell membrane. Even more interesting is that at the edges of these confluent patterns, the cells do not show localization of the protein on the part of the cell membrane located at edge of the pattern. However, this phenomenon was clearly appreciated in patterned cells on glass slides (Figure 4.3). We concluded that this is due to the edge effect (double layer of cells at
the edge of the patterns) that was also observed on wild type MDCK cells patterned on filters insert membranes on chapter 3.

Figure 4.2. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cell on agarose microwells with different confluences. MDCK_GFP-Vangl2 cells were cultured on filter insert membranes with 200 µm x 200 µm square patterns for five days. (A) In patterns with low confluency GFP-Vangl2 protein is localized on the cell cytoplasm more precisely around the nucleus (B) In medium confluent patterns GFP-Vangl2 protein is localized mostly on the cell cytoplasm and in few cells some of the protein begin to localize on the cell membrane. (C) In patterns with high confluency GFP-Vangl2 protein is well localized on the membrane of most of the MDCK_GFP-Vangl2 cells. Scales bars 50 µm. Green- GFP-Vangl2 protein, Blue-nucleus.

Figure 4.3. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cell on agarose microwells on glass. MDCK_GFP-Vangl2 cells were cultured on glass with 200 µm x 200 µm square patterns for five days. GFP-Vangl2 protein (green) is well localized on the membrane of most of the MDCK_GFP-Vangl2 cells when the 200 µm pattern is confluent. Cells on the edges of the pattern do not show localization of the protein on the cell membrane. Scale bar 50 µm. Green-Vangl2 - Blue-Nucleus
To further assess the dynamics of the localization of the Vangl2 protein over time and to understand if cells only needed to be in high confluency to localize the protein to the cell membrane, or if the culture timing was also a factor that influenced the localization of the protein to the cell membrane, 2x10^6 cell/ml of MDCK_GFP-Vangl2 cells were seeded on un-patterned insert filter membranes for 16 hours, 1, 7 and 15 days. On the first day, the cells were cultured with serum medium on top and lower compartment and for the next days with serum-free medium in the top compartment and serum medium in the lower compartment to promote apical-basal polarization. After 16 hours in culture the cells were confluent and formed tight junction, shown by the ZO-1 staining (Appendix-Figure 4.2) and most of the GFP-Vangl2 protein was still located perinuclear (Figure 4.4A). It is expected that a 16 hours time period only allow very limited polarization of the cells after reaching confluency. After one day in culture (Figure 4.4B) in some cells the GFP-Vangl2 protein is localized mostly to the cell membrane and even though cells are at high confluency in some cells the protein is still localized perinuclear. By day 7 (Figure 4.4C) and 15 (Figure 4.4D) most of the protein localized to the cell membrane. However, there is a remaining amount of protein in the cytoplasm. The cells were also cultured on patterned filter insert membrane for 1,7,15 days (Appendix-Figure 4.3). We could observe the same dynamics of the protein in the patterned cells than on the unpatteren cells. Additionally, using a live image microscope (ImageExpress Microscope, Molecular Devices ) pictures of MDCK_GFP-Vangl2 cells seeded on a 96 tissue culture plate were taken every hour in a period between 16 to 32 hours after cells were seeded. The movie created using this sequence of pictures allowed us to observe that in this period of time (16 hours) is when most of the protein relocates from the cytoplasm to the cell membrane (Movie 4.1). Finally, we could observe that in both un-patterned and patterned cells over time the amount of protein increases more in some cells than in others, which creates the heterogeneity that we detected before on the un-sorted cell sheet. Specifically, at day 7 and 15 high expressing cells were observed.
Figure 4.4. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells culture over time. MDCK_GFP-Vangl2 cells were cultured on un-patterned filter insert membranes for 16 hours, 1, 7 and 15 days. (A,B,C,D) GFP-Vangl2 localization in un-patterned cells on 16 hours, 1, 7 and 15 days respectively. Scales bars 50 μm. Green- GFP-Vangl2 protein, Blue-nucleus.

4.3.3. Assessment of epithelial cell polarization markers on MDCK_GFP-Vangl2 cells

To assess if the exogenous GFP-Vangl2 protein does or does not affect the dynamics of apical-basal polarization in MDCK_GFP-Vangl2 cells comparing with wild type MDCK cells, we assessed the typical apical polarization marker ZO-1 and the presence of cilia using an antibody against acetylated α-tubulin. 2x10⁶ cell/ml of MDCK_GFP-Vangl2 cells were seeded on un-patterned and patterned insert filter membranes for 1, 7 and 15 day. We observed that the dynamic of ZO-1 expression and localization on un-patterned MDCK_GFP-Vangl2 cell sheet (Figure 4.5 A-C) was the same as in un-patterned wild type MDCK cell sheet (Figure 4.5 D-F). Also, no difference was observed in the ZO-1 protein level and its localization between the modified cells (Figure 4.5 G-I)
and the wild type cells (Figure 4.5 J-L); in both MDCK_GFP-Vangl2 cell sheet and wild type MDCK cell sheet, ZO-1 is localized to the cell membrane in the sub-apical segment of the cells from day 1. In patterned MDCK_GFP-Vangl2 cell sheet (Appendix-Figure 4.4 A-F) the dynamics of ZO-1 expression and localization were the same as in un-patterned MDCK_GFP-Vangl2 cell sheet and wild type cells MDCK, as expected. In the case of primary cilia, 2x10^6 cell/ml of MDCK_GFP-Vangl2 cells were seeded on patterned and un-patterned insert filter membranes for 7 and 15 days. We observed that the dynamics of primary cilia formation in un-patterned MDCK_GFP-Vangl2 cell sheets (Figure 4.6 A-B) is the same as in wild type MDCK cell sheets (Figure 4.6 C-D). By day 7 we could observed cilia formation in a subset of cells as expected 10 and by day 15 primary cilia was still present. Cilia numbers in both cell populations was manually quantified on day 7 in a 50μm x 50 μm square area. Approximately, 16 cilia in wild type MDCK cell sheet and 20 cilia in MDCK_GFP-Vangl2 were present in the same area. In patterned MDCK_GFP-Vangl2 cell sheet (Appendix-Figure 4.5 A-B) the dynamics of primary cilia formation were the same as in un-patterned MDCK_GFP-Vangl2 cell sheet and wild type MDCK cells sheet, as expected.
Figure 4.5 Assessment of ZO-1 in un-patterned MDCK_GFP-Vangl2 cells and wild type cells on filter insert membranes over time. MDCK_GFP-Vangl2 cells and wild type MDCK cells were cultured on un-patterned filter insert membranes for 1,7 and 15 days and stained for tight junction marker ZO-1. Images A to F show projected stacks of ZO-1 over time. (A,B,C) ZO-1 expression on MDCK_GFP-Vangl2 cells on day 1,7 and 15 respectively. (D,E,F) ZO-1 expression on wild type MDCK cells on day 1,7 and 15 respectively. Images G to L show the z position of ZO-1 over time. (G,H,I) ZO-1 position on MDCK_GFP-Vangl2 cells on day 1,7 and 15 respectively. (J,K,L) ZO-1 position on wild type MDCK cells on day 1,7 and 15 respectively. Scales bars 50 μm. Red and Green- ZO-1 protein, Blue-nucleus.
Figure 4.6. Assessment of primary cilia formation on un-patterned MDCK_GFP-Vangl2 cells and wild type MDCK on filter insert membranes over time. MDCK_GFP-Vangl2 cells and wild type MDCK cells were cultured on un-patterned filter insert membranes for 7 and 15 days and stained for primary cilia marker Acetylated α-tubulin. (A, B) primary cilia on MDCK_GFP-Vangl2 cells on day 7 and 15 respectively. (C,D) primary cilia on wild type MDCK cells on day 7 and 15 respectively. Scales bars 50 μm. Red- Acetylated α-tubulin, Blue-nucleus.

4.4 Discussion

The generation of the MDCK_GFP-Vangl2 cells population through the use of lentivirus vector allowed a stable transduction, which creates a steady overexpressing MDCK population with one of the PCP core proteins, necessary to later execute the co-culture experiments. Further sorting of the transduced cells allowed to generate a more homogenous population of MDCK_GFP-Vangl2 cells, which gave more clarity on the GFP_Vangl2 protein localization behaviour within the cell. Additionally, tagging the Vangl2 protein with GFP protein permits us to assess the localization over
time of the exogenous Vangl2 protein within the cell, which could have not been achieved in this study with the endogenous Vangl2 protein due to the lack of commercially available robust antibody (that is effective in canine cells).

The observations that we were able to make gave important insight regarding the dynamics of the GFP-Vangl2 protein localization. In general, it was observed that over time the GFP-Vangl2 protein goes from localizing around the cell nucleus to the cell membrane. We detected that for this relocation the cell sheet confluency and extended cell culture time were important. Micropatterning the MDCK-GFP-Vangl2 cell on agarose microwells gave a better understanding of the importance of the cell sheet confluency. In micropatterns with different density (high, medium and low) cultured for the same amount of time the GFP-Vangl2 protein localize in different part of the cell. The perinuclear localization was very obvious in patterns with low and medium confluency or with cells that had no tight contact with neighbouring cells. In contrast, pattern with high confluency or with cells with tight contact with neighbouring cells, most of the GFP-Vangl2 protein was relocated to the cell membrane and the perinuclear accumulation was significantly diminished. Furthermore, at the edges of these high confluent patterns, the cells do not show localization of the protein on the part of cell membrane located at edge of the pattern. This suggests that the cells need to be in contact with neighbor cells to localize the protein on the cell membrane. On the other hand, with the over time culture experiments we could elucidate that high confluency was not enough to localize the GFP-Vangl2 protein to the cell membrane because even though the cells were confluent in the first couple of hours (16 hours) the protein still had perinuclear localization. With the sequence of pictures took in the live microscope (Movie 1.1) we could further observe that the relocation of the GFP-Vangl2 protein takes a period of time and occurs approximately in the first 32 hours of culture.

It is known that MDCK cells fully apical-basal polarize 5 to 7 day after reaching confluency\(^1\). Therefore, these results show that cells do not only have to be in tight contact with neighbour cells, but also need a certain time or level of polarization to be able to localize the protein to the cell membrane. In the literature there is no information that suggests that there is direct connection between levels of apical-basal polarization and the correct localization of the PCP core protein within the cell. Montcouquiol\(^11\) and colleagues showed that on MDCK transduced with GFP-Vangl2 construct there was a strong accumulation of the GFP-Vangl2 protein to the cell membrane at the junction between two transduced cells, which we also observed in our experimental results. Beside the cell membrane localization we also could observe some protein on the cytoplasm, which was more pronounced over time. This cytoplasmic accumulation was mostly observed on day 7 and 15 of culture. We speculate cytoplasm accumulation over time was due to saturation of the cell
membrane simply due to the protein overexpression. It has been shown that excess of Vangl2 protein in vertebrate auditory epithelial cells interfered with the restriction of the protein to the cell membrane\(^8\), which is what we observed (Figure 4.4 C-D).

The dynamics of the formation and localization of tight junction (Z0-1) on MDCK_GFP-Vangl2 cells was not affected by the overexpression of Vangl2 protein. There is no evidence in the literature that suggests that the apical-basal polarization of epithelial cells is affected in cell population with mutation in any of the PCP core protein\(^5\). However, we observed that there was a relationship between the level of apical-basal polarization with the dynamic of the exogenous PCP protein. Therefore, we decided track the dynamic of Z0-1, a marker for tight junction formation, to study if there was any connection between both of them. Furthermore, there is not a focus in the literature in studying the relationship between the apical-basal polarity and planar cell polarity, which is the reason we decided to assess tight junction formation in this modified cells. In the case of primary cilia we decided to assess just the dynamics of the cilia formation and not cilia orientation. One of the issues of using cell lines is that the length of the cilia through the cell sheet and from sample to sample varies significantly. Therefore, measuring and even observing where the cilia is pointing out is a very difficult task to perform; contrary to tissue models such as the Drosophila wing actin hair and the hair bundles in the inner ear cells where the cilia orientation is very obvious. Since assessing the directionality of primary cilia in cell lines such as MDCK was experimentally challenging we focused on the more limited readout of cilia formation. It was very insightful to observe that the dynamics of primary cilia formation were not perturbed in these PCP modified cells. It has been shown that in vertebrates some of the PCP core proteins are important for ciliogenesis. For instance, in Xenopus Dvl protein is important for ciliogenesis in epidermis multiciliated cells\(^12\). Also, it has been shown that mice with mutation in Celsr protein (protein orthologous to Drosophila flamingo) cause defect in ciliogenesis in brain multiciliated epidermal cells\(^13\). In the case of Vangl2 protein its role in ciliogenesis is not very clear. Knockdown of Vangl2 in Xenopus show defect in epidermal multiciliated cell ciliogenesis. In contrast, Song and colleagues found that mutation of Vangl1 or Vangl2 protein did not cause ciliogenesis defect in mouse ciliated airway cells and primary mouse embryonic fibroblast\(^14\). This suggests that the role or relation between the PCP core protein and ciliogenesis is still not completely understood.

Overall, our results give us an understanding of the behaviour and dynamics of the exogenous GFP-Vangl2 protein. Observing that the exogenous protein is able to go to the cell membrane demonstrates that MDCK cells, a dog’s cell, have the machinery to move and localize a mouse exogenous protein to its natural location. Furthermore, understanding the dynamic of the GFP-Vangl2 protein gave us readout to assess the effect of co-culture conditions in the modified cell
population. Hence, if we can identify a change in the localization of the GFP-Vangl2 protein on the modified cells over time, such as loss or more accumulation of GFP-Vangl2 protein to the cell membrane, this would indicate that the co-culture condition is affecting the modified cell population. Also, these data suggests that the exogenous GFP-Vangl2 protein do not affect the dynamics of apical-basal polarization and formation of primary cilia. Based on these results we expected it was reasonable to use these two markers (ZO-1 localization, cilia formation and Vangl2-GFP localization) to assess the cell sheet organization in the next set of co-culture experiments. Finally, all this information let us to decide which are the best times points to execute the co-culture experiment.

4.5 Conclusion

The generation of epithelial cell population, that allowed to visualize the behaviour of one of PCP core protein, was a valuable tool to begin to understand the behaviour a PCP core protein in an \textit{in vitro} system. This set of experiments allowed elucidating the behaviour of the exogenous mouse Vangl2 protein in MDCK cell line. The results showed that the protein is dynamic over time, and most important that has the capacity to localize to its natural location, the cell membrane. Furthermore, we could observe that the introduction or overexpression of this protein to MDCK cells did not affect any measured features of the apical-basal polarization of the cells such as formation and localization of tight junction and primary cilia formation. Finally, the knowledge acquire through out this set of experiments is important to decide time points for the co-culture experiments and give as baseline to understand the effect of co-culturing this modified cell with wild type MDCK cells.

4.6 Reference


Chapter 5.
Co-culturing patterned sheets of MDCK_GFP-Vangl2 cells and wild type MDCK cells

5.1 Introduction
Understanding the structure of the epithelium and the mechanisms that establish and control this structure is important to design strategies to engineer functional epithelium and also to develop in vitro models to study normal and diseased epithelium. Epithelial cells apically-basally and planar polarized (PCP) within the epithelium. Both of these polarizations are essential for the correct epithelium function. Apical-basal polarization is important to form the protective barrier against pathogens and allows the vectorial transport between body compartments. On the other hand, PCP is important to align or arrange structural features of tissue such as hair or cilia. Apical-basal polarization is established by preferential protein portioning to the apical and basolateral domains of the cell membrane. PCP is established by mutual inhibition of the core proteins inside the cell and by interaction of some core proteins across the cell boundaries. Specifically, in vivo experiment in Drosophila suggests that the PCP core proteins form two complexes inside the cell, one in the distal side of the cell (Fmi, Fz, Dsh, Dgo) and another in the proximal side of the cell (Fmi, Vang, Pk), due to mutual exclusion mechanism. Furthermore, it is suggested that Vang and Fz interact between two neighbouring cells, which amplify the asymmetry of the protein complexes inside the cell. These two mechanisms together creates a feedback loop that results in the asymmetrical localization within a cell of PCP signalling core proteins along the axis perpendicular to the apical-basal axis, which translates in the organization of structural features of the cell. It is important to mention that all these mechanisms are still not fully understood. In vivo experiments have also shown that modifying (overexpression or silencing) PCP core protein in a group of cells can produce organization changes or planar organization changes in neighbouring cells (genetically normal cells). This phenomenon is called domineering non-autonomy. This phenomenon shows us: (i) epithelial cells can communicate spatial information between them, and (ii) the levels of PCP core proteins within the cells are translated to the cell sheet as morphological changes. This phenomenon has been mainly studied in Drosophila, in mammalians has been mainly studied in the inner ear.
To engineer fully functional epithelium it is important to generate both types of polarization in the engineered tissue. Epithelia cell sheets spontaneously generate apical-basal polarization \textit{in vitro}. Therefore, usually apical-basal polarization is present in engineer epithelium. Furthermore, there are many studies and \textit{in vitro} models that permit to understand the mechanisms of how this polarization is generated. In contrast, planar polarization is not spontaneously generated \textit{in vitro} and there are no \textit{in vitro} models that allow to understand better this polarization. Therefore, it is important to investigate strategies that allow us to understand and generate this polarization in \textit{vitro}. We think that there could be different strategies of inducing planar polarization \textit{in vitro}: (i) physical forces such as flow; (ii) environmental clues such as substrate topography; (iii) Mimic developmental cellular signalling such as PCP signalling. In this study we focus in mimicking a developmental cellular signalling pathway, PCP signalling. \textit{In vivo}, the domineering non-autonomy phenomenon have permitted researchers to better understand PCP signalling because it produces a clear readout on how changes in the PCP core protein affect the organization in cellular structures. For instance, in the Drosophila wing each cell has a polarized actin hair pointing to the distal side of the cell. When in a group of wing cells, Vang or Fz protein are modified (overexpress or silence), the polarization or organization of the wing actin hairs of the non-modified neighbouring cells is affected (Appendix-Figure 5.1). As a first step to understand how to generate PCP signalling \textit{in vitro} we want to investigate if we can see the same domineering non-autonomy phenomenon \textit{in vitro}. Therefore, we set up a set of experiments where in a genetically distinct epithelial cell population with different level of Vangl2 protein, one of the PCP core proteins was co-cultured side-by-side on filter insert membranes. We used the parafilm insert co-culture method and the MDCK\_GFP-Vangl2 cells developed and characterized on chapters 3 and 4 respectively. Specifically, we co-cultured side-by-side on filter insert culture system MDCK\_GFP-Vangl2 cells with wild type MDCK cells over time, and evaluated if this co-culture condition would affect organizational factors such as expression of ZO-1 and primary cilia formation in both cell populations, and the localization of GFP-Vangl2 proteins on MDCK\_GFP-Vangl2 cell population.

5.2 Method

5.2.1 Co-culture experimental design

MDCK\_GFP Vangl2 cells were co-cultured side-by-side with wild type MDCK using the parafilm insert method described on chapter 3 (Figure 3.1C). Briefly, the parafilm insert with stripe array was bonded to the filter insert membrane. We then added 1 ml of PBS to the insert well, degassed the films for 5 minutes at a pressure of 30 psig and UV sterilized the films in PBS for 20 minutes. Then, fetal bovine serum was added to the filter insert and incubated at 37 °C for 30 minutes prior to seeding the first cell type. To seed the first cell type (MDCK\_GFP-Vangl2) we added
500 µL of a cell suspension of 2 × 10⁶ cells/ml making sure that the entire surface of the insert was covered by the cell suspension. The samples were incubated at 37 °C, 5% CO₂ for 3 hours. Next, the cell suspension was removed and any un-adhered cells were gently washed away. To generate the co-cultures, the parafilm inserts were removed 3 hours after seeding of the first cell type and incubated the patterned cells in fetal bovine serum at 37 °C for 20 minutes. Next the fetal bovine serum was removed and seeded 500 µl of a second cell type (wild type MDCK) at a seeding density of 3×10⁶ cells/ml. The patterns were incubated for 1 hour to allow the additional cells to adhere to the surface free from the first cell type and then thoroughly washed the substrates with growth medium to remove any un-adhered cells. Finally, 1.5 ml of serum medium were added to the top compartment and 2.6 ml of serum medium to the bottom compartment. On the next day the medium in the top compartment was switched to serum-free medium and continued the culture for up to 1 week periodically supplying the cells with fresh medium in both compartments.

The co-cultures were analysed on day 1, 5, and 7. We chose these days based on the results of the MDCK_GFP-Vangl2 cells monoculture experiments on chapter 4. To analyze GFP-Vangl2 localization on the modified cells we decided to include day 5 because it is a point in time where cells already localized the protein to the cell membrane and the cells are apical-basal polarized. For the analysis of marker of apical-basal polarization (ZO-1 and primary cilia formation) we decided to analyze up to day 7 because there were not differences between day 7 and 15.

For the live image microscope experiment MDCK_GFP Vangl2 (high transduced) cells were co-cultured side-by-side with wild type MDCK using the parafilm insert. The co-culture was done on 12 well tissue culture plates due to technical issues using insert filter on the live image microscope. As mentioned on chapter 3 we have observed the same behaviour of the GFP-Vangl2 protein on filter insert membrane as in tissue culture plates or glass slides. After 16 hour of seeding the second cell type, the cells were stained with 50 µg/ml of Hoechst for 30 min at 37°C. Then, the cells were washed with medium for one hour at 37°C. Finally, the plate was placed on the microscope and pictures were taken every hour for 14 hours for FITC channel and Dapi channel using a 10X lens.

5.2.2 Assessment of the effect of co-culture condition on cell sheet organization

To determinate the effect of the co-culture of two genetically distinct epithelial cell populations on the cell sheet organization, we assessed the apical polarization marker ZO-1, the presence of cilia using an antibody against acetylated α-tubulin, and the localization of GFP-Vangl2 protein on MDCK_GFP-Vangl2 cells. Co-cultured cells cultured for different days were fixed with 4% paraformaldehyde, washed in phosphate buffered saline, permeabilized using 0.1% triton-X100. The cells were stained with DAPI (Invitrogen), monoclonal antibodies against ZO-1 (Invitrogen, 1:100), acetylated α-tubulin (Sigma Aldrich, 1:500), and monoclonal antibodies against GFP (Invitrogen, 1:1000). Cy3-conjugated anti-mouse (1:250, Millipore, USA) and FITC-conjugated anti-
sheep (1:250, Sigma Aldrich) secondary antibodies were used for fluorescent imaging. Images were taken on a Nikon D-eclipse-C1 confocal microscope.

5.3 Results

As mentioned above, to assess the effect of co-culturing side-by-side two genetically distinct epithelial cell populations in the organization of an epithelial cell sheet we studied three different factors: (i) localization GFP-Vangl2 protein on the genetically modified MDCK population, (ii) expression and localization of ZO-1 protein throughout the epithelial cell sheet and, (iii) formation of primary cilia throughout the epithelial cell sheet.

5.3.1 Assessment of GFP-Vangl2 protein localization in MDCK_GFP-Vangl2 cells when Co-cultured with wild type MDCK

To assess the effect of co-culturing MDCK_GFP-Vangl2 cells with wild type cells in the localization of GFP-Vangl2 protein on MDCK_GFP-Vangl2 cells over time, we co-culture the cells for 1, 5 and 7 days. As controls, MDCK_GFP-Vangl2 cells were seeded on un-patterned filter insert membrane for 1, 5 and 7 days. With these co-culture experiments we wanted to assess mainly the localization of the protein at the edge or interface between the two cell populations. On the co-culture samples at day 1 in most of the cells the GFP-Vangl2 protein was localized to the cells membrane (Figure 5.1A-white arrow) and in a few cells the protein still had perinuclear localization (Figure 5.1A-red arrow). The same behaviour could be observed on the un-patterned cells (Figure 5.1D). On the interface between the two cell populations we could not see any clear disturbances in the localization of the protein. On day 5 and 7 in all the cells we could observe a really sharp expression of the protein on the cells membrane in both co-cultured and un-patterned cells (Figure 5.1B-F). However, in the co-cultured condition, as in the monoculture condition as well, we observed that the levels of GFP-Vangl2 protein increased significantly at both these later time points (5 and 7 days). The increase in the protein expression made it difficult to observe the effect in protein localization at the interface between the two populations. Therefore, we decided to co-culture lower sorted transduced cells (Appendix-Figure 5.2) to see if with these cells we could clearly observe the effect at the interface of the two cell populations. However, we also observed the high increase in GFP-Vangl2 levels in some of the low transduced MDCK_GFP-Vangl2 cells, which created the same heterogeneity observed using the high transduced cells and difficulty to observe the effect in protein localization at the interface between the two populations (Appendix-Figure 5.3). Furthermore, as mentioned on chapter 4, it was difficult to visualize the GFP-Vangl2 protein in some of the cells due to their low transduction levels. Due to lack of clarity on the three time points (day 1, 5 and 7) we decided to use the live image microscope to take
sequences of pictures in early time points (between 20 and 32 hours after the second cell type was seeded). Pictures were taken every hour for 14 hours (Figure 5.2). On the early time point frames or pictures (20-23 hours), in some MDCK_GFP-Vangl2 cells at the interface of the two populations we could observe a decrease in the protein accumulation at the cell membrane that is in contact with the wild type cells (Figure 5.3-red arrow). However, this phenomenon is not clear in all the cells at the interface.

Figure 5.1. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells co-cultured with wild type MDCK over time. MDCK_GFP-Vangl2 cells were co-cultured with wild type MDCK on filter insert membranes for 1, 5 and 7 days. (A, B, C) GFP-Vangl2 localization in MDCK_GFP-Vangl2 cells in co-cultured condition on day 1, 5 and 7 respectively. (A) White arrow shows localization of GFP-Vangl2 protein to the cell membrane and red arrow shows perinuclear location of GFP-Vangl2 protein. (B, C) White arrow shows cells with significant increase expression of GFP-Vangl2 protein. MDCK_GFP-Vangl2 cells were cultured on un-patterned filter insert membranes as controls (D, E, F) GFP-Vangl2 localization in un-patterned cells on day 1, 7 and 15 respectively. Scales bars 50 μm. Green- GFP-Vangl2 protein, Blue-nucleus.
Figure 5.2. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells co-cultured with wild type MDCK on early time point. Example of one of the pictures taken with the live microscope. Picture was taken proximally 20-23 hours after second cell type (wild type MDCK) was seeded. (A) FITC channel showing MDCK-GFP-Vangl2 population. (B) Dapi channel showing nucleus of MDCK_GFP-Vangl2 cells and MDCK wild type cells. Scale bar 100 um. Green-GFP-Vangl2; Blue-Nucleus
Figure 5.3. Close up of interface between MDCK_GFP-Vangl2 and MDCK wild type population on co-culture at early time points. (A,B,C,D) Close up of different pictures taken on the live microscope at the interface between MDCK_GFP-Vangl2 cells and wild type MDCK cells. Red arrows show decreases of GFP-Vangl2 protein accumulation at the cell membrane that is in contact with wild type cells. Blue arrows show normal accumulation of GFP-Vangl2 at the cell membrane that is in contact with MDCK_GFP-Vangl2 cells.

5.3.2 Assessment of epithelial cell polarization markers on MDCK_GFP-Vangl2 and wild type MDCK cells

To evaluate if the co-culture condition had any effect in the dynamics of apical-basal polarization in MDCK_GFP-Vangl2 cells and wild type MDCK, we co-culture the cells for 1,5 and 7 days on filter insert membranes. The ZO-1 expression was assessed for day 1 and 7, and primary cilia formation for days 5 and 7. As controls, MDCK_GFP cells were co-cultured with wild type
MDCK cells for 1,5 and 7 days. We did not observe any effect on the dynamics of the ZO-1 expression in any of the cell populations (Figure 5.4 A-D). Also, no difference was observed in the ZO-1 protein levels and its localization between the two populations. ZO-1 is localized to the cell membrane in the sub-apical segment of the cells from day 1 throughout the cell sheet (Figure 5.4 E-H). Regarding cilia formation, on day 5 (Figure 5.5 A) the amount of cilia formed was low, which is normal in the dynamics of cilia formation in MDCK cells (Appendix-Figure 5.4). Therefore, due to this low amount it was difficult to evaluate the effect of the co-cultured condition on cilia formation at this day. However, it is important to point out that the amount of cilia through the cell sheet was similar in the control co-culture (Figure 5.5C) and co-culture with modified cells (Figure 5.5A). Approximately, there was in average 2 cilia in a 50x50 um square on both samples. On day 7 we could observe an increase on the cilia amount. We did not observe any difference in cilia formation throughout the cell sheet between the co-cultured with modified cells (Figure 5.5B) and control sample (Figure 5.5D). Approximately, there was in average 10 cilia in a 50x50 um square on both samples. Finally, it is important to mention that we observed that the number of primary cilia formed in different batches of cells at the same day was slight different (data not show). So, the generation of control for each batch of experiment was important.
Figure 5.4. Assessment of apical polarization on co-cultured MDCK_GFP-Vangl2 with wild type MDCK cells on filter insert membranes over time. MDCK_GFP-Vangl2 cells were co-cultured with MDCK wild type cells (WT) on filter insert membranes for 1 and 7 days and stained for tight junction marker ZO-1. As control MDCK-GFP were co-cultured with wild type MDCK for 1 and 7 days and stained for ZO-1. Images A to D show projected stacks of ZO-1 over time. (A,B) ZO-1 expression on co-cultured cells on day 1 and 7 respectively. (C,D) ZO-1 expression on control samples on day 1 and 7 respectively. Scales bars 50 μm. Red- ZO-1 protein. Images E to H show the z position of ZO-1 over time. (E,F) ZO-1 position on co-cultured cells on day 1 and 7 respectively. (G-H) ZO-1 position on control samples on day 1 and 7 respectively. Scales bars 5 μm. Red- ZO-1 protein, Blue-nucleus.

Figure 5.5. Assessment of primary cilia formation on co-cultured MDCK_GFP-Vangl2 with wild type MDCK cells on filter insert membranes over time. MDCK_GFP-Vangl2 cells were co-cultured with wild type cells on filter insert membranes for 5 and 7 days. As control MDCK-GFP were co-cultured with wild type MDCK (A,B) primary cilia on co-cultured on days 5 and 7 respectively (C,D) primary cilia on control samples on days 5 and 7 respectively. Scales bars 50 μm. Red- Acetylated α-tubulin, Blue-nucleus.
5.4 Discussion

In domineering non-autonomy phenomenon observed in Drosophila and mice tissue, organization of cellular structures is affected in both genetically modified cells and neighboured cells with non genetically modification. In our model system we could make the following observations. First, the genetic modification in MDCK cells, through overexpression of Vangl2 protein, did not affect the expression and localization of the apical polarization marker ZO-1 in these modified cells. This finding correlates with what is found in the literature, that the apical-basal polarization of epithelial cells is not affected in cell population with mutation in any of the PCP core protein. More interesting is that in these modified cells the cilia formation was not affected. As mention in chapter 4 the role of Vangl2 protein in ciliogenesis is still not clear. This non-effect in ciliogenesis has been observed before in other mammalians cells, such as ciliated airway cells and primary mouse embryonic fibroblast. In other vertebrates it has been shown that some of the PCP core proteins are important for ciliogenesis. Therefore, one other possibility for the non-effect of Vangl2 overexpression on MDCK ciliogenesis could be the fact that we are using a mouse Vangl2 construct on dog cells. Therefore, in future work it would be worth it to study the effect of this overexpression in ciliogenesis in mouse cells.

Additionally, these two factors (ZO-1 expression and cilia formation) were also not affected in the wild MDCK type cells when they were co-cultured with the modified cells. This could suggest three scenarios: (i) Supposing that the overexpression of Vangl2 protein on modified cells is affecting the levels or localization of endogenous Vangl2 protein of wild type cells (which was not possible to assess at the moment due to the lack of a robust antibodies), this would suggest that in MDCK cells Vangl2 protein is not linked with ciliogenesis, and expression and apical localization of ZO-1 protein specifically in MDCK cells. (ii) Supposing that the overexpression of Vangl2 protein on modified cells affects the levels of other endogenous PCP core protein in the wild type cells (which again is not possible to assess at the moment due to lack of robust antibodies), this would suggest that overall PCP core protein are not linked with ciliogenesis and expression and apical localization of ZO-1 protein specifically in MDCK cells. (iii) There is a chance that even though the exogenous GFP-Vangl2 protein can localize to its natural location, for some reason this exogenous protein is not able to interact or affect the internal components of the cell and it neighbouring cells. One reason could be what we mentioned above regarding the use of a mouse protein in a dog’s cell.

One of the issues of using cell lines for this type of experiments is that the number of primary cilia formed is used as readout, which is not ideal. We observed that the numbers of primary cilia formed from batch to batch varies. This behaviour is expected because it is known that in epithelial cell lines only a subset of cell ciliate in vitro. Therefore, comparing experiments where
different batches of cells were used was confusing. The generation of control for each batch of experiment was important. The use of a cell line or even better primary cells where it is known that more than 95% of cell produce cilia each time would facilitate the analysis in cilia formation in co-culture experiments.

Assessing the effect on the localization of the GFP-Vangl2 protein on the modified cells in the co-cultured experiment was challenging. In the first set of experiment where we co-cultured the cells over time (1, 5 and 7 days) the effect on the GFP-Vangl2 protein localization on the modified cells at the interface of the two populations was not clear. On day 1, 5 and 7 at the interface we did not see any clear effect. In the last two time points we observed the increase of the number of cells with high expression of GFP-Vangl2 protein, in which the protein was not restricted to the cell membrane but it was also localized to the cytoplasm. This high expressing cells were both at the interface of the two cell population and away from the interface. When we observed this high expressing cell on the monocultures we did not think that this was going to make the visualization of the protein localization difficult, but it did.

The live image pictures of the co-cultures taken in early point (20 hour-23 hours) gave us more clear visualization of the localization of the protein at the interface between the two cell populations. In some MDCK-GFP-Vangl2 cells at the interface we could perceive that the amount of protein decreased at the cell membrane that was in contact with the wild type cell (Figure 5.3-red arrow). However, this phenomenon was not clearly observed through all the interface. This result could suggest that may be the effect that the co-culture has in the protein localization at the interface is transitory. Therefore, the time where the samples are analyzed could be crucial. This idea is consistent with what researchers have observed in studies in vivo, in which during epithelium development the localization of PCP core proteins is dynamic over time 13. When we analyzed the relationship between time and localization of the GFP-Vangl2 protein in both conditions, monoculture and co-culture (Table.1 and Table.2), we realized that the time period in which the protein is more dynamic is between 6 to 24 hours. Therefore, this early point could be the clue to better understand if there is a real influence in the protein localization at the cell interface on the co-culture experiment.
Table 5.1 Dynamics of MDCK_GFP_Vangl2 cell in Monoculture

<table>
<thead>
<tr>
<th>Timing</th>
<th>Cell Status</th>
<th>Apical-Basal Polarization Status</th>
<th>Vangl2 localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>Cell seed</td>
<td>No polarized</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>1 hour</td>
<td>Cells attach</td>
<td>No polarized</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>6 hours</td>
<td>Cells start to form a sheet</td>
<td>Start to for tight junctions</td>
<td>Cytoplasm-perinuclear</td>
</tr>
<tr>
<td>16 hours</td>
<td>Cell sheet confluent</td>
<td>Tight junctions well formed</td>
<td>Cytoplasm-perinuclear</td>
</tr>
<tr>
<td>24 hours</td>
<td>Cell sheet confluent</td>
<td>Tight junctions well formed</td>
<td>Membrane-fewer in the cytoplasm</td>
</tr>
<tr>
<td>5 days</td>
<td>Cell sheet confluent</td>
<td>Primary cilia start to appear</td>
<td>Membrane-increase in the cytoplasm</td>
</tr>
<tr>
<td>7 days</td>
<td>Cell sheet confluent</td>
<td>More primary cilia</td>
<td>Membrane-increase in the cytoplasm</td>
</tr>
</tbody>
</table>

Table 5.2 Dynamics of MDCK_GFP_Vangl2 and wild type MDCK cell in Co-culture

<table>
<thead>
<tr>
<th>Timing</th>
<th>CP1 Status</th>
<th>CP2 Status</th>
<th>Interface</th>
<th>Apical-Basal Polarization Status</th>
<th>Vangl2 localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>Cells seed</td>
<td>N/A</td>
<td>N/A</td>
<td>CP1-Not polarized</td>
<td>CP1-Cytoplasm</td>
</tr>
<tr>
<td>1 hour</td>
<td>Cells attach</td>
<td>N/A</td>
<td>N/A</td>
<td>CP1-Start to form tight junctions</td>
<td>CP1-Cytoplasm</td>
</tr>
<tr>
<td>3 hour</td>
<td>Cells start to form a sheet</td>
<td>Cells seeded</td>
<td>Starts to form</td>
<td>CP1-Forming tight junctions</td>
<td>CP1-Cytoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CP2-Not polarized</td>
<td></td>
</tr>
<tr>
<td>6 hours</td>
<td>Cells start to form a sheet</td>
<td>Cells start to form a sheet</td>
<td>Continue forming</td>
<td>CP1 Forming tight junctions</td>
<td>CP1-Cytoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CP2-Not polarized</td>
<td>Cytoplasm-perinuclear</td>
</tr>
<tr>
<td>16 hours</td>
<td>Cell sheet confluent</td>
<td>Cell sheet confluent</td>
<td>Well formed</td>
<td>CP1 and CP2-Tight junctions well formed</td>
<td>CP1-Cytoplasm-perinuclear</td>
</tr>
<tr>
<td>24 hours</td>
<td>Cell sheet confluent</td>
<td>Cell sheet confluent</td>
<td>Well formed</td>
<td>CP1 and CP2-Tight junctions well formed</td>
<td>CP1-Membrane-fewer in the cytoplasm</td>
</tr>
<tr>
<td>5 days</td>
<td>Cell sheet confluent</td>
<td>Cell sheet confluent</td>
<td>Well formed</td>
<td>CP1 and CP2-Primary cilia start to appear</td>
<td>CP1-Membrane-increase in the cytoplasm</td>
</tr>
<tr>
<td>7 days</td>
<td>Cell sheet confluent</td>
<td>Cell sheet confluent</td>
<td>Well formed</td>
<td>CP1 and CP2-More primary cilia</td>
<td>CP1-Membrane-increase in the cytoplasm</td>
</tr>
</tbody>
</table>

CP1= Cell population 1 or MDCK_GFP-Vangl2 cells; CP2=Cell population 2 or wild type MDCK
5.5 Conclusions

Recreating in vivo systems, such as domineering non-cell autonomy, that have facilitated the understanding of the mechanism to generate planar organization in the epithelium is a very valid starting point to begin to elucidate important factors that will allow us to develop a reliable in vitro system of an epithelial cell sheet with planar organization. Assessing the effect on the localization of the GFP-Vangl2 protein on the modified cells in the co-culture experiment has several challenging factors. We could observe in some cells a decrease in the amount of protein at the cell membrane that was in contact with the wild type cell at an early point, but it was not consistent throughout the interface. However, we reached to the conclusion that early time points could be the clue to better understand the effect on effect on the localization of the GFP-Vangl2 protein when co-culturing these two distinct populations. Finally, we did not observe any effect in apical-basal polarization throughout the cell sheet.

5.6 Reference

Chapter 6.
Conclusions, Challenges and Future Works

6.1 Conclusions

Overall we wanted to find strategies that allow us to create epithelium with correct structure. In this specific study we wanted to begin to study the possibility to use chemical signalling (cellular signalling) as a strategy to organize an epithelia cell sheet \textit{in vitro}. We focused in an important signalling pathway (PCP pathway), which is known to generate the planar organization of the epithelium. Specifically, we recreated the domineering non-cell autonomy phenomenon, observed in studies of PCP \textit{in vivo}, as the starting point to understand the effect of genetically pattern different cell populations in the organization of an epithelial cell sheet \textit{in vitro}. For the development of this study we: (i) developed patterning techniques that allowed us to create monocultures and side-by-side co-culture of epithelial cell populations on filter insert culture system, (ii) created a genetically modified epithelial cell population (MDCK\_GFP-Vangl2) and assessed markers of cell sheet organization, (iii) co-cultured side-by-side genetically distinct epithelial cell population (MDCK\_GFP-Vangl2 with wild type MDCK cells) and assessed the effect of this condition in the organization in the cell sheet.

1. Development of novel Micropatterning tools: The generation of techniques that allow the spatial and geometrical control of epithelial cell populations on insert filter membranes, while allowing the cells to fully mature or apical basal polarize, not only permitted us to execute our experiment, but they have the potential to become important tools to answer other biological questions that due to lack of appropriate techniques have not been answered. For instance, our methods could be useful for probing the importance of cell organization on epithelium function for applications in tissue engineering and generating relevant \textit{in vitro} models of diseased epithelium to better understand disease mechanisms.

2. Understanding the behaviour of GFP-Vangl2 protein on MDCK cells and its effect on cell sheet organization: Reagents such as robust antibodies for PCP core proteins are not broadly commercially available. Therefore, the generation of an epithelial cells population with a GFP tagged PCP core protein (GFP-Vangl2), which allowed the visualization of the protein, was a very valuable tool to begin to understand the behaviour of the GFP-Vangl2 protein in an \textit{in vitro} situation. Furthermore, we were able to elucidate different factors that influence the behaviour of the protein
within the cell, such cell confluence, cell-cell interaction and time in culture. A very interesting observation was that the GFP-Vangl2 protein only was able to localized to the cell membrane when the modified cell was in tight contact for certain time with other modified cell. This let us conclude that the GFP-Vangl2 protein needs the neighbouring cell to stabilize to the cell membrane. This observation is interesting because it suggests that the protein needs to interact with other component or protein from the neighbouring cell, which probably is other PCP core protein as the model proposes it.

We also observed high over-expression of the protein in some cells over time. This high overexpression of the protein in the some cells over time was an issue that we found and that made difficult to observe the protein behaviour at later time points. We do not clearly understand why this overexpression happened over time. To better understand this phenomenon it is necessary to complete further studies to assess if this is specific for MDCK cells or is an issue of viral transduction per se.

Finally, we did not see any effect in the expression and localization of tight junction and primary cilia formation due to the presence of GFP-Vangl2 protein in the cells. This suggests that the overexpression of MDCK-Vangl2 does not affect the apical-basal polarization of modified MDCK cells.

3. Effect of co-culturing side-by-side MDCK_GFP-Vangl2 cells with wild type MDCK cells: Recreating in vivo systems, such as domineering non-cell autonomy, that have facilitated the understanding of the mechanism that generates planar organization in the epithelium is a very valid starting point to begin to elucidate important factors that will allow us to develop a reliable in vitro system of an epithelial cell sheet with planar organization. The results of the co-culture experiment regarding its effect in the GFP-Vangl2 localization in modified cells showed an effect in some cells at the interface of the two populations at early time points. These results together with the monoculture results suggest that to localize Vangl2 to a cell membrane, the membrane has to be in contact with a neighbour cell membrane that contains sufficient levels of Vangl2 protein or may be other of other PCP core protein.

We realized that early time points were important not only to understand the dynamics of the protein within the cells, but to also give us a clearer understanding of the effect of the co-culture experiments in the localization of the GFP-Vangl2 protein. Therefore, an important conclusion we reached was that the timing is an essential factor that has to be taken into account when design experiments in which the dynamics or behaviour of a PCP core protein want to be studied.

Finally, we did not see any effect in the expression and localization of tight junction and primary cilia formation in the co-culture condition through the cell sheet, as we observed in the
monoculture of MDCK-GFP-Vangl2 cells. These results suggest that the overexpression of GFP-Vangl2 protein on modified cell population does not affect the apical-basal polarization of its neighbour wild type cell population.

6.2 Challenges

1. MDCK cells were the best option at the time we did our experiments. Their structure and behaviour is very well characterized. Furthermore, the exogenous GFP-Vangl2 protein was able to localize to the cell membrane, which we did not observe with other epithelial cell lines. However, we also are aware that this cell type has some limitation. Due to the fact that this cell line comes from dog it is difficult to find antibodies for PCP core proteins that work in these cells. Also, the exogenous Vangl2 is from mouse, which could be an issue. Therefore, it would be appropriate to verify observation that we have made using MDCK cells, such as the non-effect in ciliogenesis, in other cell type, preferentially from mouse.

2. Finding clear readouts of PCP signalling is not simple. PCP signalling controls cell morphology, therefore activation or changes in PCP signalling can only be measured by morphological changes, which can be challenging to quantify. For instance, in *Drosophila* PCP signalling happens in different tissues, however the most used tissue for PCP studies is the wing because it has a simple PCP readout. In our system we decided to use markers of apical-basal polarization, expression and apical localization of tight junctions and primary cilia formation. One of the issues of using cell lines is that the length of the cilia through the cell sheet and from sample to sample varies significantly. Therefore, measuring and even observing where the cilia is pointing out was a very difficult task to perform; contrary to tissue models such as the Drosophila wing actin hair and the hair bundles in the inner ear cells where the cilia orientation is very obvious. Therefore, we focused on the more limited readout of cilia formation. However, due to the nature of the cell line we also observed a difference in cilia formation from sample to sample, which made difficult to compare experiment where different batches of cells were used. The use of a cell line or even better primary cells where it is known that more than 95% of cell produce cilia each time would facilitate the analysis in cilia formation in co-culture experiments and maybe cilia orientation could be assessed.

6.3 Future Work

The generation of planar cell polarization in an epithelial cell sheet *in vitro* is not a simple task. Therefore, future studies will be necessary to elucidate more factors to develop an effective strategy to generate this planar polarization in engineered epithelium.
In the future to further understand the effects of co-culturing modified and wild type cell populations, it could be important to understand the behaviour of the endogenous Vangl2 PCP core protein. This could allow us to observe better the overall effect on the sheet organization of co-culturing MDCK-GFP-Vangl2 cells with wild type MDCK. If the overexpression in the modified cell has an effect in the behaviour of the endogenous protein in wild type cells, this would be a strong confirmation that the co-culturing condition is affecting the wild type cells. However, as mentioned earlier it is difficult to find an antibody for these PCP core proteins that work in dog cells. So, probably it would be necessary to try another cell line or primary cell to execute this type of experiments.

6.4 Work Significance

The epithelium is an essential tissue to create full functional organs, and furthermore the malfunction of this tissue is the cause of some human diseases such as cancer. Therefore, creating strategies to engineer epithelium is not only important for tissue engineering and regenerative medicine, but also for understanding disease processes. Nevertheless, studies in engineering epithelium are not as advanced as in other tissues like muscle, bone and nerve. Although the epithelium seems to have very simple structures, a mono or multilayer of cell over a basement membrane, the organization of the cells within the tissue is very precise and has specific properties, which are essential for the correct tissue functioning.

Through the development of this study we have realized that little work has been done in epithelium tissue engineering in terms of finding strategies to engineer a mature structure. Therefore, this work is an important step towards designing engineering strategies that will allow for generation of more mature/functional epithelium. The planar cell polarization is a structural property of the epithelium that has been overlooked in epithelium tissue engineering studies. This polarization is as important for the correct tissue function as the apical-basal polarization. A possible reason why this polarization has been overlooked is that the mechanism of how it is generated in vivo is still not clear in any species, which makes it challenging to create strategies to generate it in engineered tissues. However, this obstacle should not be a reason for not including it as design criteria for epithelium engineered tissue strategies. On the contrary, finding strategies to generate this polarization in engineer tissues could also be an invaluable tool to further understand this polarization; to our knowledge in vitro system models in which this polarization is generated in an epithelial cell sheet are not developed.
Appendix

Figure 2.1 Example of PCP defects in vertebrate and *Drosophila*. (a-b) In wild type cells in *Drosophila* wing, cells generate hairs that grow and point distally (a). In mutant cells in *Drosophila* wing, cells generate hairs without any precise orientation (b). (c-d) As happened in the *Drosophila* wing cells, in mammalian the skin hairs point distally in wild type animals (c). In contrast, in PCP mutant animals skin hairs point in different directions (d). (e-f) *Drosophila* eye is composed of a group of ommatidia arrange in particular manner; each ommatidium is composed of photoreceptor cells which have a precise arranged too (e). In PCP mutant the arrangement of the photoreceptors in the ommatidium and the arrangement between the ommatidia is disturbed. (g-h) Mammalian cells localized in the inner ear, more specific in the cochlea, have a group of sensory hairs parallel arranged (g, show in green). In PCP mutants this parallel arrangement is disturbed (h).

Figure 3.1 *Co-patterning of GFP expressing and non-GFP epithelial cells on filter insert membrane at Day 7 using fibronectin method*. (A-B) Co-culture of GFP MDCK and wild type MDCK cells generated by fibronectin micropainting method. (B) Co-culture of GFP ARPE and wild type ARPE cells generated by fibronectin micropainting method. Scale bars 200 μm. Blue-Nucleus, Green-GFP.
Figure 4.1 Unsorted MDCK-GFP-Vangl2 cells. Unsorted MDCK_GFP-Vangl2 cells were cultured on unpatterned coverslip for 5 days under serum free medium. Scales bars 100 μm. Green- GFP-Vangl2 protein, Blue-nucleus.

Figure 4.2. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells on Day 0. (A-B) MDCK_GFP-Vangl2 cells were culture for 16 hours on unpattered filter insert membranes. Scales bars 50 μm. Green- GFP-Vangl2 protein, Red-Zo-1, Blue-nucleus.
Figure 4.3. Localization of GFP-Vangl2 protein in MDCK_GFP-Vangl2 cells on patterned filter insert membrane culture over time. MDCK_GFP-Vangl2 cells were cultured on patterned (200 μm agarose microwells) filter insert membranes for 1, 7 and 15 days. (A,B,C) GFP-Vangl2 localization in patterned cells on day 0, 7 and 15 respectively. Scales bars 50 μm. Green- GFP-Vangl2 protein, Blue-nucleus.

Figure 4.4. Assessment of apical polarization patterned MDCK_GFP-Vangl2 cells on filter insert membranes over time. MDCK_GFP-Vangl2 cells were cultured on patterned filter insert membranes (200 μm agarose microwells) for 1, 7 and 15 days and stained for tight junction marker ZO-1. Images A to C show projected stacks of ZO-1 over time. (A,B,C) ZO-1 expression on MDCK_GFP-Vangl2 cells on day 1, 7 and 15 respectively. Images D to F show the z position of ZO-1 over time. (D,E,F) ZO-1 position on MDCK_GFP-Vangl2 cells on day 1, 7 and 15 respectively. Scales bars 50 μm. Red - ZO-1 protein, Blue-nucleus.
Figure 4.5. Assessment of primary cilia formation on patterned MDCK_GFP-Vangl2 cells on filter insert membranes over time. MDCK_GFP-Vangl2 cells were cultured on patterned (200 μm agarose microwells) filter insert membranes for 7 and 15 days and stained for primary cilia marker Acetylated α-tubulin. (A,B) primary cilia on MDCK_GFP-Vangl2 cells on day 7 and 15 respectively. Scales bars 50 μm. Red- Acetylated α-tubulin, Blue-nucleus.

Figure 5.1. Domineering Non-autonomous phenomenon in Drosophila wing. In the picture wing cells outline in red have a modification in a PCP core protein (Fz). Cell outline in blue consist on wild type cells (WT) that are affected by the modified cells. In affected WT cell the actin hairs look disarrange compare with the actin hair for other non-affected WT cells1.
Figure 5.2. Generation of a homogeneous population of lower transduced MDCK cells. MDCK cells transduced with GFP-Vangl2 plasmid. After cell expansion, a homogeneous population of lower transduced MDCK cells was isolated by using activated cell sorting (FACS). (a) Flow cytometer of MDCK-GFP-Vangl2 shows the broad distribution of transduced levels or GFP fluorescent levels in the population. R5 denotes a population of MDCK_GFP-Vangl2 with low transduction levels and R4 denotes a population of MDCK_GFP-Vangl2 with high transduction levels (b) Flow cytometer of MDCK_GFP-Vangl2 cells after sorting low transduced population of MDCK_GFP-Vangl2 or R5 denoted cells.

Figure 5.3. Co-culture of MDCK wild type cells with low transduced MDCK with GFP-Vangl2 cells over time. Co-cultures with low transduced MDCK cell with GFP-Vangl2 still have high expressing GFP-Vangl2 cells (A,B) Co-culture of low transduced MDCK_GFP-Vangl2 cells with wild type MDCK cell on days 5 and 7 respectively. Scales bars 50 μm. Green- GFP-Vangl2; Blue-nucleus.
Figure 5.4. *Primary cilia formation on wild type MDCK cell on day 5.* Wild type MDCK cells were culture for 5 days in filter insert membranes and stained for primary cilia using antibody against Acetylated α-tubulin. Scales bars 50 μm. Red- Acetylated α-tubulin, Blue-nucleus.

Reference