Myoepithelial cell morphogenesis and differentiation in the mouse submandibular salivary gland in development and disease

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Myoepithelial Cell Morphogenesis and Differentiation in the Mouse Submandibular Salivary Gland in Development and Disease

By
Elise M. Gervais

A Dissertation
Submitted to the University at Albany, State University of New York
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

College of Arts & Sciences
Department of Biological Sciences
2015
Organogenesis is the process by which tissues organize, gain considerable size, and undergo cellular differentiation or specialization to form fully functional organs. To study the processes involved in organogenesis of branched organs, the mouse submandibular salivary gland is frequently used as a model system, as it can undergo morphogenesis and differentiation and be genetically manipulated *ex vivo*. The mouse submandibular salivary gland undergoes a specific process of outgrowth and invagination known as branching morphogenesis which allows for the significant increase in gland size and complexity, as well as maximization of surface area for secretion of saliva. Surrounding the mouse submandibular salivary gland is a thin layer of specialized extracellular matrix known as the basement membrane that is critical for the continued growth and development of the gland. The basement membrane contains collagens, laminins, and several other structural proteins involved in the formation of a complex matrix to provide structural support and outside-in signalling. The outer layer of polarized epithelial cells that contacts the basement membrane and participates in its synthesis later differentiates into myoepithelial cells. One of the proteins known to be required for maintenance of the epithelial cellular polarity is Par-1b, a member of the PAR family of proteins. Par-1b is maintained on the basal side of the outermost polarized epithelial cells in early development, and we have shown here that it is both necessary and sufficient to drive the localized organization of the basement membrane. We have also shown that Par-1b, acting downstream of the small GTPase Rac1, is required for the differentiation of the outermost polarized epithelial cells into contractile myoepithelial cells.
In fully functional adult salivary glands, the myoepithelium embedded in the basement membrane is thought to aid in salivary secretion by squeezing the secretory units. Loss of saliva secretion in Sjögren’s syndrome, an autoimmune exocrinopathy affecting the salivary gland, result from lack of function of the myoepithelial cells in some patients. In order to gain insight into disease progression we used immunohistochemistry to examine changes in the epithelial cell subpopulations during disease progression in a commonly used mouse model, the non-obese diabetic ShiLtJ mouse and compared the same markers in human tissues. We identified significant changes in the myoepithelial population, where a subset of myoepithelial cells co-expressed a progenitor cell marker at later stages of the disease. Further investigations into the mechanism controlling differentiation of the myoepithelium in the developing mouse submandibular salivary gland may shed light on the changes seen in the myoepithelium during Sjögren’s syndrome disease progression.
Acknowledgements

First and foremost I owe a huge debt of gratitude to my mentor Dr. Melinda Larsen. Without her unconditional support through all the ups and downs inherent to scientific investigations I would not have been able to put together the document you are about to read. Mindy’s insistence that I think in question format, write formally, and make solid, well-articulated conclusions has changed the way I think and the way I present my work. Her guidance has allowed me to publish my work, receive my own funding through a pre-doctoral grant from the NIDCR at the NIH and present my work at several international conferences. I have been known to tease Mindy about her ability to pick out anything that isn’t perfectly aligned in a figure or text that is even slightly different on a poster, but I’ve come to rely on her attention to detail and have taken it as a personal challenge to show her something she can find no formatting issues with… I have not yet succeeded. Mindy has always taken the time to sit through practice talks, review outlines, and struggle through the editing of the seemingly endless number of drafts and documents that were sent her way – her patience is matched by none! I have learned so much from working with Mindy about how to be a scientist, a mentor, and a teacher, and so for all of that THANK YOU!

Thank you to the members of my thesis committee: Dr. Susan LaFlamme, Dr. Albert Millis, and Dr. Haijun Chen. Your support and guidance has kept me on track, and challenged me to think critically about my project every step of the way. Our meetings over the years have taught me to look forward to feedback of all kinds and embrace every question.
I also need to say a huge thank you to all of my lab mates, both past and present. Without the guidance of Dr. Sharon Sequeira, I would have never analyzed my data with such a critical eye, or gotten over my fear of asking or answering tough questions. Dr. Deidre Nelson has spent countless hours helping me troubleshoot and reevaluate, especially while multiplexing. Ms. Kara DeSantis has been irreplaceable in her ability to help me analyze, manage, and process data while simultaneously making me laugh, sometimes to the point of tears. I also need to acknowledge Weihao Wang, an exceptional undergraduate student who worked for 3 years in the lab to help me complete many of the experiments presented here. Wei is truly one of the most motivated, driven, and dedicated individuals I have ever met, and I am a better scientist for having worked with him!

We frequently show pictures and list names of those who have helped in the collection or analysis of data following a presentation, listing family and friends at the bottom of the page, but we very rarely take the opportunity to really thank those behind the scenes; those at home that let us vent about a stressful day, calm us down, feed us when we are hangry, and celebrate with us at every triumph, no matter how small. My parents, Barbara and Allan Gervais have been an incredible support system throughout my entire life, encouraging me and pushing me to work hard and I owe them both a huge THANK YOU! Their unwavering support has made so many of the successes in my life possible. A big thank you is also in order to Joseph Vogt, for all your patience and understanding throughout the writing process when ‘real life’ was so often put on hold. And last but not least, thank you to my extended family, grandmothers, brothers, and many friends who have helped to keep my life outside the lab as ‘normal’ as possible. Cheers!
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<td>SMG</td>
<td>Submandibular salivary gland</td>
</tr>
<tr>
<td>E#</td>
<td>Embryonic Day #</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>BM</td>
<td>Basement membrane</td>
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<td>OCCs</td>
<td>Outermost columnar epithelial cells</td>
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<td>IPCs</td>
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<td>aPKC</td>
<td>atypical protein kinase C</td>
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<td>MAP</td>
<td>microtubule associated protein</td>
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<td>Ras-related C3 botulinum toxin substrate 1</td>
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<td>Tiam1</td>
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<td>TJ</td>
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<td>GEF</td>
<td>Guanine exchange factor</td>
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<tr>
<td>GAP</td>
<td>Guanine nucleotide activating protein</td>
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<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney epithelial Cell</td>
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<td>Term</td>
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<tr>
<td>Rho-associated coiled-coil containing kinase</td>
<td>ROCK</td>
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<td>Smooth Muscle α-actin</td>
<td>SM α-actin</td>
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<td>Sjögren’s syndrome</td>
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<td>Cesium Chloride</td>
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<td>Kinase Dead</td>
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<td>Wild Type</td>
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<td>Plaque forming units</td>
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Chapter 1: Introduction: Establishment and Maintenance of Apico-Basal Cellular Polarity in the Developing Mouse Submandibular Salivary Gland

1.1 Early Gland Development and Branching Morphogenesis

During embryonic development, complex three-dimensional organs take shape and undergo dynamic and coordinated tissue morphogenesis, including the physical movement and reorganization of cells, along with cellular differentiation, or specialization. The submandibular salivary gland (SMG) undergoes a series of morphogenetic events during embryonic development collectively referred to as the process of branching morphogenesis (Grobstein, 1953; Patel et al., 2006; Tucker, 2007). Branching morphogenesis is a conserved developmental mechanism that is utilized by many other organs, including the lungs, kidneys, and mammary glands, to increase the epithelial surface area for either secretion or absorption. The embryonic mouse SMG serves as an ideal system for studying the molecular mechanisms required for branching morphogenesis as glands can be grown ex vivo on a filter at the air/media interface and can be manipulated genetically or pharmacologically over several days in culture (Figure 1.1) (Sakai and Onodera, 2008). Mouse SMG development begins in utero on embryonic day 11 (E11), as a protrusion of epithelial cells from the oral epithelium into the surrounding mesenchyme to form a primary bud and branching initiates at E12 as invaginations or clefts, form in the outer edges of the initial bud (Brien et al., 2002; Daley et al., 2011). Rapid and iterative rounds of cleft formation and elongation coupled with proliferation within newly
formed epithelial buds characterize the early morphogenesis stages (E13-E16) and yield a much larger, more highly branched structure.

After several days (beginning around E15) spherical secretory units that consist of secretory acinar cells surrounded by a layer of specialized contractile myoepithelial cells, embedded in the surrounding basement membrane (BM), begin to take shape. The outermost epithelial cells are thought to begin differentiation into the myoepithelium during late SMG embryonic development (E16-E18), and complete differentiation postnatally. The surrounding BM is a specialized form of extracellular matrix (ECM) encompassing the entire glandular epithelium in early development and the individual secretory units in the fully developed SMG and continuously provides both structural support as well as outside-in signals to the underlying epithelial cells. The secretory acinar units surround a central hollow lumen that is connected to the ductal system of the gland, into which salivary components are secreted by the acinar cells (Nakamoto et al., 2007). The myoepithelium surrounding the secretory acinar units to provide support to the salivary gland structure and help to maintain homeostasis by producing BM proteins as well as regulatory growth factors (Redman, 1994; Ianez et al., 2010; Kandagal et al., 2013; Tamgadge et al., 2013). In both the mouse SMG and the human labial salivary gland, myoepithelial cells surround the differentiated acinar cells and some of the smaller ductal structure by extending long projections that wrap around these structures (Doggett et al., 1971; Redman, 2008; Ianez et al., 2010; Amano et al., 2012). These finger-like projections are embedded within the basement membrane surrounding the acinar units, and maintain direct contact with both the acinar cells and the surrounding mesenchymal cells (Doggett et al., 1971; Redman, 2008; Amano et al., 2012).
In the postnatal and adult glands, the secretory cells deliver saliva components into the centralized hollow lumen, aided by the surrounding contractile myoepithelial cells. Saliva is then transported into the ducts and modified as it passes through the ductal system and into the oral cavity (Tucker, 2007). While the structure of the acinar unit is presumably critical for effective saliva secretion, little is understood regarding the molecular events required for the morphological changes leading to the formation of bilayered acini.
(A) Submandibular salivary glands are extracted from E13 embryos, and placed on top of a polycarbonate filter at the air-media interface. Glands are then grown in culture at 37°C, humidified incubator with 5% CO2 for up to 120 hours. (B) SMG’s can be treated with siRNA or pharmacological inhibitors by adding either directly to the culture media. (C) Adenoviral infection of SMG epithelium requires the removal of surrounding mesenchyme, and following infection, recombination of the epithelial rudiment with removed mesenchyme before culturing for up to 120 hours. (D) During SMG organ culture, SMGs undergo branching morphogenesis to gain size and complexity. Scale bar 200 µm.
1.2 Basement Membrane and its Connections to the Glandular Epithelium

During early branching morphogenesis, the outermost columnar epithelial cells (OCCs) form a sheet of polarized cells around the epithelium of the submandibular salivary gland (Figure 1.2A, yellow line). These are the first epithelial cells to establish and maintain apico-basal polarity, and they remain in direct contact with the surrounding basement membrane throughout development. The BM surrounding the developing mouse SMG is composed of several structural proteins, including, but not limited to laminins, collagens, and heparin sulfate proteoglycan (HSPG or perlecan) along with many other less abundant structural and support proteins. These proteins form an intricate web that provides structural support to the underlying epithelial cells (Yurchenco and Wadsworth, 2004; Sequeira et al., 2010), as well as outside-in signals through the cell-surface receptors anchoring the BM to the OCC cell surface. Previous work done in the Larsen lab and others has shown that the BM is essential for the continuation of branching morphogenesis, and several of its components are crucial for the continued development of the gland as well (Bernfield and Banerjee, 1982; Sakai et al., 2003; Larsen et al., 2006b; Daley et al., 2008, 2012; Rebustini et al., 2009). BM proteins are thought to be manufactured by the OCCs with possible contributions from the surrounding mesenchymal population as well. The interior glandular epithelium or interior polymorphic cells (IPC) remain disorganized through much of early development and make little or no contact with the surrounding BM.
Figure 1.2 Polarized Outer Columnar Epithelial Sheet Makes Contact with the Basement Membrane

(A) Brightfield image of an E13 gland and a cartoon representation of an E13 with lines representing the OCC layer of cells (yellow line) and the BM (green line). Scale Bar 200 µm. (B) The outer columnar layer (yellow cells) forms a sheet of cells surrounding the less organized inner polymorphic cells (gray cells) and is overlaid with the BM. (C) The BM is made of collagen IV, laminin, and many other structural components that make contact with the OCC sheet through integrins and dystroglycan. Tight junctions form along the later edges of the cells closer to the apical membrane to connect the OCCs as development progresses. (D) Par-1b is localized along the basolateral domain of the OCCs, closest to the basement membrane, while the apical PAR complex localizes to the area of tight junctions, in the apical domain.
The t-shaped trimer of laminin-111 is one of the major components of the BM in the developing SMG. Each laminin protein is made up of three subunits, and in the case of laminin-111 they are α1β1γ1; though many other laminin combinations have been identified (Miner, 2008), laminin-111 is known to be highly expressed in the developing SMG and is required for proper branching morphogenesis (Hosokawa et al., 1999). These three individual subunits wind around each other extracellularly to form the trunk of the t, and then branch apart at the top of the t to form the two arms and top piece. The two arms and the top portion of the t initially bind to other laminin proteins to form a lattice, while the tail of the laminin molecule binds to cell surface receptors including integrins and dystroglycan, anchoring the lattice to the cell surface (McKee et al., 2007). Once the laminin lattice has been laid down, other BM proteins can attach to form the complex BM structure.

Collagen IV is also found in combinations of trimers, though only three combinations of the 6 isoforms of collagen IV have been identified; α1α1α2, α3α4α5, and α5α5α6 (Khoshnoodi et al., 2008). In early SMG development, only α1α1α2 is found in the SMG where it binds to integrin receptors embedded in the OCC cell layer. Previous work in the salivary gland and in other branched organs has indicated that defects in a collagen IV assembly can be devastating to the growth and development of the organ, and in some cases, the function of the organ, as in the kidneys (Hudson et al., 2003).

Heparin sulfate proteoglycan also known as perlecan is another important component of the BM in the developing SMG. This multi-domain glycosylated protein is very large and known to serve many diverse roles throughout embryogenesis (Knox and Whitelock, 2006). Perlecan contains many domains that serve to bind it to other BM proteins as well as binding
morphogens and growth factors (Farach-Carson and Carson, 2007) as well as integrin cell surface receptors.

Integrins are present on the basal cell surface of the OCCs in the developing SMG in pairs, typically consisting of an α and β subunit in very close proximity to each other, each spanning the membrane, with each combination of integrin proteins having a specific extracellular binding partner (comprehensively reviewed by (ffrench-Constant and Colognato, 2004). Once bound to the basement membrane protein, the extracellular portion of each integrin protein undergoes a conformational change. Once this change occurs, and the integrin is bound to the BM protein, the extracellular conformational change is propagated inward through changes in the spacing between the intracellular tails allowing the propagation of outside-in signals. These signals are further propagated through changes in integrin tail interactions with intracellular proteins, including the cytoskeletal networks.

While laminin-111, collagen IV and perlecan all bind to integrins, laminin-111 also binds to another cell surface receptor called dystroglycan, to form the initial laminin lattice and activate nearby integrins (Driss et al., 2006). Dystroglycan is a cell surface receptor made up of two subunits expressed by the same gene that undergo posttranslational splicing to express both α and β-dystroglycan subunits(Henry and Campbell, 1999; Holt et al., 2000). β-dystroglycan is a transmembrane protein making intracellular contact with the dystroglycan complex that also makes contact with the cytoskeleton, and extracellularly is bound to α-dystroglycan which binds to laminin(Martin, 2003; Moore and Winder, 2010). Dystrophin and utrophin, two proteins known to form an intracellular complex with the cytoplasmic tails of β-dystroglycan propagate signals from extracellular activation of α-dystroglycan to the
Defects in dystroglycan were discovered in muscular dystrophy models, though dystroglycan is now known to be expressed throughout the body, especially in tissues with highly structured BM (Henry and Campbell, 1996). It is thought that the formation of the BM begins with laminin-111 attaching to α dystroglycan at the basal cell surface. The conformational change in dystroglycan following laminin binding pulls laminin closer to the cell surface allowing the nearby integrins to reach and bind to laminin as well (Driss et al., 2006; Berti et al., 2011). Once the lattice of laminin proteins has been laid, other proteins bind not only to laminin, but also to other integrin pairs along the cell surface.

Once the BM is established, very early in SMG development, it is constantly remodeled, adjusting to the glands growth, and reaching into newly formed clefts to support the growing outer surface of the glandular epithelium (Bernfield and Banerjee, 1982; Koda and Bernfield, 1984; Nakanishi and Ishii, 1989). Once the gland has begun functioning after birth, and ceases growth and differentiation on a large scale, both laminin-111 and collagen IV α1α1α2 are replaced by alternate isoform combinations to form the established, fully developed BM that will continue to support the myoepithelium and surround the acinar units throughout the life of the gland (Kadoya and Yamashina, 2005).

Throughout the earliest stages of branching morphogenesis, before myoepithelial differentiation is initiated around E15 (Nelson et al., 2013), the OCCs maintain their apico-basal polarity and contact with the surrounding BM. The outer most or basal surface remains rich in integrins, dystroglycan, and other cell surface receptors used to anchor the BM and receive other survival signals from the surrounding environment. The localization of dystroglycan and...
integrins that are specifically responsible for BM attachment only on the basal surface of the cell is likely due to the maintenance of cellular polarity throughout the early stages of branching morphogenesis.

1.3 PAR Family of Proteins and Cellular Polarity

The OCCs in the developing SMG maintain a relatively columnar shape until differentiation begins in the later stages of development (around E15-16). Within these columnar OCCs, several proteins are interacting to first establish and then to maintain the apicobasal polarity of the outermost cell layer.

Apicobasal polarity is established and maintained in most mammalian epithelial tissues, as well as in many lower organisms, including in their early development. Apicobasal polarity is the formation of two distinct domains within a cell with the apical domain typically facing a luminal structure used in secretory or absorptive tissues, the lateral surfaces making contact with neighboring polarized cells and the basal cell surface making contact with the surrounding extracellular matrix or basement membrane (reviewed by (Margolis, 2005). Polarity is typically established by the mutual exclusion between apically localized proteins and basolaterally localized proteins. Two groups of highly conserved apically localized proteins have been found to be responsible for the apicobasal polarization in cells from C. elegans and Drosophila to mouse and human epithelial cells. Partition defective (Par) proteins Par-3, Par-6 and atypical protein kinase C (aPKC) form a complex that is known as the apical PAR complex which localizes to the tight junctions (Giepmans and van Ijzendoorn, 2009). Tight junctions seal cells together and have frequently been used to identify the border between the apical and basolateral domain in polarized cells (Wang and Margolis, 2007). Crumbs is another apical protein found
embedded in the apical membrane that interacts with many other proteins and protein
complexes to establish and maintain apicobasal polarity (Margolis, 2005). The proteins marking
the basolateral domains of polarized cells are not as well conserved as those found in the apical
domain, but are found in many different species. Scribble and Discs Large (DLG) (studied
primarily in Drosophila) or Par-1b (also known as Microtubule affinity-regulating kinase 2
(MARK2) or EMK1 are known to be localized to the basal or basolateral domain of the polarized
cell respectively. While there is little evidence of Scribble and DLG interacting with other
polarity proteins to maintain cellular polarity in mammalian epithelial cells, they have been
shown in both Drosophila and mammalian cells to maintain the subcellular organization of
other proteins (Harris and Peifer, 2005; Margolis, 2005). Par-1b has been shown to act in a
mutually exclusive manner with members of the apical PAR complex in both polarized
mammalian epithelial cells and in Drosophila to establish and maintain cellular polarity
(Doerflinger, 2003; Goldstein and Macara, 2007).

The PAR family of proteins were initially identified in C. elegans as Partition Defective
Mutants (Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues,
1999). Under normal conditions, the PAR proteins segregate and form distinct, mutually
exclusive domains within the embryo to allow for the appropriate spindle organization and
localization for asymmetrical cell division (Etemad-Moghadam et al., 1995). PAR proteins are
also known to regulate the cellular polarity in drosophila and mammalian cells through the
formation of mutually exclusive intracellular domains and their regulation of the microtubules
(reviewed by (Müsch, 2004; Suzuki and Ohno, 2006). Using Madin-Darby Canine Kidney cells
(MDCK cells), a cell line frequently used for epithelial cellular polarity studies, several groups
have identified roles for the PAR family proteins in the establishment and maintenance of apico-basal cellular polarity in mammalian epithelial cells.

Par-1b, a serine-threonine kinase is known to regulate microtubules by regulating microtubule associated proteins (MAPs) through phosphorylation. One of these such interactions was discovered in a study of the phosphorylation of Tau, a protein implicated in the development of Alzheimer’s disease that is known to bind to and regulate microtubule dynamics (Hayashi et al., 2012). Further investigations in Par-1b’s control of microtubules indicates that Par-1b maintains dynamic instability of microtubules by controlling activation of MAPs that both stabilize and destabilize microtubules depending on the current state of the cell (Hayashi et al., 2012). Recent work in MDCK cells has also identified Par-1b as a critical player in the assembly of laminin in the BM. Par-1b is thought to regulate the intracellular components of the dystroglycan complex, specifically utrophin, further studies of which may shed light on the mechanism by which Par-1b is controlling the assembly of the BM (Masuda-Hirata et al., 2009; Yamashita et al., 2010). RNF41, an E3 ubiquitin ligase has also been identified as a phosphorylation target of Par-1b which is required for the localization of laminin receptors to the basal cell membrane of polarized cells (Lewandowski and Piwnica-Worms, 2014). Many other studies have been done on the role of Par-1b in the formation and polarity of dendritic spines and axons, however, axonal polarity is quite different from the apico-basal polarity of the OCCs in the developing SMG, and won’t be elaborated on here. Taking the aforementioned roles for Par-1b together, it is likely that Par-1b acts to regulate the organization of the non-mitotic microtubules at the basal cell surface in polarized epithelial cells and maintains cellular polarity by translating the outside-in signals from the BM to alter the microtubule organization.
Our recent work reported here has shown a critical role for Par-1b in the deposition, organization, and localization of the BM, as well as in the differentiation of the OCCs into myoepithelium, a cell type known to be in continual contact with the surrounding BM, although the mechanisms through which these processes are carried out are still unclear.

Par-1b’s basal restriction in polarized epithelial cells is maintained, at least in part, through the mutual exclusion with the apical PAR complex, made up of Par-3, Par-6, and atypical protein kinase C (aPKC). During the establishment of cellular polarity, Par-3 dimerizes and recruits Par-6 and aPKC to the newly formed, immature tight junctions, which act as an unofficial boundary between the basal and apical cellular domains (Mizuno et al., 2003). Recent data has established a requirement for Par-3 in tight junction formation (Chen and Macara, 2005) and in the front-rear polarity of migrating cells (Pegtel et al., 2007; Wang et al., 2012). Once the apical PAR complex is formed and the polarized cellular domains are established, the tight junctions begin to form – a milestone that is often used as a marker of fully developed cellular polarity (reviewed by (Vega-Salas et al., 1987; Wheelock and Johnson, 2003). To maintain these distinct domains, Par-1b phosphorylates Par-3 and destabilizes the formation of the apical PAR complex in the basal domain of the polarized epithelial cell (Benton and St Johnston, 2003) while, aPKC phosphorylates Par-1b at tight junctions, dissociating it from the membrane (Suzuki et al., 2004).

The establishment and maintenance of cellular polarity is critical to the process of branching morphogenesis in the developing mouse SMG. While the interactions between PAR proteins are critical for the establishment and maintenance of cellular polarity and the
intracellular domains, interactions between PAR proteins and other cellular components also play a critical role in polarity, differentiation, and other cell processes.

1.4 Rac1 GTPase and its GAPs and GEFs

Another class of molecules that regulates many morphogenetic processes is the Rho family of small GTPases, which play a critical role in the reorganization of the actin cytoskeleton (Hall, 1998), regulation of cell-cell adhesions (Takaishi et al., 1997; Fukata and Kaibuchi, 2001; Charrasse et al., 2003) and control of cellular motility (Nobes and Hall, 1995). Rac1 (Ras-related C3 botulinum substrate 1) is a ubiquitously expressed member of this family (Didsbury et al., 1989; Hall, 1998; Corbetta et al., 2009) and is the most abundantly expressed Rho GTPase in the mouse embryo (Wang and Zheng, 2007; Heasman and Ridley, 2008). Rac1 was first discovered as a regulator of actin polymerization in the extension of lamellipodia in migrating cells but has also been shown to regulate many other cellular functions including cell-cell adhesions through tight junctions and adherens junctions, (Takaishi et al., 1997; Izumi et al., 2004; Hirai et al., 2007), polarized cellular migration (Pirraglia et al., 2006; Migeotte et al., 2010, 2011), cellular proliferation (Hirsch et al., 2002) and basement membrane assembly in the establishment of apico-basal cellular polarity (O’Brien et al., 2001; Yu et al., 2005), and.

Rac1 acts as a molecular switch, cycling between a GTP-bound active signaling state, and a GDP-bound inactive state. In the active state, Rac1 is aided by guanine nucleotide activating proteins or GAPs that increase the rate of GTP hydrolysis. After hydrolyzing GTP, a guanine exchange factor (GEF), removes the GDP, and allows for the reactivation of Rac1. Changes in the activation state of Rac1 can switch cellular migration from random environment sensing movements to directionally persistent movement (Pankov et al., 2005). The activation state of
Rac1 requires both GAPs and GEFs, therefore, Rac1 activity can be regulated by the subcellular localization of GAPs and GEFs, and in many cases, GAPs or GEFs can recruit Rac1 to a specific cite within the cell. For example, ß-Pix, Rac1 specific GEF has been shown to recruit Rac1 to the focal adhesions and membrane ruffles (ten Klooster, 2006). Tiam-1, Vav-2, and many other Rac1 GEFs and GAPs have been implicated in the regulation of Rac1 activity through their subcellular localization as well (Schiller, 2006). Rac1 GEFs and GAPs have also been shown to bind other proteins to bring them into close proximity with Rac1 downstream effector proteins or target proteins (reviewed by (García-Mata and Burridge, 2007)). During cellular migration, GEFs at the leading edge maintain Rac1 activity, and during cellular polarization, GEF localization at tight junctions allow for localized activation of Rac1, which has been shown to be required for both of these processes (reviewed by (Lawson and Burridge, 2014).

1.5 Interactions Between PAR Family Proteins and Rac1 GTPase

Rac1 is known to play a role in a wide variety of cellular functions, but most important to our work is Rac1s role in cellular polarity. In MDCK (Madin-Darby Canine Kidney epithelial) cells, a cell line frequently used to study epithelial apico-basal polarity, the expression of a dominant negative form of Rac1 lead to the inversion of cellular polarity and a significant disruption to the laminin assembly in the surrounding basement membrane (O’Brien et al., 2001; Hamelers et al., 2005; O’Toole et al., 2011; Lawson and Burridge, 2014). These results along with other studies establish a requirement for Rac1 in apico-basal polarity in epithelial cells. More recent work has begun to shed light on the mechanism by which Rac1 may be controlling cellular polarity.
Tiam1 (T-Cell Lymphoma Invasion and Metastasis 1), is a Rac1 GEF that plays an important role in apico-basal polarity. Tiam1 binds to Par-3, one of the Par proteins forming the apical PAR complex which localizes to the immature tight junctions early in the establishment of apico-basal polarity (Chen and Macara, 2005). Once there, Par-3 recruits and binds to Tiam1. Tiam1 is required for the maturation of the tight junctions (TJ) in epithelial cells, and their maturation solidifies the boundaries between the apical and basolateral domains established polarized epithelial cells. Once Tiam1 is localized to the apical PAR complex at the TJs, it can recruit Rac1. Rac1 binds to Par-6, another PAR protein found in the apical PAR complex, which is constitutively bound to the third PAR protein of the complex, atypical protein kinase C (aPKC) (reviewed by (Mertens et al., 2006). Once activated Rac1 binds to Par-6, releasing the activation inhibition on aPKC, leading to downstream signalling cascade that results in cellular polarity (Mertens et al., 2006). Binding of Par-3 and Rac1 inactivates Par-3 in other areas of the cell, and may shed light on part of the mechanism of mutual exclusion between the apical PAR complex and the basally localized Par-1b.

Tiam1 is also playing a role on the basal side of the polarized epithelial cell through its interaction with β2-syntrophin. β2-syntrophin is a member of the dystroglycan complex found at the basal and basolateral cell membrane in polarized epithelial cells (Mack et al., 2012). Once bound Tiam1 recruits and activates Rac1 in the basal cell domain. Tiam1 is also required for the translation of signals from α3β1 integrin to activate Rac1 and alter the cytoskeleton during cellular migration (Hamelers et al., 2005). These interactions between Tiam1 and the basement membrane receptor complexes may also provide a link between Rac1 and Par-1b in the differentiation of the OCC cell layer in the developing mouse SMG.
1.6 ROCK as an upstream regulator of Par-1b

Other Rho-GTPase mediated signaling pathways have been implicated in the control of cell polarity. Rho-associated coiled-coil containing kinase or ROCK is an effector of the Rho GTPase, Rho1. has recently been identified as a critical regulator of branching morphogenesis (Daley et al., 2009) and the localization of Par-1b in the developing SMG(Daley et al., 2012). During early salivary gland development, ROCK promotes basement membrane remodeling along newly formed clefts and progressing clefts to mediate changes in tissue shape through a myosin II-dependent pathway requiring cellular contractility (Moore et al., 2005; Daley et al., 2009).

ROCK has also been implicated as an upstream regulator of PAR polarity proteins. It has been shown to regulate the apical PAR complex through which it potentially controls cellular polarity (Nakayama et al., 2008; Simões et al., 2010). ROCK acts as an inhibitor of Rac1 activation though phosphorylation of Par-3, which disrupts the apical PAR complex, Par-3s link to Tiam1 and results in the inability to activate Rac1 (Nakayama et al., 2008). aPKC is also interacting with ROCK to phosphorylate ROCK and allow for its dissociation from the cell-cell junctions. Inactivation of ROCK will prevent the phosphorylation and inactivation of Par-3 by ROCK, which leads to the loss of apical PAR complex formation, and ultimately allow cells to remain polarized (Ishiuchi and Takeichi, 2011). Additionally, recent work in the Larsen Lab has shown that the control of cellular polarity in the OCCs by ROCK occurs through a myosin II independent pathway (Daley et al., 2012). We also demonstrated that downstream of ROCK, outside-in signals from the basement membrane require functional integrin β1 to maintain the morphology and cell-cell adhesions in the outer columnar epithelial cells. Further investigation
into how ROCK is interacting with Rac1, and the PAR family of polarity proteins are needed to fully understand how each of these players fit into the establishment and maintenance of cellular polarity, the basement membrane organization and remodeling, and the differentiation of the OCCs in the developing salivary gland.

1.7 Thesis Outline and Hypotheses

This chapter has outlined the importance of the basement membrane and the cellular polarity of the outermost columnar epithelial cells surrounding the mouse submandibular salivary gland during the process of branching morphogenesis. We have described the components of the basement membrane, how they are making contact with the underlying epithelium, and described what is known about the PAR polarity proteins which are required for the maintenance of apico-basal polarity in these cells. We have also highlighted the importance of both Rac1 and ROCK as upstream regulators of the PAR family of proteins.

Taking the entire body of information outlined here together, we hypothesized that Par-1b was playing a role in the organization of the BM as it has been shown to play a role in the localization of the BM receptors in other polarized epithelial cell types (Masuda-Hirata et al., 2009; Yamashita et al., 2010; Lewandowski and Piwnica-Worms, 2014). We began investigating a way to disrupt the BM using siRNA knockdown or function blocking antibodies for dystroglycan or integrins respectively, in a salivary gland cell line; however, we had little success translating these methods into organ culture. We further investigated a role for Par-1b in the regulation of the localized assembly of the basement membrane, and in the maintenance of cellular polarity in the outer columnar epithelial cells of the SMG using a targeted siRNA in ex vivo organ culture. We followed organ culture with immunocytochemistry and western analysis
to determine changes in the localization and levels of BM proteins as well as the shape of the outer columnar epithelial cells following treatment with Par-1b targeted siRNA. We also showed the requirement for Par-1b kinase activity. These data and further analysis and discussion can be found in Chapter 3.

We also hypothesized that Par-1b's control of the BM would regulate the differentiation of the myoepithelium, which is embedded in the BM. Our investigations into the differentiation of the myoepithelium in the developing SMG have identified the requirement for Par-1b acting downstream of Rac1 in the differentiation of the myoepithelium during SMG development. We were able to manipulate both Par-1b and Rac1 using targeted siRNAs, as well as pharmacological inhibitors for Rac1 and adenoviral constructs for overexpression of Par-1b. These investigations have led us to propose a potential mechanism by which Par-1b, acting downstream of Rac1 activation is controlling the localized assembly of the BM through its control of the localization of BM receptors in the cell membrane. These investigations and further mechanistic speculations can be found in Chapter 4.
Chapter 2: Introduction: Sjögren’s Syndrome, an Autoimmune Exocrinopathy

2.1 Sjögren’s Syndrome Diagnosis

Sjögren’s syndrome (SS) is a chronic and progressive autoimmune exocrinopathy that affects the salivary glands, lachrymal glands (tear producing glands), and in some cases other excretory organs. Millions of Americans have been diagnosed with SS and nearly as many suffer from SS and remain undiagnosed. The estimated number of affected individuals ranges from 0.05%-4.8% of the population (Kassan and Moutsopoulos, 2004; Mavragani and Moutsopoulos, 2010; Goransson et al., 2011), making Sjögren’s syndrome the second most common rheumatic disease, second only to rheumatoid arthritis (Kassan and Moutsopoulos, 2004; Venables, 2004). The vast majority of SS patients are women over the age of 40 (peri- or post-menopausal, 9:1 female to male patient ratio), although SS has been diagnosed in men, younger women, and even children (comprehensively reviewed by Tincani et al., 2013). The clinical manifestations of SS include, but are not limited to, dry mouth, and dry eye, along with dryness of other mucosa including the nose, throat, and vagina, and commonly accompanied by neuropathies (reviewed by Kassan and Moutsopoulos, 2004 and Fox, 2007). SS diagnosis requires the presence of autoantibodies, several of which have been identified in SS. Anti-La (SSB) autoantibodies are present in approximately 50% of patients, and primarily primary SS patients, and Anti-Ro (SSA) autoantibodies are present in approximately 65% of patients across both types of SS (Reviewed by Venables, 2004). While both Anti-Ro and Anti-La autoantibodies are markers of SS, they have also been identified in patients with Systemic Lupus Erythematosus, Rheumatoid Arthritis, and other autoimmune disease indicating that they are likely not specific to SS. Another
common autoantibody found in SS patients is Anti-M3 an autoantibody reacting to muscarinic acetylcholine receptor 3. This autoantibody is of particular interest in current studies to find treatments for SS because it may be affecting the nervous function in the salivary gland (Venables, 2004). Systemic complications accompanying SS have been known to include extreme, chronic fatigue, as well as involvement of the kidneys and lungs (Kassan and Moutsopoulos, 2004; García-Carrasco et al., 2006). Dry mouth, in its most severe form, results in dental caries, oral infections, difficulty swallowing, eating, sleeping, and speaking (Reviewed by Kassan and Moutsopoulos, 2004 and Fox, 2007) while dry eye symptoms can lead to ocular surface damage, and the constant feeling of sand in the eyes (Bowman et al., 2004; Tincani et al., 2013). These symptoms are not independently life threatening, but often lead to a significant reduction in the patient’s quality of life, and, therefore, warrant closer investigation.

The diagnosis of Sjögren’s syndrome is particularly difficult for a number of reasons. First, mild to moderate dry mouth is commonly seen as part of the natural aging process and is a known side effect of a litany of drugs, including chemotherapy drugs, as well as radiation therapy of the head and/or neck (Umesh et al., 2013). Because of the extensive list of possible causes for dry mouth, physicians frequently overlook SS as a possible cause, extending the time to diagnosis. Second, the diagnosis of SS is frequently (in about 60% of diagnosed patients), complicated by symptoms of another autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus or other autoimmune diseases (reviewed by Tincani et al., 2013 and Patel & Shahane, 2014). Patients presenting with SS symptoms that have a preexisting autoimmune condition are diagnosed with secondary Sjögren’s syndrome. Patients presenting
with Sjögren’s syndrome symptoms without complications from another autoimmune disease are classified as primary SS patients.

The differentiation of SS diagnosis into primary and secondary Sjögren’s syndrome is where the agreement among practitioners ends. Since there are no disease-specific molecular criteria associated with Sjögren’s syndrome, establishing criteria for diagnosis has been difficult and continues to be a matter of debate. Diagnostic classification criteria for Sjögren’s syndrome were standardized in 2002 with the American-European Consensus Group (AECG) criteria (Vitali et al., 2002) (outlined in Figure 2.1). Using these criteria, a patient experiencing several months of dry eye and dry mouth symptoms (confirmed with flow rate tests), positive tests for autoantibodies characteristic of SS, as well as a salivary gland biopsy that is positive for focal lymphocytic infiltrates of a specific size will be diagnosed with SS. Four out of six tests listed in the AECG criteria must be positive for the patient to be classified as having SS, and the presence of autoantibodies, or a biopsy containing lymphocytic infiltrations is required (Vitali et al., 2002). A revision of the classification criteria used for SS was proposed in 2012 by the American College of Rheumatology (ACR) (SC Shiboski, et al., 2012). The new ACR criteria require the presence of two out of three of the following tests; an ocular surface damage test, a blood test positive for SS autoantibodies, and a salivary gland biopsy positive for lymphocytic infiltrates (Shiboski et al., 2012) (outlined in Figure 2.2). These criteria were proposed to decrease the amount of time from presentation to diagnosis, but there has been some controversy as to the benefits of and drawbacks between the ACR and AECG criteria (Pilar Brito-Zeron and Ramos-Casals, 2014). Several studies have compared these two sets of diagnostic criteria (Bowman and Fox, 2014; Cornec et al., 2014; Quartuccio et al., 2014; Rasmussen et al., 2014) and have
sparked a new conversation and possibly a third set of diagnostic criteria that looks to diagnose primary SS as a systemic disease as early as possible (Pilar Brito-Zeron and Ramos-Casals, 2014).
American - European Consensus Group
Classification Criteria for Sjögren's Syndrome Diagnosis

I. Ocular Symptoms:
   1. Daily, persistent dry eyes for > 3 months
   2. Recurrent sensation of sand/gravel in the eyes
   3. Tear substitute usage for > 3 months

II. Oral Symptoms:
   1. Daily dry mouth for > 3 months
   2. Recurrent or persistently swollen salivary glands
   3. Frequently drinking liquids to aid swallowing

IV. Histopathology:
   1. Focus score ≥ 1 (≥ 4 mm² or more than 50 lymphocytes)

V. Salivary gland Involvement:
   1. Unstimulated whole saliva flow (≤ 1.5ml in 15 minutes)
   2. Parotid sialography
   3. Salivary scintigraphy showing delayed uptake

VI. Autoantibodies:
   1. Autoantibodies present in patient serum (Anti-Ro and/or anti-La)

SS diagnosis requires any 4 of 6 symptoms above (including either V or VI) to be indicative of disease (Primary SS) OR in the presence of another autoimmune condition, either I or II, plus 2 of the remaining items (III, IV, and V) being indicative of SS (Secondary SS).

Exclusions From SS Diagnosis: Patients who have had head or neck radiation treatments, Hepatitis C infection, AIDS, pre-existing lymphoma, sarcoidosis, graft vs host disease, and/or use of anti-cholinergic drugs.

(Modified from Vitali et al., 2002)

Figure 2.1 American-European Consensus Group Classification Criteria for Sjögren’s Syndrome

A brief summary of classification criteria as outlined by the American-European consensus group. Modified from Vitali et al., 2002.
Figure 2.2 American College of Rheumatology Proposed Classification Criteria for Sjögren's Syndrome

Summary of American College of Rheumatology Proposed Classification for Sjögren's syndrome.

Modified from Shiboski et al., 2012
2.2 Sjögren’s Syndrome Treatment

After diagnosis, patients suffering from SS have few treatment options, and there is currently no cure for SS. Sialagogues including muscarinic agonists, pilocarpine hydrochloride and cevimeline hydrochloride (Kassan and Moutsopoulos, 2004), have been used with success for dry mouth and dry eye symptoms (reviewed by (Venables, 2004)). Additionally medicated eye drops are frequently used for dry eye symptoms. While these palliative treatments are effective following diagnosis, they become less effective as the disease progresses and the amount of functional salivary and lachrymal tissue remaining decreases. Patients typically resort to drinking water to alleviate discomfort while eating, speaking, and even sleeping. Some patients have used interferon alpha treatment (Kassan and Moutsopoulos, 2004) with some success. Steroid or immunosuppressant treatments are usually avoided for SS patients because the complications and side effects far outweigh the benefits reported by SS patients (Kassan and Moutsopoulos, 2004; Thanou-Stavraki and James, 2008; Bowman and Barone, 2012).

2.3 Etiology of Sjögren’s Syndrome

In order to decrease the time to diagnosis and possibly create better diagnostic tools and treatments, several research groups are investigating the cause of SS. Currently no single event is known to be responsible for the loss of salivary function or the initiation of autoimmune attack, though many hypotheses have been put forth. Due to the overwhelming majority of Sjögren’s syndrome patients being women over the age of 40, it stands to reason that there may be some hormonal aberration that is triggering autoimmunity. Several research groups have looked at the potential involvement of a slew of endocrine factors and androgen levels, hormones, hormone receptors, and other changes occurring in a most-menopausal
woman’s body that may trigger or exacerbate autoimmune conditions (Mavragani et al., 2012; Peri et al., 2012). Other groups have focused on the pre-disease phase including looking more specifically at genetic pre-disposition to disease (Venables, 2004; Peri et al., 2012), dysregulation of the immune system (Jonsson et al., 2011), serological alterations that are known to be present prior to the clinical manifestation of disease (Barrera et al., 2013), and autoantibody actions (Venables, 2004; Jonsson et al., 2011) in order to identify early changes in the body that may be early indicators of disease. Some groups focus more narrowly on the changes to the salivary and lachrymal glands that may precede disease, including an increase in epithelial apoptosis (Polihronis et al., 1998; Busamia et al., 2011), or a loss of epithelial homeostasis (Tzioufas et al., 2012; Barrera et al., 2013). Some hypothesize that infections including viral infections such as Epstein Barr Virus (Pflugfelder et al., 1993), HTLV1 (Terada et al., 1994), HIV (Itescu, 1991) or Hepatitis C (Haddad et al., 1992) may be triggering autoimmune reactions in SS patients (reviewed by Lavoie et al., 2011; Peri et al., 2012; Tincani et al., 2013), while others are working to identify environmental triggers for autoimmune conditions, including SS (García-Carrasco et al., 2006; Tzioufas et al., 2012). While research continues on each of these fronts the world over, it is very likely that the actual cause or trigger of autoimmunity will vary widely among even a small cohort of patients, and no single cause or trigger will be identified that can be applied to even a large percentage of patients suffering from SS or any other autoimmune condition.

2.4 Effects of Sjögren’s Syndrome on Salivary Epithelium

In the salivary gland, salivary hypofunction that occurs during Sjögren’s syndrome is due to lack of functional epithelium. Sjögren’s syndrome manifests as a progressive loss in saliva
secretion that often goes undetected until saliva production has been significantly reduced. In healthy salivary glands, secretory acinar cells make up spherical secretory units surrounding a hollow central lumen (reviewed by Holmberg & Hoffman, 2014 and Tucker, 2007). Secretion of saliva products involves vesicle release coupled with water flow into an acinar central lumen that is continuous with the ductal network of the gland. Several recent studies have looked at the localization of Aquaporin 5 (Aqp5), a water channel protein, located on the apical surface of the acinar cells (Ma et al., 1999; Gresz et al., 2001; Larsen et al., 2011; Nelson et al., 2013). This recent work has brought to light a rather scathing disagreement as to whether there are changes to the distribution of Aqp5 in the secretory acinar cell population in human Sjögren’s syndrome patient samples and in SS mouse models (Beroukas et al., 2001; Konttinen et al., 2005; Soyfoo et al., 2007; Steinfeld et al., 2001; Wang et al., 2009).

Parasympathetic innervation of the mouse submandibular salivary gland is present as early as embryonic day 12, with the nerves growing from the base of the duct, along the main duct and outward to reach around the end buds as they undergo branching morphogenesis (Proctor and Carpenter, 2007). This innervation is critical for the secretion of saliva in the mouse SMG, and is maintained throughout the life of the gland (Proctor and Carpenter, 2007; Holmberg and Hoffman, 2014). In Sjögren’s syndrome, the presence of Anti-M3 autoantibodies targeting muscarinic acetylcholine receptor 3 may be directly affecting the acinar or secretory units within the salivary gland. Recent studies have shown that in the presence of Anti-M3 autoantibodies, a water channel protein, aquaporin 5, cannot be trafficked to the apical membrane, thus significantly decreasing the saliva output (Lee et al., 2013). While these studies have identified a potential mechanism by which SS may be progressing, only a subset of
SS patients have Anti-M3 autoantibodies, indicating that there are likely several mechanisms by which SS is affecting the glandular epithelium.

The ductal cells modify the saliva products secreted by the acinar epithelium while it is being transported to the oral cavity. Previous studies have speculated that there is an increase in the ductal cell population accompanying a loss of acinar cells in Sjögren’s syndrome affected glandular epithelium (Daniels, 1984). However, subsequent studies have revealed considerable heterogeneity in the extent of acinar loss and ductal dysplasia in these patients (Dawson et al., 2000; Beroukas et al., 2001; Goicovich et al., 2003; Barrera et al., 2013). Therefore, there is still debate as to whether there is a progressive relative increase in the ductal population associated with SS disease progression.

Myoepithelial cells surrounding the acini and some of the ducts promote salivary gland structure by producing basement membrane and regulatory growth factors, and facilitate the secretion of saliva by contraction (Redman, 1994; Ianez et al., 2010; Kandagal et al., 2013; Tamgadge et al., 2013), and a decrease in the myoepithelial cell population has been reported in the parotid glands of some SS patients (Nashida et al., 2013). As patients are typically diagnosed late in disease and display considerable heterogeneity in tissue atrophy, cellular mechanisms contributing to disease progression are not well understood.

2.5 Mouse Models of Sjögren’s Syndrome

In order to study the potential triggers, development, and progression of Sjögren’s syndrome, many researchers in the field are using mouse models. Human tissue samples are difficult to come by as only small biopsies are taken at the time of diagnosis, and those samples that are available are extremely heterogeneous in nature. Often biopsy samples of the human
labial salivary glands contain other tissues (muscle and skin tissue) making study of the disease difficult if not impossible. Labial salivary glands are present throughout the lower lip, and are easily accessed for biopsy, but are not the primary saliva producing glands. The submandibular salivary glands, located under the bottom jaw and the parotid salivary gland situated from the bottom of the lower jaw to the lower temple in front of the ear produce the vast majority of human saliva. However, due to the location of these glands, a biopsy would be more noticeable, invasive, and potentially dangerous due to the location of the facial nerves also running along the jaw line. Biopsy of these glands is also less appealing to patients, especially in unaffected individuals, though some physicians are starting to perform these types of biopsies. Age- or sex-matched controls are also virtually impossible to come by as very few healthy individuals offer a lip or other type of salivary gland biopsy. As the difficulties of obtaining human samples and appropriate controls that are useful for a histochemical study mount, the need for a model system becomes more apparent. To this end, several mouse models of Sjögren’s syndrome have been developed for ease of studying SS.

Transgenic mouse models of SS include several cytokine overexpression models (IL-6, IL-10, IL-12, IL-14α, and BAFF) (Comprehensively reviewed by Lavoie et al., 2011). Each of these transgenic models include major changes to the immune system of the mouse and produce at least a subset of the characteristics of Sjögren’s syndrome. While each of these models is useful for teasing apart the key immune system signals that may play a role in SS disease onset and progression, they do not closely recapitulate the multifactorial nature of either primary or secondary SS seen in human patients. Knockout mice (including knockouts of Id3, PI3K, TGF-β1, and TSP-1) have also been used to study Sjögren’s syndrome lymphocytic infiltrations and
immune involvement (comprehensively reviewed by (Lavoie et al., 2011)). As none of these single gene changes have been linked directly to SS in human patients, these models are of limited use to study the progression of disease. The differences in the mouse and human immune system also become an issue in using any model that mutates a single immune system gene as the results may not translate well into the human disease studies.

Some of the more widely used mouse models of Sjögren’s syndrome include the NZB/W F1, MRL/lpr, and NFS/sld mouse models. The NZB/W F1 mouse model was the first model of spontaneous SS, and the phenotype is evident after 8 months of age (Kessler, 1968). The MLR/lpr mice develop a disease characteristic of systemic lupus erythematosus and rheumatoid arthritis, followed by a SS like phenotype around 8 weeks of age (Hang et al., 1982). This model was originally thought to not to develop Anti-Ro and Anti-La autoantibodies, but more recent studies indicate that as many as 30% of mice do develop these autoantibodies (Wahren et al., 1994). Autoimmune lymphoproliferation in these mice goes unchecked due to the genetic alterations, leading to an aggressive disease phenotype. The NFS/sld mice, requiring thymectomy at 4 weeks of age develop some characteristics of SS, including secretory dysfunction beginning at 18 weeks (Hayashi et al., 1988). This model, requiring surgery, coupled with the delayed onset of disease characteristics is difficult to use, and it does not closely mimic human disease, making it less attractive for use in a study seeking to identify early changes in the SS disease phenotype.

2.6 Non-Obese Diabetic Mouse Model of Sjögren’s Syndrome

Using the non-obese diabetic (NOD) mouse model, which develops Sjögren’s syndrome secondary to autoimmune diabetes, a three phase timeline of disease progression has been
described by Cuong Q. Nguyen and colleagues (Lee et al., 2009). Between 6 and 10 weeks of age (Phase 1), it is thought that retarded salivary gland development and increased apoptosis in the secretory cells leads to aberrant physiological and biochemical processes. In phase 2 (10-18 weeks of age) lymphocytic infiltrations become clearly identifiable in the salivary glands. By 18-20 weeks (phase 3), there is a measurable decrease in saliva and tear flow rates and symptoms of clinical disease are evident.

After a thorough investigation of the NOD mouse model genome, researchers identified several genes that were mutated that may be playing a role in the diabetes and SS phenotypes in the NOD mouse. Through replacing each mutated idd gene in the NOD mouse individually, researchers narrowed down the affected genes that were causing the SS-like disease (Brayer et al., 2000). Following identification of these two gene loci, the C57BL/6 NOD-Aec1Aec2 mouse model was created. The C57BL/6 NOD-Aec1Aec2 mouse genome carries two genes, Idd3 (Aec1) and Idd5 (Aec2) from the original NOD model (Nguyen et al., 2006), which lead to a SS-like phenotype without the complications of autoimmune diabetes. Further study of this mouse model revealed that there were differential phenotypes in male and female SS-like disease progression (Nguyen et al., 2006; Lavoie et al., 2011). Modeling primary SS, these mice develop decreased secretory function and lymphocytic infiltration of the salivary glands by 16-20 weeks of age. This model has been used extensively to study the progression and onset of primary SS, but comes with many difficulties as well. The C57BL/6 NOD Aec1/Aec2 model is not currently available commercially, and the use of this model requires a local breeding colony or collaboration with a group that has a breeding colony established. Also, since only
approximately 40% of SS patients are diagnosed with primary SS (Tincani et al., 2013), we were more interested in a model of secondary SS.

The NOD/ShiLtJ strain is commonly used as a model of Sjögren’s syndrome as it replicates many hallmarks of the human disease. NOD/ShiLtJ mice develop autoimmune diabetes around 4 weeks of age and an autoimmune exocrinopathy that shows significant similarities to Sjögren’s syndrome becomes evident between 8 and 12 weeks (outlined in Figure 2.3) (Karnell et al., 2014). Decreasing saliva secretion with SS disease progression in the NOD/ShiLtJ mouse has been previously documented to be similar to human SS disease progression (Lodde et al., 2006; Soyfoo et al., 2007) and decreased saliva secretion is evident by 16 weeks (Cha et al., 2002). As SS progresses in the NOD/ShiLtJ mouse, multiple autoantibodies develop, including characteristic anti-Ro and anti-La autoantibodies (Donate et al., 2014; Karnell et al., 2014; Lavoie et al., 2010). Lymphocytic infiltrations in the submandibular salivary glands are evident in female mice around 12 weeks of age; however, the lachrymal phenotype in the female mice is significantly delayed. In male NOD/ShiLtJ mice, the lachrymal phenotype is evident around 12 weeks of age, but the salivary phenotype is delayed. This sexual dimorphism is also evident in the C57BL/6 NOD Aec1/Aec2 and it has been suggested that the dimorphism may be linked to a region on chromosome 3 (Aec1 locus) (Nguyen et al., 2006). Although the disease progression of both diabetes and SS phenotypes has been characterized in the NOD/ShiLtJ mouse (Nashida et al., 2013), changes in the salivary epithelial cell populations have not been thoroughly examined.
<table>
<thead>
<tr>
<th>Disease Phenotype/Characteristic</th>
<th>NOD/ShiLtJ</th>
<th>Aec1/Aec2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Sjögren’s syndrome</td>
<td>Secondary SS – Autoimmune Diabetes</td>
<td>Primary SS</td>
</tr>
<tr>
<td>Anti-Ro Autoantibodies</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Anti-La Autoantibodies</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-M3R Autoantibodies</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Onset on Lymphocytic Infiltration</td>
<td>12 weeks</td>
<td>10 weeks</td>
</tr>
<tr>
<td>Loss of Saliva Flow</td>
<td>20 weeks</td>
<td>19 weeks</td>
</tr>
<tr>
<td>Commercially available</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 2.1 Comparison of NOD/ShiLtJ and Aec1/Aec2 Mouse Models of SS**

Common symptoms of SS used in diagnosis and whether or not they are present in the two mouse models compared in Chapter 5 listed here. Modified from Lavoie et al., 2010.
Figure 2.3 Timeline for NOD/ShiLtJ SS-like Disease Phenotype Progression

Timeline layout of progression of Sjögren’s syndrome like disease phenotype in the NOD/ShiLtJ, with specific disease characteristics and events along the bottom of the time line and the time points used for the study described in Chapter 5 outlined in green above.
2.7 Thesis Outline and Hypotheses

Chapter 2 details much of what is known about Sjögren’s syndrome etiology in humans and some of the most commonly used mouse models, including those used in our studies of Sjögren’s syndrome progression. We have hypothesized that there would be notable structural changes to the mouse SMG with the progression of disease that would be particularly evident as the mouse began to experience a decreased saliva flow (around 12 weeks) and in the regions of lymphocytic infiltrations. However, our investigations into the structure and morphology of the SMG in the SS mouse model has not identified any major morphological abnormalities (aside from the expected lymphocytic infiltrations) in the remaining epithelial tissues, even in regions of the tissue adjacent to lymphocytic infiltrations. We further investigated several cell type markers for the identification of ductal, acinar, myoepithelial, and progenitor cells within the epithelium. While we found no major changes in these cell types directly adjacent to the lymphocytic infiltrations at any stage of disease progression, we did find that overall, there were changes in the proportions of acinar cells to ductal cells in the remaining epithelia tissue, and that there was a marked increase in the myoepithelial marker that was co-localizing with a marker of epithelial progenitors. While we were able to identify differences in specific cell type markers, overall the latest stage of SS that we studied was not representative of human late stage disease when compared to human tissue samples.
Chapter 3: A Role for Par-1b in the Control of the Basement Membrane in the Developing Mouse Submandibular Salivary Gland

3.1 INTRODUCTION

During organogenesis, the mouse submandibular salivary gland (SMG) begins as a small epithelial protrusion and rapidly undergoes iterative rounds of outgrowth and invagination known as clefting to form separate buds and increase the secretory surface area, a process collectively known as branching morphogenesis (Larsen et al., 2006a; Daley et al., 2008, 2009; Sequeira et al., 2010). Throughout the process of branching, the glandular epithelium is surrounded by a sheet of highly polarized outer columnar epithelial cells (OCCs) (Fig 1.2). These outermost epithelial cells are making contact with a specialized form of extracellular matrix known as the basement membrane (BM) (Bernfield and Banerjee, 1982; Sakai et al., 2003; Larsen et al., 2006a; Rebustini et al., 2007, 2009; Sequeira et al., 2010)). In the SMG, the major components of the BM include laminin-111, which is laid down in an extracellular lattice-like structure (McKee et al., 2007; Miner, 2008), followed by the deposition and organization of collagen IV, perlecain, and other structural proteins (Rousselle et al., 1997; Yurchenco and Wadsworth, 2004; Sequeira et al., 2010). The BM proteins make contact with the OCCs through integrins, cell surface receptors that span the epithelial membrane in pairs, are activated when bound to their ligand, and propagate the outside-in signals from the basement membrane (Chartier et al., 2006; Kim et al., 2011). Laminin-111 is also making contact with
dystroglycan, another cell surface receptor that propagates signals from the BM inward to the cytoskeleton and other proteins forming a complex just inside the cells surface (reviewed by Miner, 2008). The localized deposition, organization, and assembly of the BM is critically important for the continued growth and development of the mouse SMG, but the mechanisms by which these processes are regulated have not been well defined in this system.

The PAR family of polarity proteins is well conserved and known to regulate cellular polarity in many systems, including C. elegans, Drosophila, and many mammalian epithelial cells types reviewed by (Böhm et al., 1997; Goldstein and Macara, 2007). Originally identified as partition defective mutants (Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999), the PAR family of proteins have been shown to regulate the front-rear polarity in motile cells (Etienne-Manneville, 2008), and the apico-basal polarity in epithelial cells through a system of mutual exclusion (Masuda-Hirata et al., 2009). The apical Par complex, made up of Par-3, Par-6, and atypical protein kinase C (aPKC), is known to be localized only to the apical or interior domain of the cell, in part through its mutual exclusion with Par-1b, a serine threonine kinase involved in epithelial apico-basal cellular polarity (reviewed by (Müsch, 2004; Suzuki and Ohno, 2006)). Par-1b has recently been shown to interact with utrophin, a membrane of the intracellular complex that translates signals from the cell surface receptor dystroglycan to the cytoskeleton (Lewandowski and Piwnica-Worms, 2014). These recent studies led us to further investigate the role of Par-1b in the regulation of the BM in the developing mouse SMG.
3.2 METHODS AND MATERIALS

3.2.1 Thawing and Carrying SIMS and HEK cells

Both SIMS, an immortalized mouse salivary ductal cell line (Laoide et al., 1996) and HEK 293 (Human epithelial kidney cells) (Graham et al., 1977) cells were thawed from common lab stocks at the start of a series of new experiments. Frozen cells were quickly moved from the liquid nitrogen freezer to the 37°C water bath to avoid slow thawing. Once totally thawed, the cells in the vial were gently pipetted into a 10 cm dish of pre-warded cell media (SIMS and HEKs: DMEMF12 with Phenol red (55 ml removed to sterile tube) with 5 ml Pen Strep (100 µg/ml penicillin, and 100 µg/ml streptomycin) and 50 ml Hi FBS (Gibco, 1082). If necessary, half of the cell containing media was moved to a second plate and each supplemented with an additional 5 ml of pre-warmed cell media to avoid overcrowding. Following thawing, the media was replaced with fresh, pre-warmed media at 24 hours, and cells were observed once a day until nearly confluent. When cells reached confluence, they were passaged as follows: Cell media was aspirated, and 5 ml of pre-warmed 1XPhosphate Buffered Saline (1XPBS) was added to the edge of the plate, swirled and aspirated off. A second 5 ml of 1XPBS was added and the process repeated. Following the second wash with 1XPBS, 1.5 ml of pre-warmed 0.25% trypsin-EDTA was added and the plate was moved to the 37°C incubator for 5-8 minutes, or until all the cells had lifted off the plate as single cells and small cell clusters. The trypsin was then neutralized by adding 10 ml of appropriate cell media. One ml of these cells was added to a fresh, pre-labelled cell culture plate containing 9 ml of pre-warmed cell media, and cells were placed at 37°C. Cell passage was completed every 48-72 hours depending on confluence with media changes every 48 hours.
3.2.2 Cell seeding, Immunocytochemistry and Preparation for Western Analysis

Cells remaining in the original plate after passage were collected in a 50 ml conical and counted using a hemocytometer to determine the approximate concentration of cells. Once the concentration of the cells was determined, 24 or 6 well cell culture plates were prepared by placing appropriately sized sterilize coverslips in each well being used for immunocytochemistry experiments. SIMS or HEK cells were seeded at $3 \times 10^5$ cells/well in a 24 well plate for ICC or $3 \times 10^5$ in a 6 well plate for western analysis.

Following the necessary time in culture, cells were fixed by adding an equal volume of 4% paraformaldehyde (PFA) (10 ml 16% PFA, 30 ml 1XPBS, 2 g sucrose) to the cell culture media for a minimum of 20 minutes at room temperature (500 µl in a well of a 24 well plate and 2 ml in a well of a 6 well plate). The media and PFA was aspirated off after 20 minutes and replaced by 1XPBS until immunostaining was completed. Following washing in 1XPBS, 500 µl of 1XPBS-0.1% Triton for cell permeabilization was added to each well for 15 minutes at room temperature. 1XPBS-0.1% Triton was replaced with 1XPBS for 5-10 minutes, then coverslips were then moved to a fresh parafilm covered plate lid with 50 µl drops of pre-made blocking solution per coverslip: 1X PBS - Tween (1XPBS-T,0.1%Tween-20) with 20% Donkey Serum (Jackson, 7-000-121) and 1 drop MOM per ml (Vector, MKB-2113)) for 1-2 hours at room temperature in a humidified chamber. After blocking, each coverslip was dipped 10 times into a beaker of 1XPBS-T, and placed cell-side down on a 20 µl drop of primary antibody solution on the parafilm-covered lid in a humidified chamber for an hour at room temperature or at 4°C overnight. After incubation in primary antibodies, the coverslips were dipped again in 1X PBS-T and placed on to 20 µl drop of secondary antibodies on a parafilm-covered lid for an hour in
humidity chamber at room temperature in a dark area. Following incubation in secondary antibodies coverslips were moved to 500 µl 1XPBS in a fresh 24-well plate for 5-10 minutes. The 1XPBS was aspirated off, and 500 µl of DAPI was added to each well for 5-8 minutes. DAPI was replaced in the conical and replaced with 500 µl 1x PBS. The coverslips were then dipped 10 times in 1XPBS solution; followed by 5 dunks in deionized water immediately before mounting. 2-3 coverslips (cell-side down) were set on each pre-labelled slide, with a drop of Fluoro-Gel (Electron Microscopy Sciences 17985-10) mounting media for each coverslip. Slides were stored at -20°C until imaging on the Zeiss Z1 Cell Observer or the 510 Meta or 710 Meta Zeiss Confocal microscopes.

Cells being prepared for western analysis were lysed as follows: Cell media was aspirated and enough ice cold sterile 1XPBS was added to each well to cover the surface. The 1XPBS was replaced once with more ice cold 1XPBS, then it was replaced with 30-50 µl of RIPA++ (10 ml RIPA buffer (Thermo-Fisher Scientific 89900) plus 1 phosphatase cocktail inhibitor tablet (Roche 04906837001) and 1 protease cocktail inhibitor tablet (Roche 11836170001) was added to each well of a 24 well plate, or 50-100 µl per well of a 6 well plate. Plates were put on ice for 20-25 minutes with mild agitation (tipping the plate side to side several times) throughout the incubation time. Cells were scraped with a flat edge scraper at the end of the RIPA++ incubation and pipetted into pre-labeled microcentrifuge tubes. Tubes were spun at high speed in a table top centrifuge at 4°C for 20 minutes, and then placed on ice. The supernatant was removed gently to a new, pre-labelled microcentrifuge tube and held at -20°C or -80°C until a microBCA was performed (see below).
3.2.3 SMG Organ Dissection

Timed-pregnant female mice (outbred strain CD-1 or ICR) were euthanized following the University at Albany IACUC-approved procedures, as previously described (Daley et al., 2009, 2011). The day of vaginal plug discovery was designated as E0. Using a sterile scalpel (#11 blades) and forceps (#5, Fine Science Tools, 11252-20), embryos were removed from the sacs into a separate 10 cm dish containing 25 ml of HBSS (Hanks Balance Salt Solution, Life Technologies 14175095). The embryonic salivary glands were removed from the lower mandible slices as previously reported (Daley et al., 2009) with a few modifications. Under a stereo dissecting microscope with a transmitted light base (Nikon SMZ645), the embryo heads were removed with an angled cut just below the lower jaw and moved to a fresh dish of HBSS. Next the lower mandibles were isolated from the heads using a cut below the upper jaw. Placing the tissue, tongue side up, the surrounding tissues were moved away until the submandibular salivary glands became visible. They were both carefully moved away from the surrounding tissues, and moved with capillary action to a fresh plate of DMEM/F12 (Thermo Fischer 21041-025) + 100 µg/ml penicillin, and 100 µg/ml streptomycin (+Pen-strep Gibco 15140-122).

3.2.4 SMG Whole Organ Culture

5-6 intact E13 submandibular salivary glands were placed on a 13 mm, 0.1 µm Nuclepore Track-Etch membrane filter (Whatman WHA110405) floating over 200 µl of culture media in a glass-bottomed microwell plate. The culture media contained DMEM/F12 + pen-strep with 50 µg/ml transferrin and 150 µg/ml ascorbic acid (+vitC +Trans), as previously
described (Rebustini and Hoffman, 2009). Pharmacological inhibitors or siRNA complexes were added directly to the culture media, and glands were grown in a humidified incubator (95% air/5% CO2) at 37°C for as long as 120 hours with media changes every 48 hours and DIC images taken every 24 hours, beginning 2 hours after plating.

3.2.5 siRNA Transfection and Pharmacological Inhibitor Treatments for SMG

siRNA constructs for Par-1b siRNA (Abcam, s65473) or NT siRNA (ABI Biosystems, 4390844,) were created by combining additive-free culture media (DMEM/F12+PenStrep) with the siRNA (500 nM unless otherwise specified) followed by the addition of 9 µl RNAiFect transfection reagent (Qiagen 301605 – discontinued Jan 2015) to achieve a final volume of 50 µl. The complex was allowed to form undisturbed at room temperature for 20 minutes.

It came to our attention that soaking the whole glands in siRNA containing media prior to culture with siRNA containing media led to better siRNA uptake. siRNA complexes were formed for 20 minutes, in a volume of 50 µl, then added to 150 µl culture media and glands were added directly to the siRNA complex containing media for 1-1.5 hours gently rocking at room temperature. Following this incubation, the siRNA containing media was added to 150 µl culture media with additives (DMEM/F12 + PenStrep + VitC + Trans) and placed in a Matek dish on a floating Nuclepore filter for whole gland culture.

The pharmacological inhibitor of ROCK, Y27632 (Calbiochem, 688000), was dissolved directly in growth media (DMEM/F12) since it is water-soluble and was added directly to the culture media at a concentration of either 70 µM or 140 µM.
3.2.6 siRNA Transfection and Function Blocking Antibody Treatment of Cells

For siRNA transfection of cells, SIMS cells were seeded 5x10^4 cells per well and allowed to form small islands before transfection with siRNA or treatment with function blocking antibodies. siRNA complexes were formed as previously described (Section 3.2.5) and 50 µl of siRNA complex was added directly to cells for 1-2 minutes, then culture media was added to final volume of 2 ml in a 6 well plate. Cells were then grown for an additional 48 hours and either fixed or lysed for use in immunocytochemistry or western analysis respectively.

For function blocking antibody experiments, SIMS cells were seeded at 3x10^5 cells per well in a 6 well plate a grown to approximately 40% confluence. Function blocking antibodies were added to the cells directly after culture media was replaced. Following an additional 48 hours in culture, they were either fixed or lysed for immunocytochemistry or western analysis respectively.

3.2.7 Amplification and Purification of Par-1b Adenoviral Stocks

Adenoviral stocks were prepared for the following viruses: Par-1b wild type, Par-1b kinase dead (kinase domain removed) and kinase inactivated (point mutation in kinase domain, K to A at position 49) as seen in the schematic of adenoviral purification procedure in Figure 3.1. Adenoviral stocks of kinase-dead and wild type Par-1b adenoviruses were provided by Dr. Anne Meüsچ (Böhm et al., 1997; Cohen et al., 2004).

Adenovirus stocks were added to HEK293 cell culture and the cells and media (crude viral lysates, CVLs) were collected after 24 hours when the cells began to look unhealthy. CVLs were stored in 10 ml aliquots at -80°C after being flash frozen on dry ice with methanol. Crude viral lysates (CVLs) were thawed in a 37°C water bath (6-8 minutes), vortexed for approximately 30
seconds, then flash frozen in mixture of dry ice and 70% ethanol (12-15 minutes). The freeze-thaw process was repeated 5 times to allow for complete cell lysis and release of viral particles. The CVLs were then spun in an Eppendorf Centrifuge 5810R at 4000 rpm, 4°C for 15 minutes in what rotor. The supernatant was collected in a fresh, pre-labelled 15 mL conical vial and kept on ice while preparing 2 Beckman centrifuge tubes (#344059) per viral prep for cesium chloride gradients by soaking in 70% ethanol, rinsing in dH₂O then drying inverted. We prepared the cesium chloride gradient by placing 2.5 ml of low density (density 1.25) CsCl in each of the prepared tubes and then slowly under-laying 2.5 ml of high density CsCl (density 1.4) using a sterile 5 ml pipet and taking care to pipette slowly to avoid mixing or bubbles. Once the gradient was prepared, 5 ml of supernatant was gently pipetted on top of each CsCl gradient and the tubes were carefully marked. The tubes were then carefully balanced using sterile 1XPBS dropwise.

Once prepared, the tubes were gently placed into the ultracentrifuge buckets (SW41 rotor and buckets) noting which pairs were exactly balanced, and the tops were tightly screwed into place. The buckets were then moved to the LE90K centrifuge and hooked into place and then spun at 35,000 for 1 hour at 4°C with slow acceleration and deceleration under vacuum.
Figure 3.1 Purification of Par-1b Adenovirus - Workflow

Adenoviral stocks are used to infect HEK293 cells for 24 hours. The cells and media are then harvested to forma crude viral lysate, which is subjected to several freeze thaw cycles to lyse the cells and release virus. The CVL is then spun to remove the cellular debris and separated on a two-step CsCl gradient. After separation, the virus is dialyzed then aliquoted for use in future experiments.
After spinning, the tubes were gently removed from the buckets one at a time and were set in a small tube clamp on a stand over a small beaker containing 10% bleach solution. We wiped the outside of each tube with 70% ethanol, and then identified the opalescent band (see figure 3.2), just below the separation of the clear CsCl and pink media. To collect the opalescent band we used a 5 ml syringe and 21 gauge 1.5” needle to gently pierce the side of the plastic tube (Figure 3.2A, arrow). The viral containing material removed by syringe was placed in pre-labeled micro centrifuge tube(s) and bands from the same original supernatant were combined. Approximately 1.5 ml was obtained from each viral preparation.

To perform the second spin, in a second set of pre-cleaned Beekman ultracentrifuge tubes we added 8.0 ml 1.33 g/mL CsCl (density 1.33) and gently overlayed the recovered bands from first spin (1-2 ml). In a second balance tube we added 10 ml 1.33 g/mL CsCl. We then balanced the tubes with sterile 1XPBS as before and placed the tubes in SW41 buckets, and screwed the lids tightly in place. The tubes were spun at 35,000 for 18 hours at 4°C with slow acceleration and deceleration and held at 4°C after spinning. The following day, ultracentrifuge tubes were carefully removed from buckets and placed in the tube clamp over a fresh 10% bleach solution, one at a time. We identified the pink/orange-colored band, at the interface between the clear and pink solutions (see Figure 3.2B) and recovered this band using a 5 ml syringe and 21 gauge 1.5” needle (Figure 3.2B Arrow). We placed the recovered virus into a pre-labelled microcentrifuge tube(s) on ice, then measured the approximate final volume of purified virus and added glycerol to a 10% final concentration. Using a syringe, we transferred the virus with glycerol from microcentrifuge tubes into a pre-soaked Pierce Slide-A-Lyzer dialysis cassette (#66380) by gently puncturing the corner of the cassette at the loading port and pushing the
virus into the cassette. The cassettes were then set up to float in dialysis buffer (for 4X stock: 556 ml dH2O, 40 ml 1 M Tris pH 7.4, 4 ml 1 M MgCl2, 400 ml glycerol) and stirred with stir bar on a magnetic plated for several minutes at room temperature until all completely dissolved. Prior to use, 1 L of the 4X stock was diluted with 3L dH2O to a final volume of 4 L for each viral prep. Buffer was then autoclaved and refrigerated covered with foil until use) for 4 hours, 1 L of buffer per hour, with 3 buffer changes at 4°C. After dialysis, the virus was gently removed using a syringe through the corner of the cassette and aliquoted into sterile microcentrifuge tubes (~10-20 µL each for working aliquots and 200-250 µL each for back up tubes to be aliquoted as needed). All aliquots were stored at -80°C and each preparation was tested on HEK and SIMS cells for potency before use in further experiments (Figure 3.3 B-E).
Figure 3.2 Cesium Chloride Adenoviral Purification

(A) Adenoviral particles separate as an opalescent band (indicated by the black arrow) after the first cesium chloride gradient and can be removed from the plastic Beckman tube with a syringe and needle. (B) After the second CsCl separation, the virus is present in a pink-orange band (indicated by black arrow) at the interface between the media (bright pink) and CsCl solution (clear) and can be removed in the same manner as following the first spin.
Figure 3.3 Validation of Par-1b Adenoviruses in HEK and SIMS cells

(A) Schematic showing the wild type Par-1b protein sequence (top panel), the area of the Par-1b sequence that is deleted in the kinase domain deletion construct (center panel), and a schematic showing the sequence for the kinase inactivation via K49A point mutation within the kinase domain (KA). (B) HEK cells infected with Par-1b wild type, KA, and KD adenoviruses show marked increase in Par-1b as seen by western analysis when compared to uninfected controls. (C) Quantification of western analysis in B shows a 3 fold increase in Par-1b levels following adenoviral infection. (D) SIMS cells infected with Par-1b wild type, K49A, and KD adenoviruses
show marked increase in Par-1b as seen by western analysis when compared to uninfected controls. (E) Quantification of western analysis in D shows a 2 fold increase in Par-1b levels following adenoviral infection in SIMS cells. Blots shown in B and D are representative of experiments repeated at least three times. Quantifications are averages of at least three experiments.
3.2.8 Adenoviral Infection of SIMS and HEK cells

To infect cells with adenovirus, SIMS or HEKs were seeded in a 6 well plate at 3X10⁵ in a total volume of 2 ml. Cells were allowed to grow for 24 hours until small islands were formed, but cells were <40% confluent. Media was aspirated off, and replaced with fresh cell culture media, and then adenovirus was added dropwise to each well (2-6 µl/100 µl, PFU not determined for CsCl purified adenoviral constructs). Cells were imaged at 24 hour intervals and fixed or lysed as above with 2% PFA for cells expressing GFP.

3.2.9 SMG Epithelial Rudiment Separation and Adenoviral Infection

10-15 intact E13 submandibular salivary glands were placed in a glass-bottomed, 50mm diameter microwell dish (MatTek Corporation, P50G-1.5-14-F) containing 200 µl of Hank’s balanced salt solution (HBSS lacking Ca²⁺ or Mg²⁺, Life Technologies) containing 0.4% (v/v) dispase (Life Technologies, cat. no. 17105-041) and incubated for 15 minutes at 37°C. After the incubation, using a sterile 200 µl pipette tip, the dispase solution was carefully aspirated out without disturbing the glands and immediately replaced with 200 µl of DMEM/F12 media containing 5% (w/v) BSA (DMEM/F12-BSA, pre-sterilized with a 0.22 µm filter) to neutralize the dispase. Under the dissecting microscope, using a pair of forceps with fine tips (#5 Dumostar, Fine Science Tools, 11295-20), the loosened mesenchyme was gently separated from around the SMG epithelial buds and ducts, taking care to leave the epithelium intact. After several glands were dissected away from the mesenchyme, using a pre-wet 200 µl pipette tip with DMEM/F12-BSA media the epithelial rudiments were gently removed into a new 50 mm diameter microwell dish containing 200 µl of DMEM/F12 + pen-strep. Then any ‘clean’ mesenchyme, containing no gland pieces, including the sublingual gland or partial buds or
ducts, were also removed to separate 50 mm diameter microwell dish containing 200 µl of DMEM/F12 + pen-strep. Epithelial rudiments were then soaked in adenoviral construct-containing media or vehicle control media for 1-2 hours at room temperature. The titer of the resulting solution was approximately 1x10^8-10^9 PFUs. Rudiments were gently separated using forceps several times during vial incubation.

Following incubation in adenoviral or vehicle containing media, the rudiments were rinsed with DMEM/F12 + pen strep, 200 µl per plate two times before plating. Once rinsed, several epithelial rudiments were pipetted on to a notched Nuclepore filter floating over 200 µl of culture media using a pre-wet P200 tip with a smooth opening to prevent glands sticking to the rough edges of the pipette tip. Excess media was avoided and removed with the pipette or with forceps using capillary action. A small amount of mesenchyme was then added to the filter in the same manner, and the excess media was removed. The rudiments were then pushed apart on the filter and pieces of mesenchyme pushed into place to surround each rudiment (See Figure 3.4D and D’). Recombined rudiments were incubated in a humidified incubator (95% air/5% CO2) at 37°C for 96-120 hours with media changes every 48 hours and brightfield images every 24 hours starting with images taken right after plating (See Figure 3.4E).
Figure 3.4 Epithelial Rudiment Adenoviral Infection

(A and A’) Whole E13 SMG’s are dissected, then incubated in dispase solution, (B and B’) and the mesenchyme is then manually pulled away from the epithelium. After soaking in adenoviral containing media for 1-2 hours at room temperature, (C) the adenoviral infected rudiment is then (D and D’) recombined with previously removed mesenchyme. (E) Brightfield images of epithelial rudiments surrounded by mesenchyme pieces grown in ex vivo culture for the indicated times. Epithelial cells undergo branching morphogenesis and mesenchymal condensation is evident as early as 24 hours in culture. Scale bars all 100 µm. Cartoon amended and images from Sequeira, Gervais et al., 2011.
3.2.10 Morphometric Analysis

Morphometric analysis was performed using brightfield images collected at each 24 hour time point throughout the gland culture. Each individual gland was marked, and marks were counted using MetaMorph™ Version 6.1 MDS Analytical Technologies software followed by graphing in Excel or GraphPad Prism (Prism 6.05, 2014). The total number of buds was normalized to the number of buds for the individual gland at the starting time point, and the entire data set was normalized to the number of buds present in the control glands for each time point.

3.2.11 Cultured SMG Immunocytochemistry

At the desired time-point, whole or recombined SMGs were fixed for 20-30 minutes with 4% PFA (2% PFA was used for any glands expressing GFP to preserve its structure and fluorescence). Following fixation, glands were washed with 1XPBS, 200 µl per plate, twice, for ten minutes per wash. Glands were permeabilized with 0.01-0.04% Triton-1XPBS-Tween (0.1%) for 15 minutes at room temperature, then rinsed twice in 1XPBS before blocking in 20% Donkey Serum in 1XPBS-3% BSA +1 drop mouse-on mouse blocking reagent (MOM, Vector Laboratories MKB-2213) for 1-2 hours at room temperature. Following blocking, the glands were again rinsed with 1XPBS twice, and then incubated in primary antibodies in the dark at 4°C overnight (See table 3.1). The next day, the glands were rinsed, and incubated in secondary antibodies for 1-2 hours, followed by rinsing in 1XPBS. Following additional rinses in 1XPBS, glands were incubated in DAPI for 8-10 minutes, and mounted on slides using imaging spacers (Grace BioLabs) and Fluoro-Gel mounting media (Electron Microscopy Sciences). For each condition, 5-
15 glands were used per experiment and experiments were repeated a minimum of three times. Immunocytochemistry was followed by imaging using the Zeiss 710 or Zeiss 510 confocal microscope to take several images of each gland from each condition.
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<th>Antibody Targeting:</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Stock Concentration</th>
<th>Dilution for ICC</th>
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**Table 3.1 Antibodies used for Immunocytochemistry**

Antibodies use for immunocytochemistry following culture of whole SMGs, epithelial rudiment or epithelial recombinations. Dilutions for antibodies were made in 3%BSA-1XPBS-T.
3.2.12 Western analysis and Quantification of Western Analysis

Glands prepared for western analysis were gently lifted off the filter and placed in a microcentrifuge tube containing about 10 µl RIPA 2++ (10 mL RIPA buffer plus 2 phosphatase tablets (04906837001) and 2 protease tablets (11836170001) per gland, plus 10-20 µl extra volume. For each condition, 5-15 glands were used and results presented are representative blots and quantifications are an average of at least 3 experiments unless otherwise stated. Glands were lysed in RIPA2++ for 20-30 minutes on ice with frequent vortexing, followed by light sonication (solicitor was set to lowest setting, and probe was set into liquid. Sonication was done in small pulses to avoid bubbles), and spinning at high speed at 4°C to remove cellular debris. Following spinning, the supernatant was removed to a fresh tube and kept on ice or frozen at -20°C until usage.

MicroBCA (Pierce, 23235) was performed on each sample (from glands or cells) to determine protein concentration. Samples were compared with a standard curve consisting of BSA from 0- 20 µg/µl followed by preparation of western samples to 10-15 µg protein per lane. 14 µl of sample was mixed with 2X loading buffer (1610737) and loaded in a single well of a 15-well (4-15%) prepared gel (Bio-Rad 456-1086) or 19 µl in a single well of a 10 well (4-15%) gel (Bio-Rad 456-1083) and gels were run for about 60 minutes at 120 volts. Once the dye front reached the bottom of the gel, it was stopped, and the gel gently moved to the transfer apparatus, and transferred to blotting paper for 50 minutes at 100 volts. After transfer, the blot was blocked with 5% (w/v) powdered milk (Carnation) dissolved in 1XTBS-Tween overnight at 4°C. After blocking the blot was cut using the protein ladders as a guide, and individual pieces were incubated in primary antibodies (See table 3.2) overnight rolling at 4°C. Blot pieces were
then washed 4 times in 1XTBS-Tween, and incubated in secondary antibodies for 1-2 hours at room temperature. Following secondary antibody incubation, blots were washed 4 times in 1XTBS-Tween, reassembled and exposed to x-ray film after incubation in either ECL or SuperSignal (Thermo Scientific 32106 or 34076 respectively). Following development, blots were scanned and the pixel intensity of control and treated bands were quantified and compared using either Excel or GraphPad.
<table>
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<th>Antibody targeting:</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Stock Concentration</th>
<th>Dilution for Western Analysis</th>
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Table 3.2: Antibodies Used for Western Analysis.

Dilutions for antibodies used for western analysis are in 1% milk - 1XTBS-T, except for GAPDH which was always prepared in 5% milk-1XTBS-T.
3.3 RESULTS

3.3.1 Manipulation of Basement Membrane with Integrin β1 Function Blocking Antibodies Salivary Gland Cells

To investigate the importance of cell surface receptors in the assembly and organization of the basement membrane (BM) in the developing mouse submandibular salivary gland (SMG), we used SIMS cells, the salivary gland ductal cell line. In order to determine whether or not assembly of collagen IV in the BM is possible without the function of integrin β1, which is present in many integrin basement membrane binding pairs. We treated a submandibular salivary ductal cell line (SIMS cells) with a function-blocking antibody targeting integrin β1. Following 48 hour treatment of SIMS cells with the function blocking antibody for integrin β1, cells were then fixed, and immunostained for actin (Rhodamine Phalloidin) and collagen IV. The cortical localization of the actin in both function blocking antibody and control antibody-treated cells identify the cell and indicate that the cells have begun to polarize. However, the aggregates of collagen IV in the IgM antibody treated control cells show the assembly of collagen IV in to web-like fibers (Figure 3.5, top panel, white arrows). These fibers of collagen IV are clearly absent from the cells treated with integrin β1 function blocking antibody, indicating that integrin β1 must be functional for collagen IV to be assembled extracellularly. Collagen IV levels in the integrin β1 function blocking antibody treated cells also seems to be decrease when compared to IgM controls, though western analysis is required to confirm changes in protein levels.
Figure 3.5 Assembly of the Basement Membrane by SIMS Cells Requires Integrin β1

SIMS cells grown in culture for 24 hours were treated with either IgM or Integrin β1 function blocking antibody and grown in culture for an additional 48 hours in culture, then fixed and stained for actin (Rhodamine Phalloidin, red) and collagen IV (green). Arrows in the top panel indicate the formation of a web-like structure containing collagen IV and the arrows in the bottom panel point to areas with little to no collagen IV expression, and no assembly of collagen IV following integrin β1 function blocking. Scale bar 50 µm.
3.3.2 Knockdown of Cell Surface Receptor Dystroglycan in SIMS Cells

Dystroglycan is a cell surface receptor for laminin-111, which is known to be a critical component of the BM in the developing SMG. Dystroglycan is a composed of 2 subunits that are expressed on the same gene, and post-translationally spliced (Henry and Campbell, 1999), therefore a single siRNA can be used to target the mRNA and knock down the levels of both subunits of dystroglycan. We began our validation of dystroglycan siRNA in SIMS cells. We transfected SIMS cells 24 hours after seeding with an siRNA for dystroglycan or a non-targeting siRNA control and allowed the cells to grow for an additional 48 hours. The cells were then lysed and western analysis performed (Figure 3.6 A-D). The results of the western analysis show approximately a 30-35% decrease in both α and β dystroglycan following siRNA treatment when compared to Non-targeting (NT) siRNA controls. These results indicate that we can manipulate the levels of dystroglycan in SIMS cells.
Figure 3.6 Quantification of Dystroglycan siRNA Knockdown in SIMS Cells

SIMS Cells were grown in culture for 24 hours, then treated with 200 nM Non-Targeting siRNA or 200 nM Dystroglycan siRNA (s64804) and grown in culture for another 48 hours. Cells were then lysed and protein concentration measured with a microBCA assay. The lysates were then analyzed via western blot. (A and C) Western blot scans show a slight decrease α and β dystroglycan following dystroglycan targeted siRNA treatment when compared to NT siRNA controls. (B and D) Quantifications of blots showed that both α and β dystroglycan are knocked down approximately 30-35% in SIMS cells after dystroglycan siRNA treatment. Blots shown in A and C are representative of experiments repeated at least three times and quantifications are averages of at least three experiments.
3.3.3 siRNA Knockdown of Dystroglycan in the Embryonic Mouse SMG

Following successful reduction of dystroglycan in SIMS cells using targeted siRNAs, we treated E13 SMGs with 500 nM dystroglycan siRNA (s64802 or s64804) to determine whether or not dystroglycan was required for the assembly of the BM in the developing mouse SMG. To knockdown dystroglycan in the SMGs siRNA was added directly to the culture media from which it is selectively taken up by the glandular epithelium (Daley et al., 2009). Brightfield images were taken at 2, 24 and 48 hours, and we saw little to no difference in branching or overall gland size (Figure 3.7 A). Morphometrical analysis indicates only a small difference in gland size after treatment with s64804 for 48 hours, though this difference is not significant and no change in the number of buds was seen after treatment with a second siRNA for dystroglycan s64802 (Figure 3.7B). We performed immunocytochemistry for β dystroglycan and collagen IV to determine if any decrease in β dystroglycan had occurred and whether or not a change in β dystroglycan would cause any disruption to the basement membrane. Immunostains showed little to no difference in the levels or staining pattern for β dystroglycan and no notable change in collagen IV levels or organization (Figure 3.7C). We repeated these experiments at both 500 nM and 300 nM as per the manufacturer’s recommendations. Following treatment for 48 hours, we again performed immunocytochemistry for β dystroglycan, collagen IV and laminin-111. At 300 nM siRNA, we saw no significant changes in β dystroglycan or in either collagen IV or laminin-111 (Figure 3.8A). We also performed western analysis for α dystroglycan and saw a 10% decrease after treatment with 300nM s64802, but no decrease following 500 nM s64802 when compared to controls (Figure 3.8B). Following several failed attempts to knockdown dystroglycan using either s64802 or s64804, we attempted to
combine the two siRNAs during complex formation, and found no change in either levels of either subunit of dystroglycan or any disruption to the BM (data not shown). We also attempted knockdown of dystroglycan with three additional siRNAs from Santa Cruz (sc-43489A, sc-43489B, and sc-43489C). Following treatment with either 400 nM or 500 nM siRNA for 48 hours, glands were lysed and western analysis was performed. Quantifications of western analysis for either α or β dystroglycan indicate little to no knockdown in all but two cases, 500nM sc-43489A and 400 nM sc43489B both show marked decreases in β-dystroglycan (Figure 3.8 C). However, the levels of α dystroglycan in the same samples is either unchanged or increased when compared to controls.
Figure 3.7 Treatment of E13 SMGs with Dystroglycan siRNA

E13 SMGs were harvested and treated with either 500 nM NT siRNA or 500 nM dystroglycan siRNA s64804 or s64802 for 48 hours. (A) Brightfield images were taken at 2, 24 and 48 hours; images shown for 500 nM siRNA concentrations of NT siRNA and s64804 only. Scale bar 100 µm. (B) Morphometric analysis of glands that were left untreated (media) were compared to glands treated with NT siRNA (n=7), s64802(n=7) or s64804 (n=8) show that treatment with s64802 is not affecting the branching of the SMG, while s64804 seems to have slight effect on branching by 48 hours. (C) Immunostaining for β dystroglycan and collagen IV indicate that the siRNA treatment has very little effect on β dystroglycan or on BM assembly after 48 hours. Scale bar 50 µm.
Figure 3.8 siRNA–mediated Knockdown of α and β Dystroglycan

E13 SMGS were exposed to 300nM NT or dystroglycan siRNA s64802 for 48 Hours. (A)

Immunocytochemistry for laminin-111 (green) and β dystroglycan (red) and collagen IV (cyan) show little to no changes in levels or localization of β dystroglycan or BM proteins following dystroglycan siRNA treatment when compared to NT controls. Scale bar 50 µm. (B)

Quantification of western analysis (averages of 2 experiments) indicates only a 10% knockdown of α dystroglycan after treatment with 300nM s64802 siRNA, and no change in α dystroglycan levels following 500nM s64802 siRNA treatment. (C) Three additional siRNAs targeting dystroglycan from Santa Cruz (sc-43489A, sc-43489B, and sc-43489C) were tested at 2 concentrations (400 nM or 500 nM) in 48 hour SMG culture with no consistent knockdown of both α and β dystroglycan together when compared to the NT siRNA control of the same concentration (averages of two experiments).
3.3.4 Par-1b siRNA Knockdown in the Developing SMG

Par-1b is known to regulate cellular polarity in many types of epithelial cells (reviewed by Müsch, 2004; Suzuki and Ohno, 2006) and we wanted to determine whether Par-1b was required for the maintenance of cellular polarity in the developing SMG. In order to manipulate Par-1b levels in the developing SMG, we utilized a Par-1b targeted siRNA. We cultured E13 SMGs for 48 hours with normal culture media, or culture media containing 500nM non-targeting (NT) siRNA or 500nM Par-1b targeted siRNA and took brightfield images at 2, 24 and 48 hours (Figure 3.9A). Treatment with Par-1b siRNA reduced the size of the epithelium over the 48 hours in culture, though morphometric analysis indicates only a moderate change in bud number when compared to NT siRNA treated controls (Figure 3.9B). Following the 48 hour culture, we lysed the glands and performed western analysis to determine whether or not Par-1b levels had been affected (Figure 3.9C). With 500nM Par-1b targeted siRNA there an approximate 50% decrease in Par-1b levels (Figure 3.9 D). These results indicate a successful knockdown of Par-1b levels in the developing SMG at a level that is not toxic to the gland.
Figure 3.9 siRNA Knockdown of PAR-1b Disrupts the Basement Membrane in SMGs Cultured for 48 Hours

E13 SMGs were cultured for 48 hours in the presence of 500 nM non-targeting siRNA (NT), or 500 nM Par-1b siRNA. (A) Brightfield images were taken at 2, 24 and 48 hours. Scale bar 100 μm. (B) Morphometric analysis indicates a slight decrease in branching morphogenesis with Par-1b siRNA knockdown at 48 hours when compared to NT siRNA controls (n=15 for each condition). (C and D) Western analysis of SMGs following 48 hour culture in the presence of NT siRNA or Par-1b siRNA indicates a decrease in Par-1b levels with Par-1b siRNA treatment of about 50% (average of 3 experiments).
3.3.5 Par-1b siRNA Knockdown Decreases Levels of Basement Membrane Proteins

We next wanted to determine whether Par-1b plays a role in the deposition, or organization of the BM in the developing mouse SMG. We treated E13 SMGs with Par-1b-targeted siRNA for 48 hours, and performed immunocytochemistry for three major components of the BM, collagen IV, laminin-111 and the heparin sulfate proteoglycan, also known as perlecan (Figure 3.10A). Each of these three major BM components was markedly reduced after Par-1b siRNA knockdown when compared to the NT siRNA control. These results indicate that Par-1b is required for the appropriate deposition of the BM. We followed ICC with western analysis for collagen IV which showed a marked decrease of approximately 45% in collagen IV levels after treatment with Par1b siRNA when compared to controls (Figure 3.10 B and C). These results indicate that Par-1b is required for the expression and organization of the BM surrounding the developing mouse SMG. However, while Par-1b siRNA treatment is sufficient to reduce the levels of Par-1b in the developing SMG, it is not sufficient to tease apart whether Par-1bs kinase activity is required for its role in the regulation of the BM deposition and organization at the basal periphery.
Figure 3.10 Par-1b siRNA Knockdown Disrupts the Basement Membrane

E13 SMGs were treated with 500 nM NT siRNA or 500 nM Par-1b targeted siRNA and cultured for 48 hours followed by fixation and immunocytochemistry or lysis and western analysis. (A) Fixed SMGs were immunostained for three basement membrane markers, perlecan (cyan), laminin-111 (green) and collagen IV (red). Glands treated with Par-1b siRNA showed a marked reduction in the BM when compared to NT control. Scale Bar 10 µm. (B and C) Western analysis for collagen IV also shows a marked decrease in collagen IV and quantification of western analysis indicates approximately a 45% decrease in collagen IV levels following Par-1b siRNA treatment when compared to NT siRNA control. Blot shown is representative and quantification is the average of at least 3 experiments.
3.3.6 Infection of SMG Recombined Rudiments with Adenovirus

In order to over express Par-1b in the developing mouse SMG, we needed to be able to infect the gland with an adenovirus construct. We attempted infection of the intact gland with a GFP expressing adenovirus, and found that only the outer edges of the mesenchyme were infected (data not shown). Because we were only interested in infecting the epithelium, and not the mesenchyme, we dissected the E13 SMG further, removing the mesenchyme from the epithelium, making it possible to infect only the epithelial rudiment by soaking it in adenovirus containing media as previously reported (Larsen et al., 2006a) (As seen in figure 3.1). Following gland infection with a GFP adenoviral construct, we recombined the infected epithelium with the previously removed mesenchyme on a Nuclepore filter and imaged the gland after 24 hours in culture (Figure 3.11A). Brightfield images overlaid with GFP images show that the viral infection was successful and contained to the epithelial compartment (Figure 3.11A, second and third panel). Next we wanted to ensure that the virus would continue to be expressed if the recombined gland was sustained in culture for a longer period of time. GFP infected epithelial rudiments were recombined and grown in culture for 72 hours, followed by fixation and immunostaining. Staining for epithelial cadherin (ECAD), marking the epithelial cells and Perlecain which surrounds the epithelial area further confirm that the GFP expression is maintained within the epithelial compartment following 72 hour culture (Figure 3.11B and C).
Figure 3.11 GFP Adenoviral infection of the SMG Epithelium

E13 SMGs were harvested, and epithelial rudiments removed from surrounding mesenchyme. Epithelial rudiments were infected with a GFP adenoviral construct, recombined with removed mesenchyme and cultured for 72 hours. (A) Brightfield, fluorescent, and overlaid images of recombined SMGs in which only the epithelial cells (outlined in white dashed lines) were infected with GFP-expressing adenovirus following 24 hours in culture. Scale bars 100 µm. (B) Confocal or brightfield images (BF) of recombined SMGs cultured for 72 hours stained for nuclei (DAPI, blue), adenoviral GFP (green), the epithelial marker, E-cadherin (red) show GFP expression is only within the epithelial compartment. Scale bars 250 µm. (C) Immunostains for nuclei (DAPI, blue), adenoviral GFP (green), ECAD (red), and perlecan (cyan), show the bud structure and the appropriate assembly and localization of the BM following 72 hour culture
after recombination. Scale bars 50 μm. Dashed white lines outline epithelium. As seen in Sequeira, Gervais et al., 2011.
3.3.7 Par-1b siRNA Knockdown Phenotype is Partially Rescued by Matrigel

In order to further investigate whether the kinase activity of Par-1b is required for the regulation of the BM we expressed a kinase dead Par-1b protein in the SMG glandular epithelium using an adenoviral construct. We infected the E13 epithelial rudiments as described above and overlaid epithelial rudiments on a Nuclepore filter with a thin layer of Matrigel. Matrigel is a tumor extract that contains several extracellular matrix proteins including laminin, collagens, and fibronectin. The exact components are proprietary, but Matrigel has been shown to be biologically active in the support of cells in culture (Kleinman and Martin, 2005). These experiments were performed alongside Par-1b siRNA treated epithelial rudiments to compare the phenotype of loss of Par-1b levels and loss of Par-1b kinase activity. Following 48 hours in culture the epithelial rudiments were fixed and we performed immunocytochemistry for collagen IV and ECAD to identify the rudiments. Following immunostaining, it became evident that the Matrigel was rescuing the effects of the Par-1b siRNA treatment and presumably the Par-1b KD adenoviral infection. The levels of collagen IV were not reduced after either treatment when compared to the NT siRNA control, although the organization of collagen IV was slightly disturbed (Figure 3.12 A). When we compared these results to the previous Par-1b siRNA treatment in whole glands (Figure 3.12B), we noted the marked difference in BM levels and localization after siRNA treatments. It is likely that the basement membrane proteins present in the Matrigel can still be organized by the epithelial cells even after knockdown or inactivation of Par-1b. The presence of this exogenous BM may also be helping to keep the outer layer of epithelial cells organized, as there is little change to the organization of the OCCs in glands overlaid with Matrigel as compared to the whole glands.
treated with Par-1b siRNA (comparing Figure 3.12 A and B). The organization of the BM provided by Matrigel is rudimentary when compared to NT siRNA controls, as evidenced by the lack tightly basally localized organization in the Matrigel-supported rudiments treated with either Par-1b siRNA or Par-1b KD adenovirus. These results indicate that Par-1b is required for the normal levels of BM proteins, and is required for the basally restricted organization, but that the BM can be organized into rudimentary web-like structure when Par-1b has been knocked down or inactivated.
Figure 3.12 Matrigel Rescues the Effects of Par-1b siRNA Knockdown and Par-1b Kinase Dead Adenoviral Infection

E13 SMG’s were harvested and further dissected to remove mesenchyme from the epithelium. The epithelial rudiment was treated with NT siRNA, Par-1b siRNA or Par-1b kinase dead adenovirus, overlaid with Matrigel and cultured for 48 hours followed by immunocytochemistry.
(A) Immunocytochemistry for collagen IV (cyan) and ECAD (red) show no decrease in collagen IV levels, and very little disruption in the localization of collagen IV following Par-1b siRNA or Par-1b KD adenoviral infection when epithelial rudiments are overlaid with Matrigel. (B) Whole glands treated with either NT or Par-1b siRNA disruption to the levels and organization of the BM (perlecan, cyan) as well as the disorganization of the outermost layer of epithelial cells, outlined in white (ECAD, red). (C) Recombined epithelial rudiments treated with Par-1b siRNA or kinase dead Par-1b adenovirus show disruption of the BM (collagen IV, red) and the outermost layer of epithelial cells, as seen in ECAD (cyan) staining. Scale Bars 10 µm throughout.
3.3.8 Par-1b Kinase Activity is Required for the Deposition and Organization of the Basement Membrane

To avoid the phenotypic rescue of Par-1b siRNA or Par-1b KD adenoviral treatments, we repeated both sets of experiments and recombined the Par-1b KD adenovirally infected or siRNA treated epithelial rudiments with the previously removed mesenchyme on Nuclepore filters and cultured the recombined glands for 48 hours. Following culture we performed immunocytochemistry for collagen IV and ECAD (Figure 3.12C). We found a marked decrease in collagen IV levels following both Par-1b siRNA treatment and Par-1b KD adenoviral infection. We also found disruption of the outer columnar layer of cells as seen by ECAD staining in glands where Par-1b was knocked down or the kinase dead Par-1b was overexpressed. These results indicate that Par-1b kinase activity is required for the columnar shape of these outer columnar cells, as well as for the expression of collagen IV. Taking these results thus far together, we have shown that Par-1b is critical for branching morphogenesis in the developing mouse submandibular salivary gland. We have also shown that Par-1b kinase activity is required for the deposition and organization of several of the major components of the basement membrane surrounding the glandular epithelium in the developing SMG. We next questioned whether or not Par-1b overexpression was sufficient to drive the deposition and organization of the BM in the SMG.

3.3.9 Par-1b is Necessary and Sufficient to Drive Ectopic Basement Membrane Deposition and Localization

Using the same recombination technique required for the Par-1b kinase dead adenoviral infection, we infected the epithelial rudiments with a wild type (WT) Par-1b adenovirus to
overexpress active Par-1b in the SMG epithelium. Following infection, the glands were plated, recombined with previously removed mesenchyme and cultured for 48 hours (Figure 3.13A). Time course images show that glands infected with WT Par-1b adenovirus show no global defect in branching morphogenesis when compared to controls. Following culture we performed immunocytochemistry for C-Myc, Par-1b, and collagen IV. The WT Par-1b adenovirus construct contains a C-Myc tag which can be used to identify the localization of WT Par-1b adenovirus expression as well as verify gland infection. Staining for C-Myc shows a marked increase in C-Myc expression throughout the glandular epithelium when compared to control glands indicating successful infection throughout the epithelium. Co-localized with C-Myc staining throughout the adenovirally infected glands is Par-1b (Figure 3.1 B). In the control glands, Par-1b localization is restricted to the outer edges of the epithelial rudiment and is diffuse in the inner polymorphic cells. However, following Par-1b overexpression via adenovirus, the expression pattern of Par-1b is markedly different; Par-1b is localized throughout the epithelium indicating that infection was successful. Par-1b appeared to localize to one side of the cell in the OCCs following the WT Par-1b overexpression, though which side of the cell Par-1b was localizing to was random instead of strictly basal. Collagen IV, which is normally localized strictly to the basal periphery of the SMG epithelium, is also localized throughout the interior of the epithelium after infection with the WT Par-1b adenovirus (Figure 3.13B). The merged images showing Par-1b and Collagen IV overlap indicate that Par-1b overexpression and localization correlates with collagen IV localization to the same side of that cell’s membrane. This data is consistent with the model that Par-1b specific membrane localization drives collagen IV expression and deposition in cells that are expressing WT Par-1b
inappropriately. These data indicate that overexpression of Par-1b is sufficient to drive ectopic localization of BM proteins.
E13 SMGs were harvested and epithelial rudiments were dissected away from the surrounding mesenchyme, and then treated with WT Par-1b adenovirus. (A) Following recombination with the previously removed mesenchyme, brightfield images were taken at 2, 24 and 48 hours and show no major differences in gland growth following WT Par-1b adenoviral infection. Scale bar 100 µm. (B) Following fixation at 48 hours, rudiments were immunostained for collagen IV (cyan), Par-1b (red) and c-Myc (green). Merged images show a co-localization of Par-1b with the overexpression of c-myc indicating the overexpression of Par-1b is due to the adenoviral infection. Overexpression of collagen IV also co-localizes with Par-1b ectopically indicating that Par-1b is driving collagen IV expression and localization. Scale Bar 10 µm.
3.4 DISCUSSION

Here we have investigated the requirements for Par-1b in the localized deposition and organization of several of the major components of the basement membrane (BM) surrounding the epithelium of the developing mouse submandibular salivary gland (SMG). Previous studies have shown that the polarity protein Par-1b is localized to the basolateral domain of polarized epithelial cells and forms a mutual exclusion with the apical PAR complex (Par-3, Par-6 and atypical protein kinase C) (Suzuki et al., 2004; Chen and Zhang, 2013). Here we have shown that basally localized Par-1b is responsible for the basally restricted deposition and organized localization of several BM proteins including collagen IV, laminin-111, and perlecan. Although we have not identified a specific mechanism by which Par-1b is regulating the localized deposition and organization of the BM, taking into account other recent work on Par-1b we would speculate that Par-1b is regulating the localization of the BM through the BM cell surface receptor localization.

Our studies began with an attempt to identify a way to disrupt the BM in the developing SMG to identify the mechanism by which its basal localization is controlled. Our goal was to be able to disrupt the BM through disrupting its cell surface receptors, thus allowing us to disrupt the BM without disrupting the structure of the SMG manually. We attempted to disrupt the BM in SIMS cells using both integrin β1 function blocking antibodies and dystroglycan siRNAs with some success. These reagents were not as successful in the SMG. We did not follow integrin β1 function blocking experiments in SIMS with tests in the SMG because the expression of integrin β1 in many BM binding integrin pairs (Hynes, 2002; ffrench-Constant and Colognato, 2004), as well as its function in cell-cell connections in epithelial tissues (Schwartz and DeSimone, 2008)
might make integrin function blocking antibodies toxic to the SMG. We also considered using function blocking antibodies for integrin α3 and integrin α6 but were concerned that a compensatory mechanism may prevent any real phenotype from being obvious as there are several integrin receptor pairs for each of the main BM components we were most interested in disrupting (Pedchenko et al., 2004; Rebustini et al., 2007; Kim et al., 2011). We also had difficulty identifying a way to confirm that the function blocking antibodies were indeed blocking the function of their targets, especially if a compensatory mechanism was having an effect. To continue along this line of investigation, we decided to instead focus on dystroglycan, a laminin receptor.

Following moderately successful knockdown of dystroglycan in the SIMS cells, we attempted to identify an siRNA to knockdown the protein levels of dystroglycan in the SMG. We tested 5 different siRNAs for dystroglycan from two different companies, and were not successful in knocking down dystroglycan. We did find a marked decrease in β dystroglycan with one siRNA from Santa Cruz, though the increase, or lack of knockdown in the α dystroglycan subunit was suspect. It is likely that knockdown of a single subunit of dystroglycan would be sufficient to disrupt its cellular activities which require the interaction of both subunits, however, the marked increase in the α subunit was concerning. Following the inconsistent dystroglycan knockdown results, we reevaluated, and decided to instead focus on the possible requirement for polarity regulators in BM assembly. We wanted to further investigate the role for basally localized Par-1b in the establishment and/or maintenance of the basally localized BM in the developing SMG.
Previous investigations have shown that the basally localized Par-1b promotes laminin accumulation within the basement membrane by restricting laminin receptor localization in MDCK cells, a kidney epithelial cell line commonly used for studies of cellular polarity (Masuda-Hirata et al., 2009; Yamashita et al., 2010). These results led us to question whether Par-1b may be controlling the deposition and organization of the BM in the developing SMG through a similar mechanism. We began by confirming that we could knockdown Par-1b using a targeted siRNA in the SMG culture system.

Following the identification of a targeted Par-1b siRNA that successfully knocked down Par-1b levels in the developing SMG, identified marked decreases in perlecan, laminin-111, and collagen IV via ICC in SMGs exposed to Par-1b siRNA for 48 hours and approximately a 45% reduction of collagen IV by western analysis (Figure 3.10 and 3.12). These results indicated that Par-1b was required for the maintenance of an organized BM at the basal periphery of the gland. Lysates made for use in these experiments included both the epithelium and surrounding mesenchyme and the amount of collagen IV found in the mesenchyme was not independently investigated. It is possible that the collagen IV present in the mesenchymal compartment which is accounted for in these experiments is the majority of what is seen following Par-1b siRNA knockdown as the ICC accompanying these data would suggest a larger than 50% knockdown of collagen IV. It is also possible that laminin-111, collagen IV, and perlecan expression is unaffected by the loss of Par-1b and that even under these conditions they are normally deposited extracellularly, but are not properly organized into the structured basement membrane. These proteins may instead diffuse away from the glandular epithelium and be degraded. We did not investigate the media directly surrounding the gland on the Nuclepore
filter, though it is unlikely that these proteins could diffuse through the surrounding mesenchyme and escape into the media due to their large size.

It is plausible given these data that following the loss of Par-1b, the formation of the laminin lattice is disrupted, thus preventing the organization of other BM components, including collagen IV and perlecans in the appropriate web-like structure surrounding the glandular epithelium. Laminin proteins are made up of trimers of laminin subunits which are assembled extracellularly. Once assembled, the t-shaped laminin trimers bind to each other to form a tight lattice and are capable of making contact with the receptors on the cell surface (Nomizu, 1995; McKee et al., 2007). Dystroglycan, one of the cell surface receptors for laminin is thought to pull extracellular laminin proteins into close proximity with the cell surface, where they can make contact with integrins (Driss et al., 2006) and other BM proteins can be assembled into the web-like structure of the BM along the already formed laminin lattice (reviewed by Miner et al., 1994; Yurchenco, 2015). This mechanism assumes that Par-1b is controlling the localization of the BM receptors for laminin-111 in the SMG as occurs in MDCK cells (Driss et al., 2006), though further experiments are needed to confirm this mechanism in the developing SMG.

We next wanted to investigate the requirement for Par-1b kinase activity in the maintenance of the BM in the developing SMG. In order to more closely investigate this requirement, we utilized an adenoviral construct expressing a kinase-dead version of Par-1b. The use of this reagent required some modifications to our typical culture system in order to infect only the SMG epithelium. The epithelium alone will not survive organ culture (Sakai and Onodera, 2008), so to this point, we had been using Matrigel, a tumor extract that has been
shown to be biologically active in culture systems (Kleinman and Martin, 2005) and that we have found to successfully support the growth of the SMG epithelium. When compared to controls, whole glands treated with siRNA for Par-1b show a marked decrease in BM, and disorganization of the outer layer of epithelial cells. In the rudiments treated with Par-1b siRNA in the presence of Matrigel, there was no obvious difference in the OCC arrangement, and though the BM was not as tightly restricted to the basal periphery, it was also not visibly decreased (Figure 3.12). The slightly more diffuse looking BM is likely due to the presence of BM proteins in Matrigel (Kleinman and Martin, 2005) and not to the overexpression of BM proteins by the epithelial cells; though without further investigation we cannot confirm this. We presume that the assembly of the BM proteins present in the Matrigel is preventing the phenotype of disorganized OCCs and the low level of BM staining previously seen in Par-1b siRNA treated glands. To account for the lack of phenotype in the Par-1b kinase dead adenovirus treated epithelial rudiments, we repeated these experiments using epithelial tissue recombinations instead of Matrigel to support the epithelial rudiment. Following Par-1b targeted siRNA treatment or Par-1b kinase dead adenoviral infection, there was a marked reduction in collagen IV surrounding the epithelium accompanied by a disruption in the organization of the OCCs. These results reinforce the conclusion that Par-1b knockdown and kinase dead Par-1b adenoviral infection phenotypes were both rescued by Matrigel. More importantly, these results indicate that Par-1b kinase activity is required for the localized organization of the BM in the developing SMG at 48 hours.

Knowing that Par-1b kinase activity is required for the organized localization of the BM, we next investigated whether Par-1b was sufficient to drive the localized deposition of the BM. We
overexpressed Par-1b using a wild type (WT) Par-1b adenoviral construct using the same recombination method used for the kinase dead Par-1b adenovirus infections. Following 48 hour culture, we found that there was no significant morphological changes in the development of the gland, however, both the levels and localization of Par-1b and collagen IV were affected. In control glands, the expression of Par-1b is restricted to the basal most epithelial cells, and within this layer, is mostly restricted to the basolateral surfaces of these cells, with very little staining seen elsewhere within the epithelium. The collagen IV in the control glands was also basally restricted, though in the recombination culture system, the enzyme (dispase) used to aid the removal of the mesenchyme does somewhat disrupt the basement membrane surrounding the epithelium. However, after recombination, the organization of the basement membrane is reestablished by 48 hours in the control glands, leading to a somewhat diffuse, basally localized expression of collagen IV. Even with the enzyme disruption of the endogenous BM, there is very little to no collagen IV seen in the interior of the epithelium in the control glands. After treatment with WT Par-1b adenovirus for 48 hours, immunostains for the c-Myc tag on this adenoviral construct indicates that the overexpression of Par-1b was due to the adenoviral infection and that infection was seen throughout the epithelial compartment. Par-1b levels are markedly increased compared to controls and, more importantly, the expression of Par-1b is seen throughout the gland, most notably in the inner polymorphic cells that do not normally express Par-1b (Figure 3.13B) after 48 hours. Following the ectopic expression pattern of Par-1b in the interior of the epithelium, collagen IV is also being expressed within the interior of the epithelium, around those cells expressing Par-1b. Merged images show that collagen IV is being expressed only around cells that are expressing Par-1b at both the exterior of the
epithelium and within the epithelium in the polymorphic cells. These results taken together with experiments done in whole gland cultures for 48 hours indicate that Par-1b is both necessary and sufficient to drive the expression of collagen IV as well as its deposition and organization ectopically.

Future experiments will be required to determine whether or not Par-1b is controlling the localization of the organized BM through control of the localization of cell surface receptors including integrins and dystroglycan. Culture in the presence of Par-1b siRNA followed immunostaining may give us some indication about the localization of integrins and dystroglycan, though identifying new, specific antibodies for these proteins may be necessary. Western analysis will also allow us to investigate whether Par-1b and more specifically Par-1b kinase activity is required for the levels of integrins and dystroglycan in the developing SMG, as well as other proteins through which Par-1b may be acting. In MDCK cells, Par-1b is shown to regulate the localization of dystroglycan in the cell membrane through its interactions with utrophin, a protein that is part of the intracellular complex formed at the cytoplasmic tail of β dystroglycan (Henry and Campbell, 1996; Yamashita et al., 2010). In a pilot study we were able to identify the connection between utrophin and β dystroglycan in SMG samples using immunoprecipitation, but were not able to identify a direct connection between Par-1b and utrophin or β dystroglycan (data not shown). However, these experiments were a first attempt at this technique, and were only performed once, so they should be repeated before determining that Par-1b is not interacting with utrophin in the SMG. It is also possible that Par-1b is regulating the localization of dystroglycan and integrins through its interactions with the microtubules. Par-1b is the mouse homolog of the human MARK2 (microtubule affinity
regulating kinase 2) and has been shown to maintain microtubule dynamics (Bré et al., 1990; Hayashi et al., 2012; Matenia et al., 2012). Par-1bs interactions with microtubule binding proteins may also play a role in Par-1bs control of the localization of the BM. We speculate that Par-1b is regulating the BM in the developing SMG through the localization of the cell surface receptors in a manner similar to that identified in the MDCK cells (Masuda-Hirata et al., 2009), though mechanistic details will require further investigation.

3.5 SUMMARY AND CONCLUSION

These studies have identified the requirement for Par-1b, a conserved serine-threonine kinase, in the developing SMG in the maintenance of cellular polarity and the maintenance of the basally localized organization of the BM. Through siRNA knockdown and infection with wild type or kinase dead versions of Par-1b adenoviral constructs, we have identified the requirement for Par-1b kinase activity in the regulation of BM organization at the basal periphery of the developing mouse SMG. We have also shown the Par-1b overexpression is sufficient to drive ectopic BM deposition and localization in the interior of the developing SMG. Further investigations are required to identify whether or not and how Par-1b is controlling the localization and organization of the BM through the BM receptors in this system.
Chapter 4: Differentiation of the Myoepithelium in the Developing Mouse Submandibular Salivary Gland

4.1 INTRODUCTION

During the development of the mouse submandibular salivary gland (SMG), the process of cellular differentiation, or specialization, begins very early, around embryonic day 15 (E15) (Patel et al., 2006). However, the mechanisms by which differentiation occurs in the SMG remain largely unclear. The differentiation of the myoepithelium, a specialized contractile epithelial cell type surrounding the secretory units, is first apparent around E15 with the expression of the protein smooth muscle α-actin (SM a-actin) (Nelson et al., 2013). When myoepithelial cells first begin to differentiate, they undergo drastic changes in cell shape or morphology in addition to cytodifferentiation. Myoepithelial cells vertically compress and eventually form thin stellate-shaped myoepithelial cells which are embedded within the BM that surrounds the secretory acinar unit with long finger like projections (Doggett et al., 1971; Redman, 2008; Amano et al., 2012). Due to the dynamic changes to the basement membrane localization from basally restricted in early stages to fully surrounding the mature myoepithelial cell, the basement membrane may be critical for myoepithelial morphogenesis and/or cytodifferentiation.

The outermost columnar epithelial cells (OCCs), which are thought to be the myoepithelial precursor cells, undergo a morphogenetic change during differentiation and appear to lose their apicobasal polarity. Par-1b, a polarity protein, is critical for branching
morphogenesis and is known to be in control of the expression, deposition, and localized organization of the BM in the developing SMG by restricting deposition to the basal side of the OCCs (Daley et al., 2012). Whether Par-1b is required for myoepithelial morphogenesis and/or differentiation is unknown. Rac1, a small GTPase, has also been shown to play a critical role in the maintenance of cellular polarity; however, if it is required for cell polarity in the SMG is unknown. In these studies, we investigated the relationship between Rac1 and Par-1b in the cellular morphogenesis and cytodifferentiation of the myoepithelium.

We have previously demonstrated that the mouse SMG organ explants that were isolated from embryos at E13 will undergo myoepithelial differentiation when grown as organ explants at the air-media interface (Peters et al., 2014). Therefore, we used ex vivo organ cultures of mouse SMGs treated with Rac1-targeted siRNAs or pharmacological inhibitors to manipulate Rac1 levels and activity respectively, or Par-1b targeted siRNA or Par-1b wild type expressing adenovirus to manipulate levels of Par-1b to investigate the role of these proteins in myoepithelial morphogenesis and cytodifferentiation. We then used brightfield imaging and confocal imaging to examine morphogenesis and protein localization and western analysis to quantify protein levels in this study that contributes to our understanding of the molecular control of myoepithelial morphogenesis and differentiation.
4.2 METHODS AND MATERIALS

4.2.1 Ex Vivo Organ Culture

Embryonic mouse SMGs were dissected from timed-pregnant female mice (strain CD-1, Charles River Laboratories, Wilmington, MA) at embryonic day 12.5 or 13 (E12.5 or E13, with the day of plug discovery designated as E0), following protocols approved by the University at Albany IACUC committee. Embryonic tissues were microdissected as previously described (Daley et al., 2011; Sequeira et al., 2013) and cultured at the air/media interface on Nuclepore Track-Etch membrane filters (Whatman WHA110405) floating on 1:1 DMEM/Ham’s F12 medium (F12) (Invitrogen 21041-025) supplemented with 150 μg/ml Vitamin C, 50 μg/ml Transferrin and pen/strep. Five or more intact SMGs or epithelial rudiments were tested in each condition with experiments repeated at least three times.

For recombination experiments, SMG epithelial rudiments removed from the surrounding mesenchyme were prepared and cultured after recombination with previously removed mesenchyme tissue pieces, as previously described (Chapter 3.2, and (Daley et al., 2009; Sequeira et al., 2013). Briefly, dissected E13 epithelial rudiments and mesenchyme were separately incubated at room temperature in DMEM/F12 containing 0, or 1-2.5 μl adenoviral construct in 100 μl culture media for 1-2 hours. Rudiments were then washed in DMEM/F12 and recombined on top of Nuclepore filters with previously removed mesenchyme. The pharmacological Rac1 inhibitor, EHT1864 (Sigma) (Onesto et al., 2008; Shutes et al., 2007) was dissolved in DMEM/F12 and used at the concentrations and times indicated. For washout experiments, inhibitor–containing media was added for 24 hours then replaced with fresh media for another 24 hours. Par-1b wild type (Par-1b WT) was a generous gift from Dr. Anne
Meusch (Böhm et al., 1997; Cohen et al., 2011; Daley et al., 2012) and were amplified in HEK293 cells, purified using cesium chloride density gradient centrifugation, and then used to infect SMG rudiments in culture, as previously described (Figure 3.1 and 3.4 as shown in (Sequeira et al., 2013). Targeted siRNAs to Rac1 (Santa Cruz Biotechnology, sc-36352) or Par-1b (Abcam, s65473) or non-targeting (NT) siRNA (ABI Biosystems, 4390844) were transfected at 400 nM or 500 nM using RNAiFect (Qiagen, 301605) according to the manufacturer’s protocol. Target knockdown was confirmed by western analysis. Morphometric analyses are representative of experiments repeated at least 3 times, with at least ten SMGs per group. Brightfield images of glands or epithelial rudiments were captured initially at 2 hours and every 24 hours thereafter using a Nikon Eclipse TS100 microscope equipped with a Canon EOS 450D digital camera under 4X objective. Quantification of branching morphogenesis in whole glands was represented as the fold-change in number of buds at each time point divided by the number of buds at time zero (2 hours) (MetaMorphTM Version 6.1 MDS Analytical Technologies).

4.2.2 Western Analysis

Western analysis was performed as previously described (Sequeira et al., 2012). Briefly, cultured SMGs were lysed for total protein and protein concentrations of the resulting supernatants were determined using a Micro-BCA assay kit (Pierce, Rockford, IL). Western blots were developed on X-Ray film in ECL or SuperSignal solution (Thermo Scientific, 32106 and 34076 respectively), scanned (CanoScan, Canon), and quantified using ImageJ software (Version 1.46r). All western analyses were repeated with lysates from three independent experiments.
An image of a representative blot is shown in each figure and quantification is the average of at least three experiments unless otherwise noted.
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<th>Antibody targeting:</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Stock Concentration</th>
<th>Dilution for Western Analysis</th>
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<tr>
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**Table 4.1 Antibodies Used in Western Analysis Experiments**

Antibodies targeting listed proteins were used at the dilutions listed in the right most column in 1% milk-1XTBS-T, except GAPDH which was diluted in 5% milk-1XTBST and were procured from the companies listed.
4.2.3 Immunocytochemistry, Quantification of Immunocytochemistry, and Confocal Microscopy

Whole-mount SMG immunocytochemistry (ICC) analysis was performed as previously described (Sequeira et al., 2013; Sequeira et al., 2012 and Chapter 3.2.11). Specimens were fixed in freshly prepared 2 or 4% paraformaldehyde containing 5% w/v sucrose, in 1XPBS for 20-30 minutes. Fixed samples were permeabilized, blocked, and exposed to primary antibodies and incubated overnight at 4°C. After washing, samples were incubated with donkey anti-species cyanine or Alexa Fluor dye-conjugated F(ab’)2 secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature, protected from light. Nuclei were stained with DAPI after secondary antibody treatment. The samples were mounted on glass coverslips with mounting media (Fluoro-Gel, Electron Microscopy Sciences 17985-10) before imaging. Laser scanning confocal fluorescent microscopy on immunocytochemistry (ICC) samples was performed using a Zeiss 710 or Zeiss 510 confocal microscope, and images were acquired at 20X or 63X magnification. All confocal images within a given experiment were captured using the same laser intensity and gain settings so that intensities of each signal could be compared across samples. Confocal images were initially processed in Zen (2012, Carl Zeiss Microscopy) and quantified using Photoshop and/or Image J.

Quantification of fluorescent pixel intensity from a single equatorial projection of confocal images (n=>5 glands per condition, 5-10 images per gland per experiment), encompassing either the entire 512x512 image was calculated using ImageJ software (FIJI version 1.49J10). Alternatively, quantification was performed using a region of interest (ROI) drawn around the epithelial bud within an image using the ECAD co-stain as a guide. The
intensity of fluorescent staining was expressed as a ratio of pixels per area and normalized to
the intensity of DAPI stain within the same image.

For cell height measurements multi-channel images were opened in Adobe Photoshop
CS6 (version 13.01x64) as individual images. Markers were placed on the SM α-actin positive
cells at the glands periphery. These marks were then copied on to the ECAD image taken at the
same time. The height, perpendicular to the edge of the gland, and cell width, parallel to the
basement membrane, were then measured in pixels. The number of µm was then calculated
from the number of pixels based on the magnification of the image used. These measurements
were then graphed using GraphPad Prism (Prism 6.05, 2014) and statistics were calculated
using VassarStats (http://vassarstats.net/).
Table 4.2: Antibodies Used in Immunocytochemistry Experiments

Antibodies targeting listed proteins were used at the dilutions listed in the right most column (diluted in 3% BSA-1XPBS-T) and were procured from the companies listed.

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<th>Catalog Number</th>
<th>Stock Concentration</th>
<th>Dilution for ICC</th>
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4.3 RESULTS

4.3.1 Rac1 is Required for Branching Morphogenesis

To investigate a role for Rac1 in the developing mouse submandibular salivary gland (SMG), we used SMG organ explants in which we manipulated Rac1 function using a targeted siRNA construct to decrease Rac1 levels or a pharmacological inhibitor for Rac1 (EHT1864, EHT), which inhibits the binding of Rac1 to GTP, thus rendering it inactive. We observed a progressive inhibition of branching morphogenesis using brightfield imaging of live explants that were treated with either Rac1 siRNA or EHT1864 for 96 hours (Figure 4.1A). Morphometric analysis indicates that Rac1 inactivation using EHT (Figure 4.1B) resulted in a statistically significant reduction in branching morphogenesis when compared to controls, and Rac1 siRNA treatment also resulted in a decrease in branching (Figure 4.1C). The reduction of branching morphogenesis using EHT1864 is reversible, as branching morphogenesis can be rescued following a washout of the inhibitor (Figure 4.2A and B). As expected, following treatment with Rac1 siRNA, western analysis shows a significant reduction in Rac1 levels after 96 hours in culture when compared to controls, and no change in the Rac1 levels was detected after 96 hours of EHT treatment when compared to controls (Figure 4.1D and E). Immunocytochemistry (ICC) confirms that there are no major changes to the levels of Rac1 after EHT treatment (Figure 4.1F) and that the siRNA treatment significantly reduces the levels of Rac1 (Figure 4.1G). These results indicate that Rac1 activity is required for the branching morphogenesis of the SMG and that the morphological changes seen after Rac1 siRNA treatments mimic those seen after pharmacological inactivation of Rac1.
Figure 4.1 Decreased Branching Morphogenesis of the Mouse Submandibular Salivary Gland with Pharmacological Inactivation or siRNA Knockdown of Rac1

(A) Representative bright-field images of E13 SMGs cultured for 96 hours under normal culture conditions, with 10 µM EHT, 500 nM non-targeting siRNA or 500 nM Rac-1 siRNA indicate that Rac1 expression and activity are both required for branching morphogenesis of the developing mouse SMG. Scale Bar 100 µm. (B) Morphometric analysis of control and 10 µM EHT treated E13 SMG’s (**) p ≤0.01, n=20 per condition) and (C) NT siRNA and Rac1 siRNA treated glands show a statistically significant decrease in the number of buds in EHT treated glands, but not in the siRNA treated glands, although there is a decrease in bud number, indicating the decreased branching morphogenesis (n=15 per condition in B and C). (D) Western analysis and (E) quantification of Rac1 siRNA or 10 µM EHT treated
glands compared to controls shows a significant decrease in Rac1 levels after siRNA treatment, but not with EHT treatment as expected. Blot shown is representative and quantification is the average of at least 3 experiments. (F and G) Immunocytochemistry for Rac1 (cyan), ECAD (green), and Nuclei (DAPI, blue) show significant decrease in Rac1 levels in the epithelium after Rac1 siRNA treatment but not with 10 µM EHT. Scale Bars 20 µm.
Figure 4.2 Effects of EHT Treatment in the E13 SMG is Reversible

(A) Representative brightfield images from E13 SMG’s under normal culture conditions, glands treated with 10 μM EHT for 48 hours, and glands treated with 10 μM EHT for 24 hours, then with normal culture media for an additional 24 hours (Washout, W/O) show that the effects of EHT can be reversed when it is removed from the culture system. Scale bar 100 μm. (B) Morphometric analysis of glands from panel A shows a significant rescue of the glands ability to branch with the washout of EHT (** p ≤0.01, n=15 per condition).
4.3.2 Rac-1 Inhibition Does Not Significantly Decrease Proliferation or Increase Apoptosis

Since Rac1 is known to regulate both apoptosis and proliferation, we considered whether Rac1’s effects on branching morphogenesis could be attributed to either of these processes. We found that there was a slight increase in staining for Cleaved Caspase 3, an executioner caspase that is activated by cleavage by both extrinsic and intrinsic apoptosis pathways, in the mesenchyme surrounding the glandular epithelium, but that there was no significant difference in the number of CC3-expressing cells within the epithelium of EHT-treated glands when compared to controls (Fig 4.3A). Since apoptosis appears not to be operative in Rac1-mediated inhibition of branching morphogenesis, we examined the levels of proliferation within the glandular epithelium. Following treatment of glands with 10 μM EHT over 48 hours, there was a reduction in the mitosis-specific histone H3 phosphorylation on Serine 10 (pHH3) when compared to controls (Figure 4.3C and D). Western analysis for pHH3 normalized to total histone H3 shows a marked decrease in pHH3 in EHT-treated glands, which can be partially rescued with EHT washout (Figure 4.3C and D). Treatment of SMG with Rac1 siRNA also shows a large decrease in pHH3 levels with western analysis (Figure 4.3E and 2F) indicating that Rac1 activity is playing a role in proliferation in the developing epithelium. Western analysis takes into account levels of pHH3 in both the mesenchyme and the epithelial compartments of the gland, so to investigate whether the decrease in proliferative cells was in the epithelial or mesenchymal compartments were performed ICC for pHH3 and ECAD, to mark the epithelium. No significant difference in the pHH3 staining pattern was observed within the
ECAD-positive epithelium or mesenchyme after treatment with EHT for 48 hours when compared to controls.
Figure 4.3 Epithelial Proliferation and Mesenchymal Apoptosis is Affected by the Knockdown or Inhibition of Rac1

(A) Immunocytochemistry for CC3 (cyan), ECAD (green), and Nuclei (DAPI, blue) following treatment with 10 μM EHT for 48 hours show no major change in CC3 staining in the epithelium, indicating there is not an increase in apoptosis with EHT treatment. Scale bar 100 μm. (B) Immunocytochemistry for proliferating cells (pHH3, cyan) after treatment with 10 μM
EHT after 48 hours shows no visible increase in the number of proliferating cells within the epithelium (ECAD, green). (C and D) Western analysis and quantification shows a marked decrease in pH3 in EHT treated cells when compared to total HH3 and control treated glands. Washout of EHT at 24 hours rescues proliferation by 48 hours in the developing SMG. (E and F) Western analysis and quantifications indicate a decrease in proliferation, marked by pH3, with EHT inactivation or Rac1 or siRNA knockdown of Rac1, indicating that Rac1 activation is required for proliferation. Blots are representative and quantifications are an average of at least three experiments.
4.3.3 Rac1 Activation is Required for the Localized Basal Deposition of the BM

Since salivary gland branching morphogenesis requires basement membrane (BM) remodeling, in the developing SMG, we questioned whether basement membrane was affected by the inactivation of Rac1. After inhibition of Rac1 with EHT, we identified a significant loss in collagen IV, a major component of the basement membrane, observed by confocal imaging of immunostained whole mount organ explants and recombined organ explants (Figure 4.4A and B, top two panels). The loss of collagen IV was also detected via western analysis (Figure 4.4D and E), which revealed a 48% reduction in collagen IV protein levels in glands treated with EHT for 96 hours.

4.3.4 Rac1 Inactivation Leads to Decreased Levels of Par-1b

Since we previously reported that the basal localization of Par-1b is critical for the basal deposition of basement membrane by the outer layer of epithelial cells in the developing SMG (Daley et al., 2012), we questioned whether Rac1 regulates Par-1b in the OCCs. Correlating with the loss of collagen IV in Rac1 inhibited glands; we identified a significant decrease in Par-1b protein levels (loss of approximately 60%) (Figure 4.4F and G) as well as a mislocalization of the remaining Par-1b protein from its basal localization in the outer epithelial cells to a more lateral localization in these cells (Figure 4.4C). These data suggest that Par-1b localization and protein levels are under control of Rac1 activation, and that the Rac1-mediated disruption of basement membrane is likely to be Par-1b-mediated. To confirm that Par-1b functions downstream of Rac1 in these processes, we overexpressed Par-1b in EHT-treated SMG organ explants. The loss of collagen IV in EHT-treated epithelial rudiments was partially rescued with the addition of a
Par-1b WT adenoviral construct (Figure 4.4B bottom panel), further indicating that Rac1 functions upstream of Par-1b in BM deposition.
Figure 4.4 Rac-1 Inactivation Results in Disruption of the Basement Membrane and Cellular Polarity

(A) Immunocytochemistry for collagen IV (green) and nuclei (DAPI, blue) following 96 hour culture of E13 SMG’s under normal conditions or in the presence of 10 µM EHT1864 indicate that Rac1 inactivation decrease levels of collagen IV. Scale bar 20 µm. (B) Immunocytochemistry for collagen IV (green) and nuclei (DAPI, blue) performed on E13 glands that were removed
from the mesenchyme, soaked in standard culture media or media containing WT Par-1b adenovirus (2 µl:100 µl), then recombined with removed, untreated mesenchyme, and cultured for 96 hours with or without 7.5 µM EHT. Glands treated with only EHT showed similar decrease in collagen IV levels as was seen in whole glands, while glands treated with only WT Par-1b adenovirus show an increase in collagen IV levels when compared to controls. Epithelial rudiments treated with EHT and WT Par-1b adenovirus show an intermediate phenotype indicating that overexpression of WT Par-1b can rescue the loss of the BM caused by the inactivation of Rac1. (C) Immunocytochemistry for Par-1b (cyan), collagen IV (green), and nuclei (DAPI, blue) performed on whole glands treated with or without 10 µM EHT for 96 hours shows a loss of Par-1b levels after Rac1 inactivation, further indicating Par-1bs control of the BM is downstream of Rac1 activation. Scale bar 20 µm. (D-G) Western analysis performed on lysed glands treated with or without 10 µM EHT followed by quantification show decreased levels of collagen IV (approximately 45%) and Par-1b (approximately 30%) with Rac1 inactivation. (H and I) Western analysis for collagen IV performed on glands treated with NT siRNA or Par-1b siRNA (500 nM) for 96 hours indicate a decrease in collagen IV levels with Par-1b knockdown (approximately 60%) when compared to control. All western blots are representative of experiments done at least 3 times and quantifications are averages of at least 3 experiments.
4.3.5 *Rac1 Activation is Required for the Cytodifferentiation and Morphological Changes During Myoepithelial Differentiation*

Since loss of Rac1 and Par-1b affected basement membrane in the developing rudiments, we questioned whether there was a defect in the maturation of the outer epithelial cells which we have previously shown to be dependent on the organization of the BM. We previously reported that these outer epithelial cells differentiate into the myoepithelial population that surround the secretory acinar cells (Nelson et al., 2013; Peters et al., 2014, 2015). After these outer most epithelial cells begin to express the myoepithelial marker protein, smooth muscle α actin (SM α-actin), they transition from a cuboidal/columnar cell shape to a stellate morphology (Figure 4.5A). Interestingly, we noted that the inhibition of Rac1 signaling prevented this shape change in the outer epithelial cell population that normally occurs following four days in culture, mimicking the *in vivo* transition (Figure 4.5A, arrows). Whereas the control-treated outer epithelial cells in the explants are undergoing a shape change to assume the more flattened and stellate morphology of a myoepithelial cell at 96 hours of culture, the cells in the EHT-treated glands remain cuboidal in shape (Figure 4.5A and B). We measured the height and width of SM α-actin positive outer epithelial cells in control glands and in glands treated with EHT to confirm that that loss of Rac1 activation was inhibiting the morphological changes in the myoepithelium (Figure 4.5B). Cells in the control glands were significantly shorter than SM α-actin positive cells in EHT treated glands, indicating the loss of Rac1 activation is inhibiting the transition of SM α-actin positive cells from the tall cuboidal shape to the flattened stellate shape of the fully differentiated myoepithelium. Cells measured in the control glands are likely in an intermediate step between cuboidal polarized cells and
stellate, fully differentiated myoepithelial cells as it would be difficult to measure the height of a fully differentiated stellate myoepithelial cell. We therefore questioned whether there was a defect in the cytodifferentiation of the myoepithelium. We investigated whether the levels SM α-actin, is changed with Rac1 inhibition using immunocytochemistry and western analysis. We found that cytodifferentiation still occurs with Rac1 inhibition; however, there is a reduction in the levels of SM α-actin detected by both ICC and by Western analysis (Figure 4.5A, C-D). These data suggests that Rac1 regulates the morphological changes that are required to form a myoepithelial cell and that there may be a link between morphogenesis and cytodifferentiation of the myoepithelial cells.
Figure 4.5 Rac1 Inhibition Disrupts the Morphogenesis and Differentiation of the Myoepithelium in the Developing SMG

(A) Immunocytochemistry for SM α-Actin (red), ECAD (green), and nuclei (DAPI, blue) performed on E13 SMG’s treated with 10 µM EHT for 96 hours shows a marked decrease in SM α-actin levels as well as a marked difference in OOC shape (arrows). Scale bar 20 µm. (B) The height and width (µm) of individual epithelial cells expressing SM α-actin (n=45 per condition) were measured in glands treated with or without 10 µM EHT for 96 hours. OCC height in the control glands was significantly smaller, about half that of the OCC height in the EHT treated glands (***)
p ≤ 0.001). Additionally, OCCs were approximately 33% wider in the control glands than in the EHT treated glands. (C and D) Western analysis was performed on E13 glands cultured for 96 hours +/- EHT treatment and indicated a decrease of approximately 40% of SM α-Actin levels in EHT treated samples. Blot shown is representative and quantification is the average of at least 3 experiments.
4.3.6 Par-1b is Required for the Cytodifferentiation and Morphological Changes in Myoepithelial Differentiation

Since Rac1 regulates the localization and levels of Par-1b, we questioned whether Par-1b was also required for the morphogenesis and differentiation of the myoepithelium. We knocked down Par-1b using a targeted siRNA in whole organ explants (Figure 4.6), and confirmed with western analysis that more than 50% of Par-1b protein levels was lost after 96 hours in culture when compared with controls (Figure 4.6C-D). We further questioned whether Par-1b plays a direct role in the differentiation of myoepithelium. Immunocytochemistry confirmed that Par-1b is required for the differentiation of the myoepithelium, as evidenced by a phenotype that very closely mimics that seen after inactivation and siRNA-mediated knockdown of Rac1 (Figure 4.6A). After treatment with targeted Par-1b siRNA, SM α-actin expression is decreased, but the cells still expressing SM α-actin have not undergone the expected morphological changes seen with differentiation of the myoepithelium in the NT siRNA treated control glands (Fig 4.6A arrows). The loss of SM α-actin protein levels seen via western analysis indicate that a far smaller proportion of cells are expressing SM α-actin or that the cells that are expressing it are doing so at a much lower level in Par-1b knockdown cells when compared to negative control (Figure 4.6E-F). We have also shown similar loss of the morphological changes with Par-1b knockdown as seen after Rac1 inactivation with measurements of cell height and width. As seen in the Rac1 inactivated glands, the Par-1b siRNA treated glands also contain SM α-actin positive cells that are significantly shorter than SM α-actin-expressing cells in the control glands (Figure 4.6B). These data indicate that Par-1b and
Rac1 are both required for the morphological changes and cytodifferentiation of the developing myoepithelium.
Figure 4.6 Par-1b is Required for Differentiation of the Myoepithelium in the Developing Mouse SMG

(A) Immunocytochemistry was performed on E13 glands grown in culture for 96 hours and treated with either NT siRNA or Par-1b siRNA (500 nM) for SM α-Actin (red), ECAD (green), Par-1b (cyan) and Nuclei (DAPI, blue) show a significant decrease in Par-1b levels along with a significant decrease in the number of SM α-actin positive cells with Par-1b siRNA treatment. Scale bar 20 µm. (B) Height and width (µm) measurements of individual epithelial cells expressing SM α-actin (n=25 per treatment) were measured in glands treated with NT siRNA or Par-1b siRNA (500 nM) for 96 hours. OCC height in the control glands was significantly smaller, about half that of the OCC height in the Par-1b siRNA treated glands (**p ≤ 0.01). Additionally, OCCs were approximately 35% wider in the NT siRNA treated glands than in the Par-1b siRNA treated glands. (C–F) Western analysis and quantification following 96 hours of Par-1b or NT
siRNA treatment shows a significant reduction (50%) in the levels of Par-1b indicating a successful knockdown, and SM α-actin (50% decrease), indicating a requirement for Par-1b expression in differentiation of the myoepithelium. Blot shown is representative and quantification is the average of at least 3 experiments.
4.3.7 Par-1b is Necessary but Not Sufficient to Drive Morphological Changes in Myoepithelial Differentiation

To confirm that Par-1b functions downstream of Rac1 in the differentiation of the SMG myoepithelium, E13 SMG explants were treated with adenoviral construct to drive WT Par-1b expression in glands in the presence or absence of the Rac1 inhibitor, EHT for 96 hours. To accomplish this, explants were microdissected to remove the mesenchyme from the epithelial rudiments. The epithelial rudiments were then exposed to Par-1b WT adenovirus for 2 hours, followed by recombination with untreated mesenchyme either in the presence or absence of EHT inhibitor. The glands that were treated with both EHT and wild type Par-1b adenovirus were markedly larger than the EHT-treated glands, indicating that an increase in Par-1b can compensate for the loss of Rac1 activity during the process of branching morphogenesis (Figure 4.7A). Treatment with EHT showed a decrease in branching morphogenesis and overall gland size at each 24 hour increment over 4 days, similar to the effect in whole organ explants, while the Par-1b adenovirus-treated glands showed very similar branching and overall gland size when compared to untreated control rudiments (Figure 4.7A). When these glands were immunostained, we confirmed that the glands treated with Par-1b adenovirus showed a marked increase in Par-1b staining and the EHT-treated rudiments showed a marked decrease in Par-1b when compared to untreated controls (Figure 4.7B). In the glands treated with Par-1b WT adenovirus the levels of SM α-actin were markedly higher than in the EHT treated glands, but in the glands treated with both Par-1b WT adenovirus and EHT, there was only a moderate increase in levels of SM α-actin as seen in ICC (Figure 4.7B). We quantified the staining of SM α-actin and Par-1b with in the glandular epithelium by drawing a line around the edge of the
ECAD stain in each image set, and quantifying the pixel value within only the epithelial areas. The results indicated that there was a decrease in both SM α-actin and Par-1b levels following EHT treatment, and that levels of both Par-1b and SM α-actin were slightly increased over controls following treatment with Par-1b WT adenovirus (Figure 4.7D). However, in glands treated with both EHT and Par-1b WT adenovirus, there was an increase in Par-1b levels, but no change in SM α-actin levels when compared to EHT treatment alone (Figure 4.7D). These results indicate that while Par-1b overexpression can rescue the defects in branching morphogenesis in Rac1 inhibited epithelial rudiments, overexpression of Par-1b is likely not sufficient to drive expression of SM α-actin in the presence of Rac1 inhibition. We also quantified the shape of SM α-actin positive cells in each condition to determine whether Par-1b WT overexpression could rescue the loss in cell shape change in the myoepithelium during Rac1 inhibition. After 96 hours, there were no marked differences in cell height or width with EHT, WT Par-1b adenovirus or a combination of both treatments. These results indicate that EHT is not affecting the changes in cell shape required for the morphological changes previously seen in whole glands (Figure 4.5B). These results led us to look at a later time point to accommodate the delay in gland growth typically seen with recombination experiments.
Figure 4.7 Myoepithelium Morphogenesis and Differentiation with Overexpression of Par-1b in the Presence of Rac1 Inactivation in the Developing SMG at 96 hours

(A) Representative brightfield images taken at 2 hours and at 24 hour increments thereafter of E13 epithelial rudiments recombined with mesenchyme after 2 hour soak in either normal culture media or media containing Par-1b WT adenovirus (2 µl:100 µl) show that EHT treatment reduces branching morphogenesis, but this loss in branching and overall decrease in gland size is partially rescued by the addition of Par-1b WT adenovirus. (B) Immunocytochemistry for SM...
α-actin(red), Par-1b (cyan), ECAD (green) and Nuclei (DAPI, blue) shows a reduction in SM α-actin and Par-1b following EHT treatment, and a slight increase in both Par-1b and SM α-actin following WT Par-1b adenoviral infection. Treatment with both WT Par-1b adenovirus and EHT showed an increase in Par-1b, but no change in SM α-actin over levels seen in EHT alone. (C) Measurements of cell height (black bars) and cell width (gray bars) show no significant differences following treatment with WT Par-1b adenovirus, EHT or both treatments when compared to controls (n ≥ 220 cells per condition). (D) Quantification of the ICC in the epithelium (total pixels per channel in epithelial area only traced using ECAD co-stain and normalized to DAPI) show that overexpression of WT Par-1b in the presence of EHT does not increase the levels of SM α-actin over that of EHT alone (n= 20 for control, 22 for Par-1b WT and 24 for EHT and EHT+WT).
4.3.8 Myoepithelial Differentiation in 120 hour SMG Recombinations

To better understand whether or not Par-1b is acting downstream of Rac1 in the cytodifferentiation and morphological changes seen during the differentiation of the myoepithelium in the SMG, we cultured recombined epithelial rudiments in the presence of EHT, EHT + WT Par-1b adenovirus or WT Par-1b adenovirus alone for 120 hours to ensure that differentiation was occurring. Following 120 hours in culture with media changes every 48 hours, there were few obvious changes in gland size and development (Figure 4.8A). Immunostaining of epithelial rudiments treated with EHT, WT Par-1b adenovirus or both treatments or grown under control conditions showed an increase in Par-1b with WT Par-1b adenoviral treatment with or without EHT, however, at 120 hours, we did not see the marked decrease in Par-1b following EHT treatment that we had seen previously (Figure 4.8 B). Par-1b seems to be expressed in the interior of the gland following EHT treatment, but not in the control gland, and to a lesser extent in the combined treatment. We also saw the expected decrease in SMα-actin following EHT treatment that was largely unchanged with the addition of WT Par-1b adenovirus (Figure 4.8B). These results corroborate results from earlier time points which indicated that while WT Par-1b overexpression is sufficient to slightly increase the levels of SM α-actin in recombined SMGs, it is not sufficient to increase SM α-actin levels in the presence of Rac1 inhibitor. Quantifications of immunocytochemistry images following 120 hour culture of recombined glands further complicates the results indicating that even in the presence of EHT, Par-1b levels are markedly increased. These results may be pointing to an alternate mechanism of Par-1b control in the inner polymorphic cells, where Par-1b levels seem to have increased with EHT treatment following 120 hour culture.
Figure 4.8 Myoepithelium Morphogenesis and Differentiation with Overexpression of Par-1b in the Presence of Rac1 Inactivation in the Developing SMG at 120 hours

(A) Representative time course images taken at 2 hours and at 24 hour increments thereafter for 120 hours of E13 epithelial rudiments recombined with mesenchyme after 2 hour soak in either normal culture media or media containing WT Par-1b adenovirus (2.5 µl:100 µl) show that EHT treatment reduces branching morphogenesis, but this loss in branching and overall decrease in gland size is partially rescued by the addition of WT Par-1b adenovirus. Scale bar
100 μm. (B) Immunocytochemistry for SM α-actin (red), Par-1b (cyan), ECAD (green) and Nuclei (DAPI, blue) following 120 hour culture shows a reduction in SM α-actin and Par-1b following EHT treatment, and a slight increase in both Par-1b and SM α-actin following WT Par-1b adenoviral infection. Treatment with both WT Par-1b adenovirus and EHT showed an increase in Par-1b, but no change in SM α-actin over levels seen in EHT alone. Scale Bar 20 μm. (C) Quantification of the ICC in the epithelial areas shows that overexpression of WT Par-1b in the presence of EHT does not increase the levels of SM α-actin over that of EHT alone (n≥50 cells per condition).
4.3.9 ROCK Inhibition Does Not Affect Myoepithelial Differentiation

Previous work in the Larsen lab has identified ROCK as an upstream regulator of Par-1bs control of the BM expression, deposition, and organization during SMG development (Daley et al., 2012). We questioned whether ROCK’s regulation of Par-1b localization was required for differentiation of the myoepithelial cell population. Since previous experiments had been terminated at 48 hours and a longer time in culture was required to detect myoepithelial differentiation, we cultured E13 SMGs for 120 hours with normal culture media or in the presence of ROCK inhibitor Y27632 and followed with immunocytochemistry. While the overall structure of the gland seems to be disrupted by ROCK inhibition (Figure 4.9A), previously shown to significantly impact branching morphogenesis (Daley et al., 2012), there seems to be little to no impact on levels or localization of Par-1b or SM α-actin (Figure 4.9B) following ROCK inhibition for 120 hours. Par-1b is very tightly basally localized in the control glands, and seems to be present in the flattening myoepithelial cells, also positive for SM α-actin. This very tight basal restriction of Par-1b and the flattening of the SM α-actin positive cells may be slightly disrupted in the ROCK-inhibited glands, though cell height and width measurements of the SM α-actin positive cells has not been done. Quantification of immunocytochemistry and cell height and width measurements, as well as a repeat of this experiment will be necessary for a better understanding of whether or not ROCK continues to act upstream of Par-1b’s control of the BM at this late stage in SMG development.
Figure 4.9 Myoepithelial Differentiation is Unaffected Following 120 hour Inhibition of ROCK

E13 glands were grown in culture for 120 hours in the presence of 70 µM Y27632 to inhibit ROCK activity or grown under normal culture conditions. (A) Brightfield images were taken at 2 hours and every 24 hours thereafter until 120 hours and show a morphological change in glands treated with Y27632. Scale bar 100 µm. (B). Following fixation at 120 hours, glands were immunostained for SM α-actin (red), ECAD (green), Par-1b (cyan) and Nuclei (DAPI, blue). ICC results show no significant changes in the levels or localization of Par-1b or SM α-actin following ROCK inhibition for 120 hours. Scale bar 20 µm.
4.4 DISCUSSION

These studies have identified a role for Rac1 and Par-1b in the morphogenesis and differentiation of myoepithelial cells in developing mammalian salivary glands. Using a pharmacological inhibitor of Rac1, and a Rac1-targeted siRNA, in the mouse submandibular salivary gland (SMG) organ explants, we observed significant defects in epithelial branching morphogenesis that could be partially attributed to decreased proliferation but not increased apoptosis. At any given time point investigated (48, 72, or 96 hours in culture), the number of pHH3 positive cells, was not significantly lower in EHT treated glands when compared to controls. However, the maintenance of a lower number of proliferative cells over the course of several days in culture will ultimately lead to a smaller epithelial area overall and the number of pHH3 positive cells per area of the gland was not measured. Additionally, on a cellular level, we detected defects in myoepithelial cell morphogenesis and cytodifferentiation in response to Rac1 siRNA knockdown or Rac1 inhibition. In contrast to control SMG, in which the outer epithelial cell population began to express SM α-actin and vertically compressed as they began to form myoepithelial cells, Rac1-inhibited glands showed a reduced level of SM α-actin as well as a defect in vertical cellular compression that was statistically significant.

Since the outermost cells undergoing morphological changes are in constant contact with the basement membrane (BM) and Par-1b is known to be required for the basal deposition and the organized localization of basement membrane proteins in these cells (Daley et al., 2011), we further investigated a function for Par-1b in myoepithelial differentiation. Rac1 inhibition decreased levels of Par-1b, and Par-1b siRNA knockdown recapitulated the Rac1 inhibition phenotype in the differentiation myoepithelial cells. Par-1b siRNA knockdown
reduced myoepithelial cellular morphogenesis, resulting in a statistically significant loss of vertical cellular compression when compared to controls. Levels of SM α-actin were also decreased with Par-1b siRNA knockdown and overexpression of Par-1b only slightly increased myoepithelial cytodifferentiation. However, overexpression of Par-1b seemed to have little effect on cellular morphogenesis, relative to controls. Further, Par-1b overexpression in the presence of Rac1 inhibitor EHT did not rescue either the cytodifferentiation or morphological changes seen in the control glands, indicating that Rac1 is likely not acting directly through regulation of Par-1b or that Rac1 has an abundance of downstream effector proteins in this pathway making it impossible for Par-1b alone to rescue Rac1 activity deficiency. It is possible that due to the delayed growth typically seen in epithelial recombination experiments (which are required for the use of adenoviral constructs, including WT Par-1b adenovirus) the changes in cell height attributed to the morphological changes in myoepithelial differentiation have not yet begun in the control gland after 96 hours in culture. To further investigate this possibility, we repeated the 96 hour experiments and grew the glands to 120 hour time point. Following immunocytochemistry at 120 hours, we again saw a smaller difference in the SM α-actin levels with EHT treatment, which may indicate that the glands are able to overcome Rac1 inhibition with extended time in culture, even with media changes including fresh inhibitor every 48 hours. Further, Par-1b levels were increased with the use of Par-1b WT adenovirus as expected, but Par-1b levels were also increased with EHT treatment at 120 hours throughout the interior of the developing gland. This ectopic expression of Par-1b may be due to the polarization of the inner polymorphic cells as they begin to differentiate, a process that may not be under the control of Rac1, and may be linked to the loss of cellular polarity in the surrounding
myoepithelial cells, which we have shown is exaggerated with Rac1 inhibition. Additional experiments are currently underway to investigate whether these results are repeatable, whether the changes in SM α-actin positive cell shape are consistent with what we have previously reported at the 96 hour time point, and whether a longer culture time point will allow us to further investigate changes to cell height under several treatment conditions.

Since we have identified Par-1b as a critical regulator of the differentiation of the myoepithelium in the developing mouse SMG, and our studies have shown that Rac1 is necessary but may not be acting directly upstream of Par-1b in this process, we wanted to further investigate the possibility that there is a different direct upstream regulator of Par-1b in this process. ROCK has previously been identified as an upstream regulator of Par-1b localization and Par-1bs control of the basement membrane deposition and localized organization (Daley et al., 2012). Though we have not elucidated the direct link between ROCK and Par-1b, we were interested in the possibility that ROCK may be upstream of the Par-1bs role in myoepithelial differentiation. Following 120 hour culture in the presence of normal culture media or culture media containing 70 µm Y27632, we found no major differences in the localization or expression of Par-1b or SM α-actin. While the gland has undergone less branching, the localization and expression of both Par-1b and SM α-actin seem unaffected by this level of ROCK inhibition at this time point. A higher concentration of Y27632 (140 µm) was used previously in 48 hour gland culture (Daley et al., 2012), but to ensure the survival of the glands for 120 hours in culture, we began these studies with the lower concentration. We have also previously seen marked differences in Par-1b localization in the SMGs treated with 140 µm Y27632 for 48 hours with the basal restriction of Par-1b being lost (Daley et al., 2012). E lower
concentration of Y27632 may not be reducing the activity of ROCK enough to allow the localization of Par-1b in the inner polymorphic cells of the gland as previously seen. It is also possible that ROCK activity is preventing the expression of Par-1b in the interior of the SMG epithelium in early SGM development, but at these later stages is no longer controlling Par-1b. After 120 hour culture, Par-1b is localized within the SM α-actin expressing myoepithelial cells, which have undergone vertical compression in the control glands. While the localization of Par-1b to the SM α-actin positive cells is consistent after 120 hours of 70 µm Y27632 treatment, measurements of cell height and width will be required to confirm whether or not the vertical compression of these cells has been affected. Further investigation into the potential changes to the BM, Par-1b, and SM α-actin levels and localization as well as the morphological changes seen with myoepithelial differentiation at a higher concentration of inhibitor are underway.

Myoepithelial differentiation has long been of significance in the cancer field due to the incidence of myoepithelial-derived tumors, but little is understood regarding the development of this cell type. Some work has been done to identify stages of myoepithelial differentiation in the rat breast epithelium (Radnor, 1972) and more recent work has looked into the development of the myoepithelium in salivary glands (Ogawa, 2003), but very little is known about the signaling pathways and mechanisms driving the differentiation of the myoepithelium in any mammalian epithelial system. Our data indicates that Rac1 and Par-1b are both involved in the regulation of the myoepithelial cell type during early salivary gland development. Our data to date is consistent with Par-1b acting independently of Rac1 or Par-1b being one of many possible downstream effector of Rac1 that is independently insufficient to rescue the Rac1 inhibition phenotype. If Rac1 does regulate Par-1b, it is likely not a direct interaction.
However, Rac1 may influence the apical PAR protein complex to indirectly regulate basement membrane assembly due to the mutual exclusion of the apical and basal par complexes. Rac1 may be acting upstream of Par-1b through its interactions with the apical PAR complex, either directly or via the Rac1 guanine exchange factor, T-lymphoma invasion and metastasis 1 (Tiam1). Tiam1 can directly bind to Par-3 (Chen and Macara, 2005; Mertens et al., 2006; Nakayama et al., 2008) and is recruited by Par-3 to the tight junctions where Rac1 binds to Par-6, leading to activation of atypical protein kinase C (aPKC) (Munro, 2006). Alternatively, Rho GTPase-mediated signaling pathways have previously been demonstrated to regulate morphogenetic behaviors in developing salivary glands. Since the Rho effector protein, ROCK1 (Rho-associated coiled-coil containing kinase), regulates Rho localization of Par-1b to control the deposition and organization of the BM surrounding the epithelial compartment of the developing mouse SMG (Daley et al., 2012), the regulation of Par-1b by Rac1 may be mediated by Rho signaling or Rho signaling may be the primary regulator of Par-1b.

The mechanism through which myoepithelial cells differentiate from an initial cuboidal/columnar cell type is not fully understood. Although the outer epithelial cells are the first epithelial cell type to polarize, we noted that under normal conditions, as myoepithelial cells start to differentiate in vivo, the cells appear to lose apico-basal polarity as they vertically compress to assume a stellate morphology and assemble circumferential BM around themselves. Par-1b, is known to be restricted to the basolateral domain in polarized epithelium by the apical PAR complex, containing Par-3, Par-6, and aPKC (Suzuki et al., 2004). This mutual exclusion is critical for the maintenance of epithelial apico-basal polarity and the appropriate localization of BM deposition and organization to the basal cell surface (Masuda-Hirata et al.,
Par-1b is also known to regulate dystroglycan, a Laminin -111 cell surface receptor (Masuda-Hirata et al., 2009), which is also thought to work in concert with integrins to allow for the organization of the BM (Driss et al., 2006). Taking these findings together, we speculate that during the cell shape change transition, levels of both Par-3 and Par-6 are decreased, allowing for the movement of Par-1b along the lateral edges of the cell to encircle the cell (see schematic in Figure 4.10). As Par-1b moves, the localization of dystroglycan and integrins is expanded along the lateral edges of the cell as well, allowing for the deposition and organization of the BM in the adjacent extracellular space, surrounding the outer epithelial cell. Along with the movement of the BM and its receptors around the lateral edges and eventually around the entire cell, there are presumably changes to the cytoskeleton as the cell changes shape from a tall cuboidal cell to a flat, stellate myoepithelial cell. Par-1bs control of the microtubules through phosphorylation of microtubule associated proteins (MAPs) (Drewes et al., 1998; Chen et al., 2006; Hayashi et al., 2012) may play a role in this transition as well to further control the differentiation of the myoepithelium, though we have not investigated this possibility. This transition would allow the cells to become embedded within the BM, while maintaining contact with the interior cells which are differentiating into the secretory acinar cell population. Further investigation into the signalling mechanism by which Rac1 and or ROCK are controlling Par-1b and the deposition and localization of the BM is required to fully understand the mechanism of myoepithelial differentiation in the developing mouse salivary gland.
Polarized outer columnar epithelial cells expressing Par-1b (indicated by blue lines in the second panel) and other apically localized Par proteins maintain their polarized structure. However, when the apical Par proteins are down regulated, the cells begin to undergo morphological changes. Par-1b can localize along the lateral edges of the cells and eventually around the entire cell membrane, taking with it the cell surface receptors for the basement membrane, including integrins and dystroglycan. The movement of these receptors which are thought to be under the control of Par-1b would allow for the movement of the BM to surround the cell, and may also affect the cells overall shape. Eventually the myoepithelial cell will form a stellate shape and be wrapped around the spherical secretory unit (top panel, right most image shows cross section), embedded within the basement membrane of the salivary gland to aid in secretion.

**Figure 4.10 Model of Differentiation of the OCCs into Myoepithelial Cells**
4.5 SUMMARY AND CONCLUSIONS

We have shown here that both Rac1 and Par-1b are required for the cytodifferentiation of the myoepithelium as well as the vertical compression that presumably begins the morphological changes leading to a flattened, stellate myoepithelial cell. Rac1 is also necessary to maintain levels of Par-1b protein, which would indicate that it is upstream of Par-1b in these processes. However, overexpression of wild type Par-1b with an adenoviral construct is not sufficient to rescue the differentiation of the myoepithelium caused by a Rac1 inhibition. These results indicate that Rac1's control of Par-1b is likely not direct. These results taken together with previous research indicate that Rac1 may be controlling Par-1b through its interactions with the apical Par proteins, which are known to inactive Par-1b in the apical domain of the polarized epithelial cell (Suzuki et al., 2004). We speculate that the differentiation of the myoepithelium is mediated by Par-1b's control of the cell surface receptors for basement membrane proteins, including integrins and dystroglycan. By moving these receptors along the lateral edges and eventually around the surface of the entire epithelial cell as Par-1b localization changes, the basement membrane would be able to encapsulate the differentiating myoepithelial cell as it changes shape from a cuboidal OCC to a flattened stellate myoepithelial cell. Further investigation is required to determine whether ROCK is acting directly upstream of Par-1b in this process, as we have seen ROCK activation is required for the appropriate localization of Par-1b in previous work (Daley et al., 2012). We would also like to further investigate Rac1's role in this process, which will likely involve the apical Par complex as well.
Chapter 5: Sjögren’s Syndrome Disease Progression in the NOD\ShiLtJ Mouse Model as Compared to Human Late Stage Disease

5.1 INTRODUCTION

Since the progression of SS can be analyzed in mouse models, we evaluated two mouse models of SS, the NOD- Aec1/Aec2 and the NOD/ShiLtJ model. In these histological studies, we compared the structure and organization of the submandibular salivary gland (SMG) epithelium first from male NOD- Aec1/Aec2 mice at 8 and 12 weeks to age and sex matched parental strain control mice (C57BL/6J) to determine whether there are early changes to the gland in this primary Sjögren’s syndrome (SS) disease model. Additionally, we compared the structure and organization of the SMG epithelium from female NOD/ShiLtJ mice at 8, 12, 18, and 22 weeks of age to sex- and age-matched parental strain control (CD-1) samples. These time points were examined in order to investigate changes in the SMG epithelium during early, mid, and late stage disease as previously identified in studies documenting detection of lymphocytic infiltrates and saliva flow rates as hallmarks of disease progression (Lodde et al., 2006; Karnell et al., 2014; Lavoie et al., 2010) (Figure 2.3). Female mice were used exclusively in the study of NOD/ShiLtJ mice since a delayed phenotype in male mice has been shown in this model system. The SS phenotype is known to occur earlier in the SMG of female mice than in other salivary glands or lacrimal glands, and the SS phenotype is seen significantly earlier in female SMGs than in male salivary glands in the NOD/ShiLtJ mouse model (Jayasinghe et al., 1990; Perez-Moreno,
Jamora, & Fuchs, 2003; Xuan et al., 2013). We confirmed disease progression in the NOD/ShiLtJ animals by identifying and measuring the size of focal lymphocytic infiltrates within the SMG using B220 (CD45R), a marker for B cells. We also examined laminin-111 and collagen IV, both markers of proteins found in the basement membrane, a specialized form of extracellular matrix surrounding the acinar units within the SMG; salivary androgen binding protein α (SABPA) and Mucin 10 (Muc10), both submandibular secretory proteins; Aquaporin 5 (Aqp5) as a marker of secretory acinar epithelium, cytokeratin 7 (K7) as a marker of the ductal epithelium, and smooth muscle α-actin (SM α-actin), Calponin and Sm22α as markers of the myoepithelial population. We also looked at cytokeratin 5 (K5), which is a reported basal epithelial cell and progenitor cell marker in salivary glands and other branched organs (Knox et al., 2010; Rios, et al., 2014; Vitale-Cross et al., 2004; Zuo et al., 2014). To evaluate potential for human disease relevance, we also interrogated some of these epithelial cell markers in human SS and non-SS patient samples and in autopsy samples derived from labial salivary gland biopsies.
5.2 METHODS AND MATERIALS

5.2.1 Mouse Tissue Samples

Tissues from 8 and 12 week old male NOD- Aec1/Aec2 were shipped in paraffin blocks from Dr. Seunghee Cha at the University of Florida, College of Dentistry. Age- and parental strain-matched female C57Bl6/6J control mice (Charles River, Wilmington, MA) were received by the University at Albany animal facility at 7 or 11 weeks of age, and tissues were harvested when the mice reached 8 and 12 weeks respectively. At 8 or 12, weeks of age, mice were euthanized following the University at Albany IACUC-approved procedures, as previously described (Daley et al., 2009, 2011), and the tissues harvested (matching the tissues sent from the University of Florida) included submandibular salivary glands, parotid glands, lacrimal glands, spleen, thymus, heart, lung, leg muscle, pancreas and kidney. Tissues were collected from each of 3 mice per time point. Tissues were chopped into small pieces immediately following harvest, incubated overnight in an excess of 10% Neutral Buffered Formalin (Sigma Aldrich, St. Louis MO), then moved to 70% ethanol for storage until paraffin embedding by Histoserv Inc. (Germantown, MD).

Non-Obese Diabetic (NOD) ShiLtJ female mice (Jackson Laboratories Labs) and control female CD-1 mice (Charles River) were received by the University at Albany animal facility at 7 weeks of age. Due to previous reports of stress affecting the autoimmune phenotype (Lodde, et al., 2006; Cha, Peck, & Humphreys-Beher, 2002), both the NOD/ShiLtJ and CD-1 control mice were housed under sterile conditions, kept on a 12 hour/12 hour light/dark cycle, and the room was entered only once per day by facility staff. Although mice were initially fed sterile (autoclaved) chow (20% fat) and water ad libitum, NOD/ShiLtJ mice were switched to
dampened chow at approximately 18 weeks of age due to a wasting phenotype (scruffy fur, lethargic behavior, and overall decline in activity and health) in a small subset of mice (two mice; one at 17 weeks and one at 19 weeks). Tissues were harvested from the 19 week mouse as previously described and tissue samples were included in the microarray, but not in any data analysis thereafter. This phenotype may have been due to significantly decreased saliva flow shown previously in this mouse model (Jonsson et al., 2006), but we cannot confirm this as a cause as we did not measure saliva flow in these mice. However, following the transition to dampened chow, all of the NOD/ShiLtJ mice showed increased activity and improved fur coat quality, indicative of improved general health. This was done as per the suggestion of the mouse facility director and no mention of this wasting phenotype was found in the literature.

At 8, 12, 18, or 22 weeks of age, mice were euthanized as described above, and submandibular salivary glands were removed from each mouse. We also collected lacrimal glands, the thyroid, thymus, heart, pancreases, a single kidney and a large piece of tissue from a lung and a section of rear leg muscle (See Tables 5.1 and 5.2). We collected tissues from a total of 6 mice per time point for each mouse strain. Tissues were chopped into small pieces immediately following harvest, and fixed and stored as described above.
### Table 5.1 Tissue Information for NOD/ShiLtJ and CD-1 Mouse 8 and 12 Week Samples in TMA

Mouse tissues from either CD-1 or NOD\ShiLtJ were collected from either 8 or 12 week old animals. Tissue type, age, strain, block number (one block per mouse), and the number and designations of spots used on the TMAs are listed here.

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Table 5.1 Tissue Information for NOD/ShiLtJ and CD-1 Mouse 8 and 12 Week Samples in TMA
### Table 5.2 Tissue Information for NOD/ShiLtJ and CD-1 Mouse 18 and 22 Week Samples in TMA

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Mouse tissues from either CD-1 or NOD\ShiLtJ were collected from either 18 or 22 week old animals. Tissue type, age, strain, block number (one block per mouse), and the number and designations of spots used on the TMAs are listed.
5.2.2 Human Tissue Samples

Human tissue samples were obtained from patients that were seen at the University of Oslo or at the Oslo University Hospital, Oslo, Norway. All tissue acquisition was performed according to protocols and procedures approved by the Norwegian Committee for Ethics in Research. Labial gland biopsies were processed at the University of Oslo diagnostic pathology labs by fixation in 4% formalin, followed by embedding in paraffin to generate formalin-fixed, paraffin-embedded samples. The formalin-fixed, paraffin-embedded tissue specimens were transferred to a research biobank at University of Oslo. Cadaver tissue was obtained from donors without SS or other rheumatic disease from Oslo University Hospital, The Intrinsic Institute and transferred to the biobank. All patient records were prepared and maintained at the University of Oslo biobank, and samples were de-identified before being sent to the Larsen Laboratory.

Paraffin blocks were obtained containing labial salivary glands from seven autopsy tissue donors (5 male, 2 female, ages ranging from 58-73 at time of death, average = 65), eight patients diagnosed with Sjögren’s Syndrome (SS) (all female patients, ranging in age from 48-84, average = 60), and from six patients presenting with a combination of three or more SS symptoms but lacking autoantibodies and therefore did not ultimately meet the 2002 American-European Consensus Group criteria (Vitali et al., 2002) for SS (Non-SS) (all female patients, ranging in age from 42-61, average = 52). Patients were given identifiers (names with the same first letter for all samples from a group) for ease of use during image processing (Table 5.3).
Table 5.3 Human Tissue Information

A tissue microarray was constructed using tissues from each of 7 autopsy tissue donors, 6 patients that presented with oral dryness but were not ultimately diagnosed with SS, and 10 Sjögren’s syndrome diagnosed patients. Age of patient at the time of sample collection, patient’s sex and the number of spots on the array where the tissue was used is listed.
5.2.3 Mouse Tissue Microarray Construction and Selection

Tissue microarrays (TMAs) were constructed from either human and mouse tissue samples as seen in Figure 5.1. The Aec1Aec2 mouse tissue TMA (not shown) was designed to accommodate five SMG spots from 8 and 12 week Aec1Aec1 and control mice from at least 2 mice per condition and time point. We also included several of the other tissues harvested to prevent the salivary gland tissues from being along the edges of the array, and to aid in orientation or the array. The NOD/ShiLtJ mouse tissue TMAs were designed (Tables 5.4-5.6) to accommodate 12 CD-1 and 12 NOD/ShiLtJ SMG tissue cores for each of 2 time points (8 and 12 week tissues on one microarray and 18 and 22 week tissues on another), representing duplicate or triplicate samples per mouse. Three CD-1 embryonic day 16 tissues, two post-natal day 1 tissues, and two post-natal day 5 (P5) SMG tissues were used as positive controls for IF on each array. Several control tissues were also included on each array so that the edges of the array would contain a variety of tissue types not critical to the study as edge spots were more susceptible to tissue loss through slide processing. The TMAs were designed so tissue types were randomized and asymmetrically placed to aid in section orientation. Each corner of the microarray contained a vastly different tissue type with one corner containing no tissue for ease of orientation by eye when placing the section on the microscope. Tissue blocks were labeled with numbers and these numbers included on a grid template of the microarray. The tissue in the paraffin blocks was circled using the tip of a needle to indicate the location of the tissue we wanted to use to make up the microarray.

103 tissue cores (1.5 mm diameter) were removed from each FFPE block and placed into an acceptor block (containing pre-punched 1.5 mm holes) to generate the TMA, including one
spot with no tissue, (as previously described Gerdes et al., 2013; Nelson et al., 2013) by Pantomics (Richmond CA) (See Fig 5.1). Twenty 5 µm sections were cut from each TMA paraffin block and placed on slides. We selected slides for use in this study based on the criteria that all tissues were present, there was no damage to the wax surrounding the tissues, and none of the tissue samples were curling or rolling at the edges.
Figure 5.1 Construction of a Tissue Microarray

Formalin-fixed, paraffin-embedded tissues are removed from the donor tissue block using a 5 mm punch. The core is then placed into a hollow core in the acceptor block to form the tissue microarray (TMA). This block is then slightly warmed to allow the paraffin block and cores to stick together. The block is then sectioned and 5 µm serial sections are placed on slides. Using the corner of the TMA that does not contain tissue for orientation, the slide is stained with DAPI to mark the nuclei, and the nuclei are imaged using a 5X objective. Several images are stitched together using the microscope software. Each spot is visible by its nuclear stain, and is circled using the microscope software (red circles in bottom panel). The spots are then selected in order for automated imaging starting with the top right spot using a serpentine pattern (marked by arrows in bottom panel), and ending with the blank spot on the lower right corner. The same pattern is used for each round of imaging and the tissue spots are numbered in the order in which the images are taken.
Table 5.4 Mouse TMA 1 Layout

Layout of the mouse tissues on the microarray containing 8 and 12 week samples (TMA 6 and 8). Each TMA layout was designed to avoid placing all of one tissue type together, with few SMG tissues at the edges to avoid tissue loss of all one type, and with 3 different tissue types in the corners for orientation, including one corner with no tissue.
### Table 5.5 Mouse TMA 2 Layout

Layout of the mouse tissues on the microarray containing 18 and 22 week samples (TMA 7 and 9). Each TMA layout was designed to avoid placing all of one tissue type together, with few SMG tissues at the edges to avoid tissue loss of all one type, and with 3 different tissue types in the corners for orientation, including one corner with no tissue.

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Table 5.6 Microarray Spot Number Guide

Each tissue spot on each microarray was given a number by the computer software based on the order in which the images were taken. These spot numbers were embedded in the titles for each image taken for that spot for ease of use. Both mouse tissue microarrays were number in the same way with the TMA number (also in each image name) to designate which TMA the image had come from.
5.2.4 Human Tissue Array Construction

The human TMA was designed by Deirdre Nelson and contained 15 labial salivary gland autopsy tissue spots, eight non-SS tissues spots, and 16 SS tissue spots, with one to three cores used from each patient (Table 5.7 A&B). Four adult CD-1 mouse SMG tissue spots were also included in the human tissue TMA as positive control tissues for immunofluorescence (IF). This array was constructed by Pantomics as previously described.
Table 5.7 Human Tissue Microarray Layout and Spot Numbers

(A) The layout of human tissues in the microarray using the patient identifiers (TMA 5), assigned for ease of use. (B) Spot numbers used in the image file names corresponding to each tissue spot on the microarray.
5.2.5 Tissue Microarray Preparation Multiplexed Immunofluorescence and Imaging

TMA slides were baked at 60°C for 1 hour, then deparaffinized (Histochoice, 100% ethanol, 95% ethanol, 70% ethanol, twice each for 10 minutes per incubation then 1XPBS, 1XPBS-0.1% triton, 1XPBS, also each 10 minute incubations), and subjected to antigen retrieval, blocking (10%BSA in 1XPBS-0.5%Tween-20, 20% Donkey Serum), and subjected to multiplexed immunofluorescence, using MultiOmyx methods, as previously described (Gerdes et al., 2013; Nelson et al., 2013). Briefly, slides were incubated in DAPI for 8 minutes, and then mounted with glycerol mounting media. They were then placed on the microscope and a 1.25X image set was captured of the entire slide. Each tissue spot was identified in the order in which the software would take each image, and a set of 20X images and 40X images were collected for the DAPI, Cy2, Cy3 and Cy5 channels. The slide was soaked, tissue side down in an excess of 1XPBS until the coverslip floated away, then the slide was rinsed in fresh 1XPBS, and incubated overnight in primary antibodies at 4°C in a humidified chamber. The next day, the slides were rinsed with fresh 1XPBS, and incubated in secondary antibodies for 1 hour at room temperature in a dark humidified chamber. After rinsing and re-mounting, the slide was returned to the microscope, and images were collected in all 4 channels again after verifying correct exposure times for both Cy3 and Cy5. Images were taken at the same exposure time for every round in Cy2 and DAPI. After the images were collected, the slide was again soaked to remove the coverslip, and the slide rinsed. To remove the signal from the first round of staining, the tissues were exposed to a bleaching solution to chemically inactivate the CY dyes. The effectiveness of this procedure was validated each round with an additional set of images following mounting.
This process was repeated with pairs of directly conjugated antibodies (1 hour incubation in a dark humidified chamber at room temperature) until all antibodies desired were imaged.

Images were collected from each of 104 spots on each mouse TMA slide and 44 spots on each human TMA slide using a fully automated Olympus IX-81 microscope with a 20X Plan Apo 0.75 NA objective or a 40X UPlan SApO 0.95 NA Objective using software developed by GE Global Research, as previously described (Gerdes et al., 2013; Nelson et al., 2013).

5.2.6 Multiplexed Immunofluorescence Antibodies Used

Both mouse and human TMA slides were subjected to multiplexed immunofluorescence with antibodies to detect the proteins listed in Tables 5.8 and 5.9 respectively, following the workflow seen in Figure 5.2.
Table 5.8 Mouse TMA Antibodies List

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Chanel</th>
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Table 5.8 Mouse TMA Antibodies List

All antibodies used on the Mouse tissue microarrays, including protein, species of the primary antibody, the company and catalog number for each antibody (if available), the channel in which it was used, the Larsen Lab designation for the direct conjugates made for our use by General Electric (GE), and the dilution of the antibody are listed here.
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<th>Catalog Number</th>
<th>Chanel</th>
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</tbody>
</table>

Table 5.9 Antibodies Used for the Human TMA

All antibodies used on the Human tissue microarray, including protein, species of the primary antibody, the company and catalog number for each antibody, the channel in which it was used, the Larsen Lab designation for the direct conjugates made for our use by General Electric (GE), and the dilution of the antibody are listed here.
Protein 1

Collect Images

Inactivate Fluorophore

Protein 1

Collect Bleached Images

Apply Directly conjugated antibody to protein 2

Protein 2

Collect Images

Overlay Images with Image Processing Software
Figure 5.2 Multiplexed immunohistochemistry Workflow

Multiplexed Immunohistochemistry (IHC) begins with indirect antibodies specific for a protein of interest (protein 1), followed by a secondary antibody specific for the species of the primary antibody. After staining, the samples are imaged, then the fluorophore is chemically inactivated, and the samples are re-imaged to ensure removal of any signal. The samples are then re-stained with another antibody for a protein of interest (protein 2) using a primary antibody directly conjugated to a fluorophore, images are collected and the process is repeated. Following several rounds of imaging, the individual images can then be aligned and overlaid using image processing software.
5.2.7 Virtual Hematoxylin and Eosin (H&E) images

DAPI and background images were processed by GE Global Research (Niskayuna, NY) to construct virtual H&E images (VH&E). Nuclei were pseudo colored purple and non-nuclear tissues pseudo colored pink to create a hematoxylin and Eosin-like image, as previously described (Gerdes et al., 2013; Nelson et al., 2013).

5.2.8 Fluorescent Image Processing for Qualitative Analysis

Images were manually examined to ensure the tissue sample remained intact and was in focus prior to image processing and quantification. Black and white images captured using an IX-81 Olympus microscope were adjusted using Adobe Photoshop CS6 (version 13.01x64). Each set of images was optimized independently for presentation by adjusting the levels (levels were adjusted identically for each image showing the same stain). Images were resized to 600dpi, 0.75 inch square images prior to assembly into composite figures in Photoshop. Multiple channel overlaid images were constructed by linking the stained image to the DAPI image acquired at the same time, manually registering the DAPI images, eliminating the black pixels from each image, and pseudocoloring each channel within the overlay.

Focal infiltrate measurements were performed using the distance measurement tool in Photoshop CS6 using calibrated images. Graphs were constructed using the average foci diameter for each mouse in Prism (GraphPad).
5.2.9 Fluorescent Image Quantification

Immunofluorescence images were processed for pixel quantification using freeware ImageJ (version 1.47) to quantify the number of pixels present in each image by Kara DeSantis with assistance from Nicholas Pagendarm. For the mouse TMA, samples were excluded from quantitative analysis if less than 50% of the imaged area included SMG tissue. Background subtraction was performed on each image to be used prior to quantification with rolling ball background subtraction with a value of 50 pixels. In order to quantify only epithelial areas within the NOD tissues, a blinded counter identified five square regions of interest (ROI) measuring 225 by 225 pixels within ECAD-stained areas of each tissue sample using the fixed ROI selection function in ImageJ. The total number of pixels for each stain of interest within each ROI (five per image) was imported into Microsoft Excel. Total pixel counts per ROI were averaged per image (five ROIs per image), and then averaged per mouse (two to six spots per mouse). Results were graphed together with a calculated standard error of the mean (SEM) based on the number of mice included in the group. Statistical analyses were completed using freeware (VassarStats) to perform a two-tailed Student’s t-test using the average pixel counts from images from individual mice. Images of human tissues could not be quantitatively analyzed due to the small number of heterogeneous tissue samples.

Red and green channel overlaid images were processed for quantification using freeware ImageJ 2 (FIJI version 1.49J10) (Schindelin et al., 2012) to quantify the number of red, green and yellow pixels present in each image overlay. Images were registered using the FIJI registration function called “linear stack alignment with SIFT”. Background subtraction was performed on individual raw images prior to quantification using the “background subtraction”
function with a value of ten pixels, followed by manual thresholding to match the original image. Three color overlay images were made using the “color merge” function. Image quantification was completed in ROIs (selected as previously described, using the “fixed region of interest selection” function) sequentially using the “Color Image 3D” histogram function. The total number of pixels within each ROI was imported into Microsoft Excel. Total pixel counts per ROI (five per image) were averaged, and then averaged per mouse (two to six spots per mouse). Results were graphed together with a calculated standard error of the mean (SEM) (GraphPad). Statistical analysis was completed using VassarStats where a two-tailed Student’s t-test was performed using the averages of the individual mice. Images of human tissues were not quantitatively analyzed due to the small number of heterogeneous samples.

Epithelial area quantification was performed using ImageJ 2, FIJI. To quantify only epithelial areas within the NOD tissues, a blinded counter identified five square regions of interest (ROI), in ECAD⁺ areas, as described above. Within each ROI, ducts positive for K7 and acini positive for Aqp5 were manually encircled using the “freehand selection tool”. For each ROI, positively staining areas (um²) were measured for each marker and the values were exported into Microsoft Excel, and then graphed using Prism (Graphpad). Ductal areas positive for K7 and acinar areas positive for Aqp5 were summed separately per ROI, representing the total epithelial area positive for each marker. Summed ROI area values were averaged per image (five ROIs per image), and then averaged per mouse (two to six spots per mouse).
5.3 RESULTS

5.3.1 Immune Focal Infiltrates are Not Accompanied by Gross Structural Changes in the NOD/ShiLtJ Epithelium.

To compare structural differences between the submandibular salivary glands (SMG) from the Aec1/Aec2 and the parental strain, C57Bl6/6J, we embedded samples in a tissue microarray (Figure 5.1) and stained the tissue microarray for DAPI and ECAD (Figure 5.3) and overlaid the stains. In these histological studies, we compared the structure and organization of the submandibular salivary gland (SMG) epithelium from male NOD-Aec1/Aec2 mice at 8 and 12 weeks to age-matched female parental strain control mice (C57BL/6J). No significant difference was found in the structure of the epithelial tissue at either 8 or 12 week sample. Additionally, we detected no evidence of lymphocytic infiltrates by comparison of the DAPI-stained images. Our collaborator was not able to provide us with any additional later stage samples since the mice were not breeding efficiently. Since the Aec1/Aec2 mouse model was not commercially available, breeders were not available, and no phenotype was detected in the samples that we were provided, no further analyses of this mouse strain were performed.

After much deliberation and discussion with other groups currently using the Aec1/Aec2 model, we decided to switch our focus to a model of secondary Sjögren’s syndrome that was commercially available and much less difficult to maintain, the NOD/ShiLtJ mouse model. In a structural analysis of NOD/ShiLtJ and CD-1 mouse SMG, we compared virtual hematoxylin and eosin (VH&E) images, prepared from representative samples derived from glands of six of each strain of mice at 12, 18, and 22 weeks of age. Images from 8 week NOD/ShiLtJ and CD-1 samples for each stain set are shown in Figure 5.4; however, no quantitative analysis was
conducted based on the lack of morphological changes in the NOD/ShiLtJ samples at this time point. In the NOD/ShiLtJ SMG samples (Figure 5.4), large accumulations of densely packed nuclei were clearly discernible in diseased tissues but were not found in the CD-1 samples. We also prepared VH&E images from available human labial salivary glands derived from SS, non-SS and autopsies. Similar nuclear accumulations were present in the SS labial glands but were absent in human autopsy tissues with small aggregates of densely packed nuclei present in the non-SS patient samples (Figure 5.5). Using immunofluorescence (IF) for both the mouse and human tissues, we confirmed that the areas of densely packed nuclei were E-cadherin (ECAD) negative (Fig. 3 and Fig. 4). Nuclear accumulations within the NOD/ShiLtJ tissues were positive for CD45R, a cell surface protein found on most B cells and some T-cells (Figure 5.6A). CD45R+ lymphocytic infiltrates were identified in the NOD /ShiLtJ tissues as early as 12 weeks, while no CD45R+ cells were identified in the CD-1 control samples at any age. No CD45R+ infiltrates were found in the 8 week NOD/ShiLtJ tissues (data not shown). To quantify the lymphocytic focal infiltrates in the NOD/ShiLtJ tissues, infiltrates were measured at their widest point using the nuclear stain and CD45R localization pattern. In the NOD/ShiLtJ tissue samples, lymphocytic infiltration size shows a significant increase with disease progression between 12 and 18 weeks and a non-significant increasing trend between 18 and 22 week old samples (Figure 5.6B). Surprisingly, examination of the parenchymal tissues both proximal and distal to the lymphocytic infiltrations revealed that the epithelium in the NOD/ShiLtJ SMG tissues was very similar to the control SMG tissues at all ages, with no disruptions in overall tissue structure, according to VH&E (Figure 5.4) and ECAD (Figure 5.6A) images. To confirm lymphocytic infiltrations in the human tissues, we used an antibody to detect CD4, a T helper cell marker
(Figure 5.7A). The large accumulations of nuclei in the SS tissues as well as some accumulations within the Non-SS samples were CD4+, and a few CD4+ cells were found scattered throughout the autopsy labial salivary gland samples. The infiltration size measurements from the SS tissue samples show a significantly larger focal infiltrate size as compared to the non-SS tissues (Figure 5.7B). No focal infiltrates were detected in any of the human autopsy tissues samples (data not shown). These data confirm disease progression in the NOD/ShiLtJ mice, demonstrate that the infiltrates are less heterogeneous in this mouse model than in SS patients, and demonstrate there is no widespread disruption of epithelial tissue structure up to 22 weeks of age in the NOD mouse model. To investigate changes within specific epithelial cell subpopulations, we examined several markers for specific epithelial cell types with in the salivary gland.
Figure 5.3 Lack of Morphological Changes Detected in the Aec1/Aec2 Mouse Model at 8 and 12 weeks

DAPI and ECAD images were overlaid and showed no major structural or morphological changes in the epithelium of the SMG at 8 and 12 weeks of age when compared to age matched control female C57Bl/6J mice.
Figure 5.4 NOD/ShiLtJ and CD-1 Mouse Submandibular Salivary Gland Morphology

Mouse SMG virtual H&E stains for two representative tissue samples from 12, 18, and 22 week old CD-1 (healthy control) and NOD/ShiLtJ female mice demonstrating increasing focal infiltrates with disease progression (indicated by black arrows). 12 weeks CD-1 n=6, 12 week NOD n=6, 18 week CD-1 n=6, 18 week NOD n=5, 22 week CD-1 n=5, 22 weeks NOD n=5. Scale Bar, 100 μm.

(As seen in: Gervais et al., 2015).
Figure 5.5 Human SS, Non-SS, and Autopsy Labial Salivary Gland Morphology

Human labial salivary gland virtual H&E stains for three representative samples from autopsy (control), Non-SS patients (not diagnosed with SS due to lack of presence of autoantibodies), and diagnosed SS patients, demonstrating increased focal infiltrates in SS diagnosed patients (indicated by black arrows). SS, n=8, (average age 60, all female patients); non-SS, n=6 (average age of 52, all female patients); and autopsy, n=7 (average age 65, 5 male and 2 female patients). Scale Bar, 100 µm. (As seen in: Gervais et al., 2015)
Figure 5.6 Lymphocytic Infiltrates in NOD/ShiLtJ SMG

A. Nuclear accumulations were identified as lymphocytic infiltrations using an antibody targeting CD45R (red) in the NOD/ShiLtJ mouse SMG. Areas of infiltrate are positive with DAPI staining (blue) but are not positive for the epithelial marker, E-cadherin (ECAD) (green). Scale bar, 100 µm. B. Lymphocytic infiltrations were measured at their widest point. Progressive increases in largest lymphocytic infiltration size are apparent between 12, 18, and 22 weeks in the NOD/ShiLtJ tissues; no infiltrations were detected in CD-1 tissues at any age. (As seen in: Gervais et al., 2015)
Figure 5.7 Lymphocytic Infiltrates in Human Labial Salivary Glands

A. Lymphocytic infiltrations were identified using an antibody targeting CD4 (red) in the human SS tissues. Areas of infiltrate are positive with DAPI staining (blue) but are not positive for the epithelial marker, ECAD (green). Scale bar, 100 µm. B. Lymphocytic infiltrates were measured at their widest point. Measurements of infiltrates in human SS samples had a wide range of sizes, while few Non-SS tissues contained infiltrates; No infiltrates were found in any autopsy tissues. (As seen in: Gervais et al., 2015)
5.3.2 Unchanged Basement Membrane and Secretory Markers in the NOD/ShiLtJ

Epithelium

Due to the large disruptions in the epithelium by the lymphocytic infiltrations with disease progression in the NOD/ShiLtJ between 12 and 22 weeks, we hypothesized that there would be significant disruptions to the basement membrane (BM) or specialized extracellular matrix known to surround the secretory acinar units within the epithelium, especially in areas directly adjacent to the focal infiltrate. Previous studies have indicated an increase in matrix metalloproteinases (MMPs), an enzyme responsible for the breakdown of basement membrane and extracellular matrix proteins, in prenatal development of the SMG and in adult SMGs in other SS disease models (Cha et al., 2001) further supporting the idea that the BM may be disrupted with disease progression in the NOD/ShiLtJ. There is also evidence of significant disruption in the BM in the lachrymal gland with SS disease progression in male mice (Schenke-Layland et al., 2008). We stained the mouse and human microarrays for both laminin-111 and collagen IV (Figure 5.8, human data not shown). The high degree of heterogeneity in both the Laminin-111 and Collagen IV stains in all of the mouse and human samples made a thorough analysis of these data impossible, which is likely due to sensitivity of the antibody epitopes to paraffin-processing. Neither of these markers showed any clear trends with disease progression or between the healthy and disease samples, but neither marker was excluded from the areas directly adjacent to the lymphocytic foci in either the mouse or human disease tissues samples. These data indicated that the epithelial tissues directly adjacent to the lymphocytic foci may be unaffected by the growth and progression of the foci. Neither the collagen IV nor laminin-111
immunohistochemical staining was successful on the human tissue microarray (data not shown).

We also attempted to examine two secretory markers, Mucin 10 (Muc10) and salivary androgen binding protein α (SABPA), both proteins secreted by the submandibular salivary gland acinar cells that we hypothesized might decrease with disease progression (Figure 5.9). We expected that as the production of saliva decreased (not measured in this study, but previously shown to be decreased by 16 weeks, (Lodde et al., 2006), we would see a marked decrease in the staining for Muc10 and SABPA. Although the Muc10 staining was inconsistent across the CD-1 tissues, no clear trend was found. These results made analysis of the NOD/ShiLtJ samples impossible with no control samples for comparison. There seems to be an increase in the Muc10 staining in the NOD SMG when compared to the heterogeneous staining in the CD-1 tissues at each time point, though these results could not be quantified due to the high degree of heterogeneity and high level of background. The SABPA stain was also inconsistent, but in a different way. While the Muc10 stain varied between samples of the same condition, the SABPA stain was variable within single tissue sections. These staining artifacts also rendered this set of data useless for quantitative analysis. The antibody for SABPA was not applied to the human TMA since SABPA is not represented in the human genome, and the staining for Muc10 was also very heterogeneous (data not shown).
Figure 5.8 Heterogeneous Staining for Basement Membrane Proteins in Mouse SMG TMA

CD-1 and NOD/ShiLtJ mouse SMG tissue sections were subjected to fluorescent immunohistochemistry for laminin-111 and collagen IV (both green), together with DAPI staining (blue) for nuclei. Staining for both basement membrane markers was inconsistent within tissues and between tissue samples. Scale bar, 100 µm.
**Figure 5.9 Inconsistent Staining for Secretory Proteins in Mouse SMG.**

CD-1 and NOD/ShiLtJ mouse SMG tissue sections were subjected to IF for Mucin 10 (Muc10) and salivary androgen binding protein α (SABPA)( both green), together with DAPI staining (blue) for nuclei. Staining for both secretory proteins was inconsistent within tissues and between tissue samples and SABPA is particularly prone to edge effect staining. Scale bar, 100 µm.
5.3.3 The NOD/ShiLtJ Epithelium Exhibits a Decrease in Ratio of Acinar to Ductal Tissue

Currently, there is disagreement as to whether there are changes in the distribution of the Aqp5+ acinar cell population in Sjögren’s Syndrome and in SS mouse models (Beroukas et al., 2001; Konttinen et al., 2005; Soyfoo et al., 2007; Steinfeld et al., 2001; Wang et al., 2009). Aquaporin 5 (Aqp5) is a water channel protein found at the apical surface of polarized secretory acinar cells within the salivary gland epithelium (Ma et al., 1999; Gresz et al., 2001; Larsen et al., 2011; Nelson et al., 2013). We observed that Aqp5 is expressed throughout the secretory acinar epithelium, with no apparent differences in the distribution of Aqp5+ acinar cells in the NOD/ShiLtJ tissue relative to control tissue (Figure 5.10A). To quantify levels of Aqp5, we randomly selected epithelial ROIs and quantified the total number of Aqp5+ pixels within these areas. We observed a slight decreasing trend in the levels of Aqp5 in the NOD/ShiLtJ samples at both 18 and 22 weeks of age when compared to CD-1 age matched controls (Figure 5.10B). We also performed immunofluorescence to detect Aqp5 in human labial gland biopsy samples (Figure 5.11A). We found very heterogeneous expression of Aqp5 within all groups of human tissues; quantification of Aqp5 did not show significant differences between SS, non-SS, and autopsy samples (data not shown). To investigate changes to the ductal epithelium within the SMG of NOD/ShiLtJ mice, we used antibodies specific for cytokeratin 7 (K7). Cytokeratin 7 is an intermediate filament protein that is expressed by the apical cells of the stratified ductal epithelium which has been used previously as a ductal marker in the SMG (Knosp et al., 2012; Nelson et al., 2013). We observed that K7 is expressed by ductal cells throughout the epithelium in both the NOD/ShiLtJ tissue and control tissues (Figure 5.10A). Although focal
infiltrates were frequently periductal, the structure and cellular localization of the K7 staining seemed unaffected by the proximity to the foci. In the NOD/ShiLtJ tissues, however, increased K7 staining was observed at both 18 and 22 weeks as compared to age-matched CD-1 control SMGs. No differences were detected at 12 weeks (data not shown). To quantify levels of K7 we randomly selected epithelial ROIs and quantified the total number of K7+ pixels within these areas, where we detected increased levels of K7 staining in the NOD/ShiLtJ tissues as compared to the CD-1 controls (Figure 5.10C). We also performed IF to detect K7 in human tissue samples and observed no obvious changes in K7 localization patterns correlating with SS (Figure 5.11A).

We also did not observe any quantifiable changes in K7 staining in the human SS tissues relative to non-SS and autopsy tissue samples (data not shown). Given the significant increase in levels of K7 and the decreasing trend in Aqp5 levels in the NOD/ShiLtJ mice at both 18 and 22 weeks, we questioned whether there was a change in the relative ratio of acinar tissue to ductal tissue. To quantify this, we compared the surface area occupied by Aqp5+ acini and K7+ ducts within ECAD+ epithelial areas. We identified a statistically significant increase in the K7+ surface area of SMG epithelium in the NOD/ShiLtJ mouse relative to the CD-1 control that was mirrored by a coordinate decrease in the Aqp5+ epithelial area (Figure 5.10D). This change in the composition of epithelial area is independent of the epithelial cell population that is observed to be replaced by lymphocytic infiltrates with advancing disease progression in both the NOD/ShiLtJ mouse model and the human SS tissues. These results indicate that there is a decrease in the ratio of secretory acinar cell to ductal epithelial cell surface area in the NOD/ShiLtJ SMG relative to control SMG, which may be a contributing factor to the reported decrease in saliva production in this mouse model (Lodde, et al., 2006; Soyfoo et al., 2007).
Figure 5.10 Significant Increase in K7-Positive Ductal Population in the NOD/ShiLtJ SMG

(A) NOD/ShiLtJ and CD-1 mouse SMG tissue sections were subjected to IF for the ductal marker, K7 (green), acinar marker aquaporin 5 (Aqp5) (red), and epithelial marker Na/K-ATPase (gray) together with DAPI staining (blue) for nuclei. A significant increase in K7 and slight decrease in Aaqp5 correlating with disease progression was observed in the NOD/ShiLtJ mouse samples as compared to the CD-1 control tissues. Scale bar, 100 µm. (B) The number of pixels were measured within regions of interest (ROI) for K7 and (C) aquaporin 5. (D) The epithelial area (µm²) covered by K7 or Aqp5 staining was measured in 22 week samples by quantifying the
number of pixels within the image for each stain. A significant increase was seen in the percentage of the epithelial area covered by ductal cells as compared to acinar cells. *p ≤0.05, ** p≤0.01, and *** p≤0.001. Samples compared included CD-1 18 week (n=6), NOD/ShiLtJ 18 week (n=5), CD-1 22 week (n=5), and NOD/ShiLtJ 22 week (n=5). (As seen in: Gervais et al., 2015, graphs provided by Kara DeSantis and Nick Pagendarm).
Figure 5.11 Immunofluorescence of Human Labial Salivary Glands for K7, Aqp5, K5, and SM α-Actin

(A) Human labial salivary gland samples were stained for K7 (green) to mark the ductal population, which seems to increase in the SS samples, aqp5 (red) to mark the acinar population, and Na/K-ATPase (gray) to mark the epithelial areas of the tissue along with DAPI (blue) to mark nuclei. (B) Human salivary gland samples were also subjected to staining for K5 (green) to mark the putative progenitor population and SM α-Actin (red) to mark the myoepithelium along with DAPI (blue) to mark nuclei. Areas of the epithelium co-positive for K5 and SM α-Actin (yellow) were found in autopsy tissues, and in some of the Non-SS and SS
tissues. SS, n=8, (average age 60, all female patients); non-SS, n=6 (average age of 52, all female patients); and autopsy, n=7 (average age 65, 5 male and 2 female patients). Scale bar, 100 µm.
(As seen in: Gervais et al., 2015).
5.3.4 The NOD/ShiLtJ Epithelium Exhibit an Increase in K5 Expression in the Myoepithelium and Basal Ductal Cells

In both the mouse SMG and the human labial salivary gland, myoepithelial cells surround the acini and some of the ducts by extending long projections that wrap around the structures (Doggett et al., 1971; Redman, 2008; Amano et al., 2012). Myoepithelial cells were identified using an antibody to detect smooth muscle α-actin (SM α-actin) in regions of the tissue that were also positive for ECAD. In the NOD/ShiLtJ tissue samples, when compared to CD-1 control samples, the localization of SM α-actin was largely unchanged, including in the immediate vicinity of large focal infiltrates (Figure 5.12A). We also noted the overall unchanged localization of two other markers of the myoepithelium, Calponin and Sm22alpha (SM22α) in the NOD tissues when compare to the CD-1 controls (Figure 5.13), also with no changes in the epithelial areas directly adjacent to the lymphocytic foci. Due to the marked similarity between these three markers, we continued our investigations with only SM α-Actin. When the levels of SM α-actin staining were quantified, however, a slight increase in overall SM α-actin staining was seen at 18 weeks and a significant increase in SM α-actin was seen at 22 weeks in the NOD/ShiLtJ, as compared to age-matched CD-1 control samples (Figure 5.12B). In the human labial salivary glands we observed no obvious changes in SM α-actin+ myoepithelial cell localization (Figure 5.11B); however, quantification of levels of SM α-actin showed decreased levels in both the non-SS and SS tissues as compared to autopsy (data not shown). Since a subset of SM α-actin+ myoepithelial cells also express cytokeratin 5 (K5) in mouse SMG (Nelson et al., 2013) and mammary glands (Gusterson et al., 2005), we examined whether a subset of the expanded SM α-actin+ population may also express K5. Close comparison of the K5 and SM
α-actin staining patterns within the NOD/ShiLtJ SMG and control samples revealed a seemingly higher incidence of K5 and SM α-actin co-localization in the myoepithelium of NOD/ShiLtJ than control tissues at both 18 and 22 weeks (Figure 5.12A, yellow regions). To quantify SM α-actin⁺/K5⁺ overlap, we examined ROIs of the epithelial regions excluding areas containing large ducts (identified using ECAD). An increase in SM α-actin/K5 co-positive epithelial regions was identified in the 22 week NOD/ShiLtJ compared to age-matched controls (Figure 5.12D). We also quantified the SM α-actin⁺/K5⁻ (Figure 5.12E) and SM α-actin⁻/K5⁺ (Figure 5.12F) populations within these ROIs in the epithelium outside of large ductal areas, where we found that SM α-actin⁺ myoepithelial cells did not always express K5, but most of the K5⁺ cells do also express SM α-actin thus identifying them as myoepithelial cells. In some human patient samples SM α-actin/K5 co-positive cells were detected, however, more commonly we saw a loss of SM α-actin staining in the human diseased tissues (Figure 5.11B). Interestingly, we also observed an increase in K5⁺ basal cells in the large ducts that are not myoepithelial cells (Figure 5.12A). Quantification of the K5 staining in the NOD/ShiLtJ showed a slight increase at 18 weeks and a statistically significant increase at 22 weeks when compared to CD-1 controls (Figure 5.12C). In the human SS samples, a similar increase in K5 staining concentrated in the basal cells of the ductal epithelium was also seen (Figure 5.11B), indicating that this feature of SS is recapitulated to some extent in the NOD/ShiLtJ model.
Figure 5.12 Identification of a SM α-actin/K5 Co-positive Cell Population in the NOD/ShiLtJ SMG

(A) CD-1 and NOD\ShiLtJ SMG tissues were stained for K5 (green) and SM α-actin (red) and images were registered and merged with DAPI (blue). An increase in SM α-actin/K5 co-positive areas were detected in 18 and 22 week NOD/ShiLtJ SMGs (yellow). (B-F) Numbers of pixels were measured in regions of interest (ROI) within the epithelium of mouse NOD/ShiLtJ and CD-1 SMG for (B) SM α-actin, and (C) K5 and (D) SM α-actin/K5 co-positive (yellow). Total pixel levels for single positive cell populations were also quantified (E) SM α-actin+/K5- and (F) SM α-actin-
/K5+. CD-1 18 and 22 week n = 5, NOD 18 week n=6, NOD 22 week n=5. *p ≤0.05, and **
p≤0.01. All scale bars, 100 μm. (As seen in: Gervais et al., 2015, graphs provided by Kara
DeSantis and Nick Pagendarm).
Figure 5.13 Staining for Markers of the Myoepithelium in Mouse SMG

CD-1 and NOD/ShiLtJ mouse SMG tissue sections were subjected to IF for Smooth Muscle 22 alpha (SM22α) and Calponin (both green), together with DAPI staining (blue) for nuclei. Staining for both myoepithelial cell markers very closely mimics SM α-Actin staining and no further analysis of these data was performed. As with the SM α-Actin staining, there seems to be an increase in both markers at 22 weeks in the NOD/ShiLtJ SMGs when compared to control CD-1 SMGs. Scale bar, 100 µm.
Figure 5.14 Immunofluorescence of 8 Week Mouse Submandibular Salivary Glands for B220, ECAD, K7, Aqp5, K5, and SM α-Actin

Mouse SMG samples were stained for DAPI to mark the nuclei in all images, ECAD (green) to mark the glandular epithelium, (A) B220(CD45R) (red) to mark B cells; (B) K7 (green) to mark the ductal population, Aqp5 (red) to mark the acinar population, and Na/K-ATPase (gray) to mark the epithelial areas of the tissue; (C) K5 (green) to mark the putative progenitor population and SM α-Actin (red) to mark the myoepithelium bottom panel. Areas of the epithelium co-positive for K5 and SM α-Actin (yellow) were found in both CD-1 and NOD/ShiLtJ tissues. There are no
marked changes in any of these markers at 8 weeks so further analysis of tissues from 8 weeks was not performed. Scale bar, 100 µm.
5.4 DISCUSSION

Here we have investigated changes within cell populations within two mouse models of Sjögren’s syndrome. In our original study of the Aec1/Aec2 mouse model of primary Sjögren’s syndrome, the male SMG samples showed no SS phenotype at 8 or 12 weeks of age as seen by the lack of lymphocytic foci in the 12 week tissues (Figure 5.3). It has been previously shown that the SS SMG phenotype in the male mice is delayed compared to female mice (Nguyen et al., 2006). The difficulties obtaining the Aec1/Aec2 mice and the delayed phenotype deterred further study using this model. We instead switched to a model of secondary Sjögren’s Syndrome, which is more prevalent with human patients (about 60% of diagnoses, (Tincani et al., 2013)).

Here we report several changes to subpopulations within the SMG epithelium in the NOD/ShiLtJ mouse model of Sjögren’s syndrome. Upon confirming that the NOD/ShiLtJ mice have a SS-like immune infiltration we expected to find that, similar to disease progression in the human salivary glands, the epithelial architecture directly adjacent to the lymphocytic infiltrates in the NOD/ShiLtJ tissues would be disrupted, while the epithelium farthest from the foci would remain relatively intact. However, upon closer investigation we found that the tissue architecture up to 22 weeks (as determined by VH&E stains) and the ECAD positive epithelial distributions along with the localization of collagen IV and laminin-111 staining directly adjacent to the lymphocytic foci indicated that there was no visible disruption to the epithelium closest to the foci compared to the epithelial areas further away. Though we would have liked to further investigate changes in collagen IV and laminin-111 in the progression of SS in the NOD tissues, our initial validation of these antibodies in IHC resulted in a high degree of
heterogeneity. We went to great lengths to troubleshoot this particular issue trying multiple different antibodies for both proteins, as well as multiple fixation conditions and multiple tissue samples from both mouse and human donors in an attempt to get a consistent BM stain. However, the stains shown in Figure 5.8 were representative of the wide variety of staining patterns we saw. It is plausible that the high degree of heterogeneity in these stains is due to a degradation of the BM in the progression of SS as is seen in the lachrymal glands ((Schenke-Layland et al., 2008)). However, the control tissues do present a similar challenge and indicate that this may not be the case, at least in the mouse tissues.

We also compared the Aqp5+ acinar, K7+ ductal, and SM α-actin+ myoepithelial populations which were not obviously disrupted in tissue areas directly adjacent to the lymphocytic infiltrations. Our observations that epithelial architecture is not significantly disrupted in the NOD/ShiLtJ mouse up to 22 weeks of age is consistent with another recent report (Mellas, et al., 2014), and suggests that the presence of the advanced lymphocytic infiltrates may not directly disrupt the epithelium at this stage of disease progression. In the human SS affected tissues, we observed that large areas of the epithelium were disrupted surrounding the focal infiltrates and show an amorphous morphology, consistent with other reports (Goicovich et al., 2003; Molina et al., 2006). This difference in epithelial architecture between the mouse model and human disease may indicate that the 22 week NOD/ShiLtJ phenotype does not truly mimic late stage human Sjögren’s Syndrome, and indicates that the later stages of disease progression require further investigation in this model.

Our investigation into the epithelial cell subpopulations led to the identification of several subtle changes within the epithelial compartment of the NOD/ShiLtJ SMG, many of
which were mirrored in the qualitative analysis of the human patient samples. In the NOD/ShiLtJ model we identified a statistically significant decrease in the ratio of the surface area of epithelium comprised of secretory acinar cells/ductal cells, which may contribute to deficits in saliva flow in the NOD/ShiLtJ mouse, though our staining for two secretory markers does not further strengthen this point due to the high degree of heterogeneity of these stains. These data do however suggest that changes in the ductal to acinar area ratios are an early event in the progression of SS in the NOD/ShiLtJ model. The changes we observed in the ratio of epithelial area covered by Aqp5$^+$ acinar cells as compared to K7$^+$ ductal cells are consistent with previous reports of dysplastic ductal expansion in SS (Ihrler, et al., 2000; Konttinen et al., 2006) and non-quantitative studies that identified preferential loss of the acinar population in favor of the ductal population in SS affected salivary glands (Daniels, 1984; Konttinen et al., 2005). We also found a novel increase in the myoepithelial SM α-actin staining in the NOD/ShiLtJ SMGs as compared to controls. In the human samples, consistently decreased SM α-actin was present in the SS tissues as well as non-SS xerostomic controls as compared to autopsy, consistent with previous reports in the NOD/ShiLtJ parotid gland that indicate decreased myoepithelial populations in SS (Nashida et al., 2013). Again, this may reflect that 22 weeks in the NOD/ShiLtJ model is not representative of human late-stage disease, or may indicate gland-specific differences in disease progression since the human biopsies we queried are minor labial glands rather than submandibular glands. We also report an increase in the levels of the basal epithelial marker K5 in a subset of the myoepithelium and in the stratified ducts, consistent with previous reports of expansion of cells expressing basal cytokeratins in labial gland epithelia in SS patients (Palmer, et al., 1986). This may be indicative of an aberrant
differentiation state within the myoepithelium in parallel with expansion of the basal K5 and suprabasal K7 ductal cell expansion, which could lead to the dysplastic epithelium characteristic of more advanced disease in SS patients. Further, since K5+ cells can function as SMG epithelial progenitors (Knox et al., 2010) and myoepithelial cells can participate in regenerating acinar structures in SMG damaged by ductal ligation (Cotroneo et al., 2008), these expanding K5+ cells in the NOD/ShiLtJ model may be indicative of an intrinsic tissue renewal and/or repair mechanism that is activated in the disease state. Thus, anti-inflammatory and/or directed differentiation therapeutic strategies may facilitate an expanding K5+ population to restore function to the regenerating epithelium for SS patients in the future.
5.5 SUMMARY AND CONCLUSIONS

It is clear from these data that what has previously been identified as late stage Sjögren’s syndrome in the NOD/ShiLtJ mouse model (22 weeks), is not consistent with the phenotype seen in diagnosed human patients. The degree of lymphocytic infiltration significantly increased from 8-22 weeks, indicating that there is disease progression during this time, however, there are no major morphological or structural changes seen in the mouse tissues as were seen in the human tissues. However, upon closer investigation of the epithelium, we found that the ECAD-positive epithelial distributions as well as the distribution of Aqp5+ acinar, K7+ ductal, and SM α-actin+ myoepithelial populations were not obviously disrupted, including tissue areas directly adjacent to the lymphocytic infiltrations. Here we also report changes to subpopulations within the SMG epithelium in the NOD/ShiLtJ mouse model of Sjögren’s syndrome. We identified a statistically significant decrease in the ratio of the surface area of epithelium comprised of secretory acinar cells/ductal cells, which may contribute to deficits in saliva flow in the NOD/ShiLtJ mouse and suggest that changes in the ductal to acinar area ratios are an early event in the NOD/ShiLtJ model. We also identified a novel increase in the myoepithelial SM α-actin staining in the NOD/ShiLtJ SMGs as compared to controls. Further study of later stages of disease progression (after 22 weeks of age) may lead to a more complete timeline of the disease progression in these mice, and a clearer picture of what these early changes may imply for the course of disease establishment and progression toward true late stage disease.
Chapter 6: Summary and Conclusions

The studies reported here have investigated both the embryonic development of the mouse submandibular salivary gland (SMG) as well as the progression of Sjögren’s-like disease in the adult NOD/ShiLtJ mouse model. We have identified a role for Par-1b in the maintenance of cellular polarity in the outer layer of SMG epithelial cells early in gland development, including the requirement for Par-1b kinase activity for the maintenance of the organized localization of the basement membrane. The requirement for Par-1b has also been established in both the cytodifferentiation and morphological changes in the differentiation of the myoepithelium in the developing mouse SMG. Further, we have identified Rac1 but not ROCK as a contributor to differentiation of the myoepithelium and showed that Rac1 regulates Par-1b protein levels. We have also identified changes in the submandibular salivary gland of the NOD/ShiLtJ mice, including changes in three myoepithelial markers.

In the embryonic mouse SMG, the communication between the BM and the underlying sheet of polarized epithelial cells is critical for the glands’ continued growth and development. We have shown here that the kinase activity of Par-1b, a conserved, basally localized serine threonine kinase, is regulating the localized organization of the BM surrounding the SMG epithelium. We have also shown that this control over the organized localization of the BM continues throughout development and may be playing a role in the differentiation of CCCs into the myoepithelium. We have shown that loss of Par-1b via siRNA knockdown prevents the vertical compression of the OCCs that occurs as they transition into myoepithelial morphology as well as decreasing the levels of the myoepithelial marker SM α-actin. It is plausible that Par-
1b’s control over the BM localization is required for the morphological changes seen with myoepithelial differentiation.

These studies have also identified Rac1 as an upstream regulator of Par-1b in the differentiation of the myoepithelium. Following Rac1 inhibition or siRNA knockdown, we see a phenotype similar to that of Par-1b siRNA knockdown in the differentiating myoepithelial cells, including the loss of vertical compression and levels of SM α-actin. These results expanded our view of the potential mechanism of differentiation. It is unlikely that Rac1 is directly controlling Par-1b, as the over expression of Par-1b does not completely rescue the myoepithelial differentiation in Rac1-inhibited glands. However, it is likely, given many previous studies that Rac1 is interacting with the apical PAR complex indirectly via Tiam1 (reviewed by (Chen and Zhang, 2013; Taylor et al., 2014)). By inactivating the apical Par complex, Rac1 could be clearing the way for the movement of Par-1b around the lateral and eventually to the apical domain of the OCC. This movement of Par-1b may be the mechanism by which the tight basal restriction of the cell surface receptors for the BM is released, and the receptors migrate around the entire OCC surface. APAR-1b is also likely affecting the microtubules within the polarized epithelial cells during this transition in cell shape to regulate the placement of BM receptors. Since Rac1 directly regulates actin rearrangements, Rac1-dependent actin-mediated cell rearrangements may be required for myoepithelial morphogenesis. This possible mechanism would explain how the highly polarized cuboidal epithelial cells form flattened myoepithelial cells embedded within the basement membrane.

While in developing glands, the myoepithelium seems to be differentiated from the outermost polarized epithelial cell layer, the turnover of myoepithelial cells in a diseased
salivary gland may occur through a slightly different mechanism. Sjögren’s syndrome is an autoimmune exocrinopathy effecting the salivary glands and lachrymal, or tear producing glands. Specific changes to the glandular epithelium leading to the marked decrease in saliva production have thus far not been definitively identified. In order to investigate several markers of the basement membrane and markers of specific cell types, we used the NOD/ShiLtJ mouse model of Sjögren’s syndrome (SS). Using salivary glands harvested from mice at four different time points corresponding to progressing severity of disease, we attempted to identify major changes to the glandular epithelium with disease progression. Though we did not find major changes to the morphology of the gland, we did identify a marked increase in the levels of SM α-actin, a marker of myoepithelial cells. Interestingly, the cells expressing SM α-actin were also expressing K5, a marker of putative progenitor cells. These results taken together may indicate that the diseased salivary gland may be attempting to regenerate a population of cells that are critical for saliva production. It is possible that as in the developing SMG, the myoepithelial cells begin to differentiate first, followed by the differentiation of the acinar cells and the formation of a complete secretory unit. During disease progression, secretory units may be lost due to direct attack by the immune system, apoptosis, or by the replacement of epithelial tissue with lymphocytic infiltration. It is therefore possible that the sections of the submandibular salivary gland most affected by this damage are reverting to the developmental mechanism that originally allowed for saliva secretion. The increased differentiation of myoepithelial cells may be the first step in the salivary glands attempt to reboot differentiation to assemble new secretory units and increase saliva output. This potential mechanism may also explain the high degree of heterogeneity in the basement membrane staining in these tissues. It is likely that the
basement membrane is degraded with the entry of lymphocytic cells, and possible increase in MMPs with the progression of SS. The increase in myoepithelial marker positive cells may also be an attempt to replace the BM that has been lost during the progression of the disease and maintain tissue homeostasis as myoepithelial cells are known to produce BM proteins.

In order to better further link these studies; solidify a mechanism for myoepithelial differentiation and identify patterns of change in the basement membrane, Par-1b and Rac1 in SS, further studies are required. Investigations of localization and levels of integrin and dystroglycan following manipulation of Par-1b and Rac1 will shed light on whether or not the differentiation of the myoepithelium is occurring as we have predicted here. Further investigations of both Par-1b and Rac1 in SS were particularly difficult without antibodies validated for immunohistochemistry in both human and mouse. Further investigations into alternate antibodies and methods of investigation are required to further investigate changes in Par-1b and Rac1 in the Sjögren’s syndrome mouse model.
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