ISOLATION, CHARACTERIZATION, AND FUNCTIONAL ANALYSIS OF
HUMAN SALIVARY GLAND MYOEPITHELIAL CELLS FOR USE IN
TISSUE REGENERATION

by

Daniel R. Zakheim

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fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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Daniel R. Zakheim

Approved:
Randall L. Duncan, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved:
Robert L. Witt, M.D., F.A.C.S.
Professor in charge of thesis on behalf of the Advisory Committee

Approved:
Robin Morgan, Ph.D.
Chair of the Department of Biological Sciences

Approved:
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved:
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education
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LIST OF ABBREVIATIONS

2D: two-dimensional
3D: three-dimensional
α-SMA: alpha smooth muscle actin
ACh: acetylcholine
ATP: adenosine triphosphate
β-AdrR: beta-adrenergic receptor
BM: basement membrane
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
CCh: carbachol
CK14: cytokeratin 14
Col IV: collagen type four
DMEM: Dulbecco’s modified eagle’s medium
ECM: extracellular matrix
epCAM: epithelial cell adhesion molecule
FBS: fetal bovine serum
FN: fibronectin
GAG: glycosaminoglycan
HA: hyaluronic acid
HA-SH: thiolated hyaluronic acid
IP3: inositol triphosphate
LamI: laminin type 1, 1, 1
M3-mAChR: M3 muscarinic acetylcholine receptor
PEG: polyethylene glycol
PEGDA: polyethylene glycol diacrylate
Pln: perlecan
RPMI: Roswell Park Memorial Institute Medium
SMMHC: smooth muscle myosin heavy chain
ABSTRACT

Cancer of the head and neck accounts for nearly 50,000 cases annually, and radiation treatment for these cancers can have deleterious effects on patients. Individuals who undergo radiation therapy to treat head and neck cancer often suffer necrosis of salivary gland acini, the glands fluid-secreting units of the gland. This leads to problems such as severe dry mouth, difficulty swallowing, digesting, and dental caries. The current remedies available to these patients are largely unsatisfactory, so we have proposed the regeneration of salivary glands as a new approach to treating radiation-induced symptoms. During my graduate work, I have reported the isolation of human salivary gland myoepithelial cells, one of the three major epithelial cells types that comprise the gland. These cells have been characterized phenotypically and functionally, and future work will involve their incorporation in our three dimensional system for salivary gland regeneration.
Chapter 1
GENERAL INTRODUCTION

1.1 Salivary Gland Structure and Function

The salivary glands play a crucial role in maintaining oral homeostasis, and aid important processes ranging from digestion and swallowing, to dental health. There are three major salivary glands, each present as a pair; the parotid gland, located anterior to the ear, produces a serous, protein-rich fluid (See Figure 1.1). Secretory units of the parotid gland stain heavily with H&E, and nuclei appear more rounded. The parotid gland is responsible for producing the bulk of saliva during stimulated secretion, with its products making up ~50% of total saliva output while stimulated. Parotid glands produce about 20% of total secreted saliva while at rest. The sublingual gland contributes a mucin-rich fluid to overall saliva content. H&E staining is weaker in these glands, and secretory nuclei tend to appear more flattened due to the dense, mucinous fluid produced by its acini. Submandibular glands found in the ramus of the mandible provide a balanced sero-mucous mix, and as such, are the primary contributor to overall saliva content during non-stimulated secretion (~65% of total output). Also lining the oral cavity and pharynx, 600-1000 minor salivary glands provide additional mucin-rich fluid to supplement products of the major glands.
Figure 1.1 **Schematic of major salivary glands.** The parotid gland can be found anterior to the ear, submandibular gland in the ramus of the mandible, and sublingual gland just beneath the tongue.
The primary protein component of salivary fluid is the glycolytic enzyme alpha-amylase; this protein aids digestion by facilitating the breakdown of complex starches into simple sugars. In addition to the production of digestive enzymes, secreted immunoglobulins and lysozyme in saliva help prevent the accumulation of unwanted bacteria in the oral cavity. Other components of salivary fluid consist of select peroxidases, phosphatases, hydrolases, and dehydrogenases, each of which plays an additive role in digestion of consumed fats and starches. Beyond its critical role in digestive processes and oral immunity, salivary fluid serves as a lubricant to prevent dry foods from getting trapped in and subsequently damaging the mouth. The liquid medium also facilitates efficient binding of gustatory agonists in food to their associated receptors on the tongue; insufficient fluid production by salivary glands hampers an individual’s ability to taste. Dental health is also improved and maintained by the production of salivary enzymes. Foods that get trapped between the teeth are often broken down into smaller constituents and washed from the oral cavity, a process that resists the accumulation of plaque and formation of dental cavities. Taken together, the broad range of processes involved in maintaining oral health, which receive either primary or supplemental support from salivary glands is impressive.

**Salivary Gland Morphology and Major Cell Types**

The salivary glands arise during embryonic development through a process known as branching morphogenesis. Following the protrusion of an epithelial stalk and end bud into the surrounding mesenchyme, and subsequent integration and
communication with proximal stromal, vascular, and neural components, the early salivary glands begin to mature\textsuperscript{64}. Repeated rounds of epithelial cleft formation, progression, and subsequent differentiation of specialized cell types produces a complex branched architecture designed to maximize epithelial surface area for efficient fluid production and secretion. The three major cell types comprising salivary epithelium are acinar cells, which form the secretory units, ductal cells that form ducts for fluid transport, and stellate myoepithelial cells which envelope the secretory units\textsuperscript{21}. The contribution of each of these cell types with respect to salivary gland structure and function will be discussed next in detail.

Acinar cells are responsible for synthesis and secretion of primary saliva. These cells form diverse junctional complexes with one another, and collectively take on a spherical shape in mature tissue. Secretory acinar spheroids are polarized structures, with specialized fluid and ion transport channels localized to appropriate cellular domains for vectorial transport. Fluid and protein must enter the acinar lumen in order to ensure effective transport through the ductal network, a process requiring coordinated, uni-directional secretions\textsuperscript{2}. Fluid and protein secretion by acinar cells is regulated by neural input, both sympathetic and parasympathetic\textsuperscript{39,64}.

Fluid secretion by acinar cells is initiated through the widely conserved mechanism of cytosolic calcium release by activation of muscarinic, adrenergic, and purinergic receptors. Upon neural exocytosis of acetylcholine, norepinephrine, or select purines and subsequent stimulation of basally localized receptors on the acinar
cell membrane, activation of a specific class of Gq-coupled GPCRs takes place. This signaling cascade mediates the release of calcium from ER stores via the conventional phospholipase C (PLC)-dependent production of inositol triphosphate (IP3); IP3 binding its receptor on the ER membrane triggers the release of calcium ions into the cytoplasm, which activate calcium-dependent K+ and Cl- channels on the acinar cell membrane. Cl- influx across the basolateral membrane is driven by a gradient established by basal Na+/K+ exchangers, and ultimately results in intracellular chloride accumulation above its equilibrium potential. Chloride ions subsequently exit the acinar cell through its apically localized channels, followed by Na+ from the interstitium to maintain electroneutrality. It is this net accumulation of NaCl in the acinar lumen that drives fluid flow from the interstitium towards the acinar lumen (See figure 1.2). Primary saliva is then modified further as it travels through the ductal system. With respect to protein production, stimulation of adrenergic neurotransmitter receptors on the basal membrane of acinar cells is the primary driver of protein exocytosis.
Figure 1.2. Ion Flow During Fluid Secretion. This schematic shows important receptors, and respective contributions to ion flow during fluid secretory process by each of these receptors in polarized salivary gland acinar cells (Ambudkar, 2014).
Ductal cells are simple cuboidal in shape, and come together to form hollow tubes called ducts through which saliva is transported from secretory units towards the oral cavity. There are three different classifications of ducts in the salivary gland; intercalated and striated ducts are primarily intralobular and of smaller diameter, while excretory ducts are generally extralobular with larger diameters. The cells that comprise salivary ductal networks perform one primary role in addition to serving as a conduit for fluid flow. NaCl reabsorption by ductal cells takes place continuously during the process of saliva transport towards the oral cavity\textsuperscript{103}. Concomitant with NaCl absorption from the ductal lumen is secretion of KHCO\textsubscript{3} by ductal cells into the lumen. Absorption takes places more rapidly than secretion in ductal cells; hence the final saliva product is a hypotonic solution\textsuperscript{96}.

Myoepithelial cells are the least abundant and most poorly understood of the three major salivary gland epithelial cell types\textsuperscript{64}. Their name derives from a unique dual expression of both smooth muscle and epithelial-specific proteins\textsuperscript{25}; these cells can be highlighted beautifully in tissue however, as the expression of smooth muscle cytoskeletal filaments such as alpha-SMA are limited to the myoepithelium. Found in lacrimal, mammary, and salivary glands, even their localization appears somewhat unique in the salivary gland. Unlike the other exocrine organs in which they are found, myoepithelial cells of the salivary gland stain minimally in the ductal network; their presence in salivary tissue appears specific to secretory lobules, with which they form direct cell-cell contacts.
These cells are large, with only a few capable of wrapping around each secretory unit, and stellate in shape (See Figure 1.3). They are postulated to contract in response to neurotransmitter stimulation as a means of aiding fluid propagation\textsuperscript{59}; while this topic has been addressed in other tissues, there exists no experimental evidence to support such claims about salivary gland myoepithelial cells. They remain a field open to broad future interrogation.
Figure 1.3. **Location of myoepithelial cells in salivary gland tissue.** These cells are large, with multiple cytoplasmic processes extending from the cell body. Myoepithelial cells form contacts with secretory lobules in tissue, establishing cadherins junctions and gap junctions (image adapted from studyblue.com)
Salivary Glands in Head and Neck Cancer: Current Therapies and Treatments

Cancer of the head and neck is a major health issue, accounting for approximately 3% of all malignancies in the United States Annually. There are a few different options available to the oncologist for treating head and neck cancers, which can be used in isolation or combination depending on the nature of the patients’ malignancy. Chemotherapeutics offer one potential approach to combating a tumor, and can target individual cells and inhibit one or multiple processes crucial to their growth, such as DNA replication. Surgery also presents an effective way to remove large masses, but will often leave residual cancerous cells behind. While a number of approaches exist whereby these types of cancers can be combated, threatening malignancies often require some form of radiation therapy. When radiation therapy is delivered to a specific region of the body, high energy x-rays kill many of the cancer cells in the area that is treated, thereby shrinking the size of a tumor and slowing its growth. This is considered a localized form of treatment, as only the specific region of the body designated to receive radiation will be affected. Radiation does not only target cancer cells, however, and is capable of inflicting significant damage to healthy tissue as well; herein lies a major problem.

While efforts are commonly used to combat the deleterious effects of radiation therapy in areas that do not require therapy, damage is often unavoidable in regions of the head and or neck that receive direct exposure. The salivary glands commonly lie in the direct line of treatment, and while ductal structures are relatively resistant to
radiation, acinar cells seem to be particularly sensitive. After a week of radiation treatment, a significant reduction in saliva production is common, with slight variation in overall fluid production taking place during the initial phases of treatment. By the later stages of treatment (radiation can last for 6-8 weeks), radiation delivered to the salivary glands generally causes complete necrosis of the secretory acinar units, leading to severely diminished or even no salivary fluid output. While local nerves are not particularly radiosensitive, acinar cell necrosis can reduce innervation of the gland; this is particularly dangerous, as prolonged denervation has been implicated in irreversible necrosis of the gland. The problems that arise from insufficient saliva output are vast, and can be associated with any of the number of oral health matters with which the salivary glands are associated. Xerostomia, or severe dry mouth, is the most common malady; this can impede processes such as digestion, swallowing, dental health, and speech, among others. The quality of life is drastically reduced in patients who suffer from xerostomia as a result of radiation therapy for head and neck cancers, and few treatments are currently available to ameliorate its broad-reaching side effects.

There are a few common approaches to managing discomforts associated with xerostomia; these include simple remedies such as the constant consumption of water, to the application of artificial saliva substitutes. While effective as lubricants, the therapeutic effects of these agents are ephemeral, and require continuous administration. Some pharmacological agents are available, with primary mechanisms
of action requiring agonistic effects on neurotransmitter receptors that regulate the conventional secretory pathways. Pilocarpine, one common therapeutic agent, derives from the leaves of tropical American shrubs of the genus *pilocarpus*.

The extracted compound acts as a parasympathetomimetic, non-selectively stimulating muscarinic acetylcholine receptors upon topical application. In theory, the use of pharmacological secretagogues seems like it could be a particularly effective approach. However, as discussed previously, radiation therapy usually destroys the fluid-secreting units of the gland. The potential benefits of neurotransmitter receptor agonists disappear with necrosis of the salivary acini\textsuperscript{70}. As such, the search for more effective therapies against radiation-induced xerostomia remains imperative. One promising approach that will be discussed further involves tissue regeneration; this would incorporate the use of patient-derived autologous cells as a means of restoring tissue function after radiation.

### 1.2 Principles of Tissue Engineering: Hyaluronic Acid Hydrogels and Three Dimensional Cell Encapsulation

**Three-dimensional cell encapsulation using Hyaluronic Acid-based Matrices**

A crucial pre-requisite for any tissue-engineered organ is a scaffold that supports survival, proliferation, and differentiation of the cells it is used to house. The scaffold must also be non-immunogenic to its eventual host, and capable of supporting the passive diffusion of oxygen and other small molecules both to and from the site of
implantation\textsuperscript{96}; common scaffolds are often hydrophilic, cross-linkable polymers capable of forming semi-permeable networks to facilitate the removal of toxic waste secreted by cells, as well as entry of nutrients to support their continued growth. An effective building block that has been used for three-dimensional encapsulation of human salivary gland cells is hyaluronic acid (HA), a linear, non-sulfated glycosaminoglycan found ubiquitously in the extracellular matrix of numerous tissues\textsuperscript{35,104}. HA is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine joined by $\beta1,3$ and $\beta1,4$ glycosidic bonds, respectively. As a polymer, HA is unbranched, polyanionic and largely hydrophilic, taking on highly viscous, gel-like properties when hydrated and thermally crosslinked\textsuperscript{18}; thus, HA matrices used for the three-dimensional encapsulation of cells are often given the term “hydrogel”.

**Hyaluronic Acid Biosynthesis and Biological Function**

Hyaluronic acid biosynthesis, as well as its structural and functional nature in living organisms is somewhat unique from other glycosaminoglycans (GAGs). GAGs are primarily synthesized in the golgi, but HA’s synthetic enzymes are localized to the cell membrane. Each isoform contains multiple binding sites for UDP sugars, which serve as the substrate during HA biosynthesis\textsuperscript{45}; UDP-bound HA monomers can sequentially bind HA synthetic enzymes on the inner plasma membrane, facilitating concomitant polymerization and translocation of growing HA chains. Interestingly, HA can function both intracellularly and extracellularly; HA synthases utilize an
endogenous transmembrane pore to thread growing HA chains through the plasma membrane into the extracellular space.

There are three different isoforms of HA synthase (HAS), each responsible in part for the diversity of biological function attributed to their final enzymatic product. HAS1 & 2 are responsible for producing higher molecular weight HA, while HAS3 produces shorter HA chains of lower mass. Variation between the transcriptional profiles of different cell types influences the amount of each HAS isozyme that is ultimately observed\textsuperscript{94}. HA synthases are also susceptible to post-translational modification, with phosphorylation at particular residues altering enzymatic efficiency; adjusting HAS production and catalytic efficiency at multiple levels provides a means to fine-tune the relative abundance, size, and subsequent bioactivity of the molecule in a tissue-specific manner. The diverse modes by which HA influences cell behavior cannot be overstated; coined hyaladherins, a number of cellular transmembrane receptors are capable of binding extracellular hyaluronic acid. CD44 & RHAMM-mediated cell attachments to HA can influence cytoskeletal dynamics during cell migration and proliferation, which is highlighted by HA’s crucial role in development\textsuperscript{18}. HA can also signal through specific toll-like receptor (TLR) isoforms to mediate inflammatory responses, cell survival and apoptosis. The observed influence of HA in a particular tissue is the combinatorial product of differential HA receptor expression, and the concentration and size of HA chains present in its extracellular matrix.
Hyaluronic Acid-Based Hydrogels as a Scaffold for Tissue Engineering Applications

The term scaffold has been defined in a biological context as a temporary structure whose primary role is to support the growth of cells and tissues. As such, a scaffold must possess properties that sufficiently accommodate the processes necessary for cell survival, proliferation, communication, migration and coordinated rearrangement during tissue morphogenesis, to name a few. With these requirements in mind, the scientific community has outlined a number of parameters that should be considered when designing or choosing the appropriate scaffold. First, its surface should promote attachment sites required for growth, as well as initiation and maintenance of differentiated phenotypes. The scaffold must be non-immunogenic and non-toxic, given that it should ultimately be suitable for implantation into humans.

Porosity and mechanical rigidity of a scaffold are of paramount importance; a matrix requires adequate space to facilitate events such as cellular infiltration during neovascularization and innervation (salivary glands are both highly vascularized and innervated), while providing the strength necessary to ensure continued growth of seeded cells from the time of implantation to successful host integration. As a consequence, choosing materials with the capacity for controlled modification are particularly useful when fine-tuning the properties of the scaffold to meet cell type and tissue-specific demands.

Hyaluronic acid is a particularly attractive candidate for tissue engineering applications, because of its endogenous expression and biocompatibility in many
tissues, its broad capacity for functional modification, and relatively simple chemical composition. It is currently being explored in the context of regenerative medicine for a number of tissues \(^{26}\), including dermal filling, osteoarthritis treatment, salivary gland tissue regeneration, and targeted delivery of small molecules. Hyaluronic acid can undergo a variety of functional modifications, primarily on the carboxylic acid moiety of D-glucuronic acid, or hydroxyl group of N-acetyl-d-glucosamine, as a means of generating cross-linkable properties. HA can be photocrosslinked, thermally crosslinked, or crosslinked using freeze-drying techniques, depending on the nature of its chemical modifications. With respect to salivary gland tissue engineering, thiolated HA (HA-SH) has been used in combination with a diacrylated polyethylene glycol (PEGDA) crosslinker for the three dimensional culture of human salivary gland acinar cells\(^{73}\).

Hyaluronic acid functionalized with reactive thiol groups can be combined with mono or di-acrylated small molecules in what is known as a Michaels-type addition, to generate covalently crosslinked hydrogel networks. PEGDA is one potential crosslinker (See Figure 1.4), and has provided a non-cytotoxic method for the three-dimensional encapsulation of primary human salivary gland acinar cells. This specific reaction is accomplished by thermal crosslinking at 37 degrees C; salivary gland acinar cells grown in HA-SH/PEGDA hydrogels were able to migrate efficiently through the network, with encapsulated single cells forming spherical acini-like structures capable of a fluid-secretory response to neurotransmitter stimulation, as
well as synthesis and secretion of the salivary enzyme α-amylase. HA-SH/PEGDA hydrogels permitted the long-term culture of acini-like structures, and provided sufficient protection to maintain their structural integrity when implanted in vivo.
Figure 1.4. **Schematic of HA-SH PEGDA Hydrogel Crosslinking.** This image highlights a Michael’s type addition reaction between HA-thiol and PEGDA used to synthesize hyaluronic acid based hydrogels for three-dimensional cell encapsulation.
Also of great interest, acrylated peptide sequences that are commonly found in the native salivary gland ECM can also be incorporated into HA-based hydrogel networks. This introduces the capacity to provide encapsulated salivary gland acinar cells with physiologically relevant ECM signals without significant alterations to the HA-based matrix that would likely occur if it were crosslinked in the presence of large, bulky ECM proteins. Mono-acrylated peptide sequences bind covalently to modified HA chains, then dangle from the crosslinked HA network in what is referred to as “pendant” incorporation. For example, amino acid sequences such as RGD, YIGSR, IKVAV, which are commonly found in basement membrane proteins such as Collagen IV & Laminin, can provide integrin binding sites within an HA matrix that functionally mimic cell signaling events initiated by basement membrane proteins. In addition, the stiffness of a hydrogel is usually increased upon incorporation of high concentrations of whole ECM proteins. In the context of salivary gland regeneration, encapsulated cells must still proliferate, migrate, branch, and differentiate. These cells will secrete abundant ECM during such processes, increasing the stiffness of and remodeling the original matrix. As such, the starting matrix must be softer than that which cells see in the microenvironment of native tissue; a matrix that is too soft or too stiff can hinder cell viability and dynamics during epithelial organ regeneration. Consequently, the addition of small peptides to a scaffold can be of great utility when trying to maintain simplistic, but instructive matrices for three-dimensional cell encapsulation.
1.3 Mechanisms & Regulation of Smooth Muscle Contraction

The molecular dynamics underlying myoepithelial cell contraction have yet to be investigated, however it is postulated that similar events occur in myoepithelial cells to those in smooth muscle cells. Their expression of contractile machinery and upstream initiators of contractile events mirror one another; as such, this discussion will refer to what is understood in smooth muscle cells as a window into mechanisms regulating myoepithelial cell contractile behavior.

Cellular contractility is regulated by coordinated rearrangement of the actin cytoskeleton by a class of motor proteins belonging to the myosin superfamily. First discovered in highly contractile skeletal muscle\textsuperscript{99}, \textit{in vitro} experiments with myosin revealed the capacity for its head domain to generate actin filament sliding. Both skeletal muscle and smooth muscle myosin have heavy chains that dimerize to form a unit with two force-generating heads. Since its initial discovery, new myosin isoforms have been discovered which function as monomers, primarily in vesicular trafficking events; these distinct isoforms are now known as myosin I & II, reflecting their respective number of associated head domains\textsuperscript{67}. Today, there are at least nine known myosin types capable of yielding at least 30 different isoforms.

The principal molecular motor that regulates cellular contractile events is myosin II. In its functional state, myosin II exists as a hexamer, with two heavy chains of approximately 2000 amino acid residues each and four regulatory light chains.
bound to its neck domain. The globular actin-binding head domain has ATPase activity, and is located at the N-terminus of myosin’s heavy chain, a trait that is evolutionarily conserved among myosin heavy chain isoforms. Interestingly, MHC’s nucleotide-binding site closely mirrors that of the ras superfamily of GTPases, suggesting a common evolutionary origin. The length of myosin heavy chain’s tail domain varies widely across different isoforms, permitting diversification of binding partners with respect to both heavy chain dimerization and specificity of cargo interactions. The precise spacing of hydrophobic residues along extended α-helical domains of the MHC tail promotes a coiled-coil conformation, supporting efficient dimerization with other myosin II tails. The tail regions of myosin II dimers can bundle to form thick filaments with many protruding heads, embodying the quintessential structure of contractile filaments.

The ATPase activity of MHC’s head domain produces energy and conformational changes to the protein that ultimately drive myosin’s “power stroke”, a force which propels the head approximately 5nm towards the + end of its associated actin filament. Concomitant cycling of ATP binding and hydrolysis by thousands of myosin heads drives the cytoskeletal re-modeling required for a smooth muscle cell to contract (See Figure 1.5.).
Figure 1.5. **Acto-myosin crossbridge cycling**: the “power stroke”, which occurs continuously in a contracting cell provides the force required for cytoskeletal remodeling during contraction (Link to image: [http://upload.wikimedia.org/wikipedia/commons/](http://upload.wikimedia.org/wikipedia/commons/))
1.4. The Mammary Gland Myoepithelial Cell

Location and Morphology in Tissue

Of the exocrine tissues in which myoepithelial cells are found, the most thorough characterizations have taken place in studies utilizing mammary glands as a model system. Unlike the salivary and lacrimal glands, which undergo terminal differentiation by early postnatal stages, terminal mammary gland differentiation is unique and reflective of the functional role of the tissue\(^{43}\). While tear film production and salivation are required throughout life, the primary functional role of mammary tissue, lactation, occurs only around the time of pregnancy. As such, the ductal architecture of the mammary gland does not undergo extensive growth until puberty, while alveolar growth and expansion is most prominent during pregnancy\(^{102}\). The mammary gland only reaches its true terminally differentiated state during lactation. The partitioning of mammary myoepithelial progenitors and their role in mammary development will be discussed, but descriptions of functional roles performed by the mammary myoepithelial niche will be limited to what is observed in adult tissue.
Figure 1.6. **Schematic of Mammary Gland Organogenesis.** The pre-pubertal and pubertal mammary epithelium is less branched and comprised of many highly proliferative cap cells, with myoepithelial cells encapsulating both the ducts and terminal buds. B) The pregnant and lactating mammary gland undergoes more extensive branching, ultimately establishing a bilayer composed of luminal ductal or acinar cells surrounded by the mammary myoepithelium, which makes direct contacts with the underlying basement membrane (Gajewska et al., 2013).
The mammary epithelium consists of two phenotypically distinct layers of cells, luminal (ductal and acinar) and basal myoepithelial (See Figure 1.6.). In terminally differentiated mammary tissue, there exists a complex tubular ductal network through which milk is ejected by the secretory alveoli, ultimately reaching the outside the body. Myoepithelial cells are present in both ducts and alveoli, but coordinate differently and acquire visibly distinct morphologies depending on their location in tissue. The ductal myoepithelium forms a continuous monolayer surrounding the luminal compartment, with its cells taking on spindle-like morphologies capable of forming rings around the ducts. Alveolar myoepithelial cells are stellate, and form cell-cell contacts with secretory units as they spread and encircle the alveolus\(^1\). The alveolar myoepithelium does not form a continuous layer, leaving some luminal cells capable of establishing direct contacts with the underlying basement membrane. It has been postulated that expansion of the secretory units during pregnancy and lactation occurs more rapidly than myoepithelial cell proliferation, resulting in the observed discontinuity\(^9\). 

Myoepithelial cells are responsible for basement membrane synthesis, and actively deposit fibronectin, collagen IV, nidogen, and select laminin isoforms. They also express and signal through integrins which serve as basement membrane receptors, including alpha isoforms 1,2,3 and 6, and beta isoforms 1 and 4\(^7\). Integrin expression in myoepithelial cells is significantly higher than in acinar cells, as are cytoplasmic adhesions proteins alpha-actinin and vinculin. Alpha-actinin binds to and
offers support for fibrillar actin filaments, while vinculin can bind the cytoplasmic domains of integrins at the site of focal contacts, anchoring them to the actin cytoskeleton. The robust expression of focal-adhesion-associated proteins in addition to those involved with cytoskeletal support seems appropriate; the myoepithelium likely helps maintain tissue architecture during fluid production and secretion. Further, integrin expression has been shown to diminish in select carcinomas, which has been hypothesized to play a role in tumor invasiveness. In addition to basement membrane adhesions, communication with the underlying stroma takes place primarily through the myoepithelium.

The primary source of cell-cell and cell-ECM attachments by the myoepithelium is desmosomes and hemidesmosomes, respectively (See Figure 1.7.). Characteristic of stratified epithelia, the basal mammary compartment expresses cytokeratins 5, 14, and 17. Specific cytokeratin isoform arrangements in the myoepithelium are an important determinant of tissue architecture, as they provide cytosolic attachment sites for desmosomal adhesions.
Figure 1.7. **Acinar / Myoepithelial Cell Interactions in Glandular Tissue.** (Top) Schematic showing relative position of acinar cells and myoepithelial cells in human breast tissue. Notice that myoepithelial cells are situated between the secretory acini and the underlying basement membrane. (Bottom) Representative adhesions systems and cytoskeletal networks present in the mammary epithelium.
Similar to those present in lacrimal and salivary glands, mammary myoepithelial cells can be highlighted distinctly in tissue due to their expression of smooth muscle contractile elements alpha-smooth muscle actin and smooth muscle myosin heavy chain (SMMHC). These cells are visibly contractile, and contribute to milk ejection and propagation through ducts during lactation\textsuperscript{32}.

**Acquisition of the myoepithelial cell phenotype in mammary tissue**

Luminal and basal epithelial cells of the developing mammary gland begin to take on phenotypically distinct characteristics early during organogenesis. By embryonic day 15 in the mouse mammary gland, the basal cell marker p63 is only present in two to three basally restricted cell layers\textsuperscript{54}. Smooth muscle cytoskeletal and contractile elements, however, do not appear during embryonic development. The expression of SMA and caldesmon, a protein commonly associated with smooth muscle contractility, were generally observed in newborn rats\textsuperscript{17}. It appears that myoepithelial cell differentiation, in rodent studies, begins to take place early in post-natal development as part of a continuous maturation process that lasts through puberty. In the human mammary gland, expression of smooth muscle cytoskeletal elements appears as early as 22 weeks into pregnancy; in addition to the differential time course of smooth muscle phenotype acquisition, other developmental differences have been observed between human and rodent mammary glands. Some of these differences have been attributed to phenotypic disparity between adult rodent and human mammary glands; mature rodent mammary tissue has a significantly larger
population of adipocytes, while human breast tissue appears to have a more fibrous microenvironment\textsuperscript{76}. Given these distinctions, some have attempted to create human mammary cell culture systems as a means of identifying better representative processes in human mammary organogenesis.

Pasic et al (2011) used different extracellular signaling molecules to examine their effects on multipotent progenitor cells in a human mammary organoid model\textsuperscript{63}. The relevance of this system to in situ development was well-established, as ductal morphologies and branched architectures reminiscent of what is found in native tissue were recapitulated with high fidelity using this in vitro system. While there is experimental evidence implicating notch signaling in the differentiation of luminal cells, factors driving myoepithelial cell differentiation are not as clear. Pasic et al. examined the effects of different HER1 ligands on progenitor cell differentiation, as EGF receptor expression is ubiquitous in developing mammary tissue. While previous EGFR knockout studies in mice implicated stromal expression of the receptor in ductal development, no significant role was established in the epithelium\textsuperscript{101}. This was not the case for these human mammary developmental studies, however; soluble epidermal growth factor (EGF), a common HER1 ligand, was found to induce a vast expansion of the myoepithelial lineage in human mammary organoid models. The differentiation and proliferation of human mammary myoepithelial cells occurred by sustained HER1 activation through ERK-RSK signaling, as ERK-RSK inhibition prevented expansion of the myoepithelial cell population. In this study, it was
postulated that myoepithelial cells in adult human tissue may arise from a bipotent progenitor pool of K8+K14+ cells. Increased myoepithelial cell differentiation in response to soluble EGF coincided with a decrease in K8+K14+ progenitors.

Integrin adhesions and signaling are yet another crucial determinant of mammary epithelial cell fate, but their relative contribution in establishing the myoepithelial lineage is less well understood than mechanisms of luminal cell differentiation. B1 integrin has been postulated to play a role in myoepithelial differentiation and phenotypic maintenance. However, B1 integrin deletions did not inhibit establishment of a basal cell layer with markers indicating successful myoepithelial cell differentiation. Rather, the effect of this deletion seemed to be more pronounced with respect to contractile function; absence of the alpha3beta1 integrin isoform resulted in sustained myosin light chain phosphorylation. Myoepithelial cell failure to cycle efficiently between contracted and relaxed states impaired lactation and milk ejection.

The mammary myoepithelial precursor: Applications in Tissue Regeneration

It has been suggested that luminal epithelial cells give rise to the mammary myoepithelium. To examine the developmental origin of myoepithelial cells, Pechoux et al. used human luminal epithelial and myoepithelial cells that were separated using immunomagnetic sorting for cell-type specific surface glycophospholipids, yielding pure populations of cytokeratin 18+19+ luminal cells and SMA-positive myoepithelial cells. The cells were maintained in isolated culture conditions, and
different supplemented mediums conducive to the growth of either the luminal epithelial population or myoepithelial population were use to expand each in culture. Media was then switched in a subset of each population, to determine if one cell type could serve as a precursor for the other. Myoepithelial cells were unable to take a luminal epithelial cell phenotype regardless of culture conditions; however, a subpopulation of luminal cells appeared capable of gradually acquiring the myoepithelial phenotype, indicating that luminal cells can serve as precursors to differentiated myoepithelial cells, but not the other way around.

While it appears possible for myoepithelial cell differentiation to occur in populations of isolated luminal cells, the developmental origin of the myoepithelium is likely a subpopulation of basal epithelial progenitors. Interestingly, the basal epithelial niche has long been a topic of importance as a result of numerous studies that have demonstrated its regenerative capacity. Mouse mammary cells marked with a retrovirus were able to regenerate functional mammary tissue when implanted into the mammary fat pad, earning these cells the term mammary regenerating units (MRUs)\textsuperscript{41}. It was noted that basal cells with high expression of alpha-6 integrin were particularly efficient at repopulating the mammary fat pad. This study marked the first time that evidence for a mammary stem cell might exist, and garnered attention due to the attractiveness of a stem cell population serving as a potential origin for breast cancers. Stingl et al. (2006) used a multiparametered sorting protocol to isolate a unique population of cells from the adult mouse mammary gland; these cells too, were able to
regenerate a complete mammary gland within six weeks of in vivo transplantation and perform multiple symmetrical self-renewal divisions\textsuperscript{89}. Termed mammary stem cells, they appeared phenotypically unique, and capable of generating different mammary progenitor populations in cell culture.
Figure 1.8. **Capacity for Mammary Gland Tissue Regeneration.** Shackelton et al. (2006) demonstrated the capacity for a single LacZ\(^{+}\)Lin\(^{-}\)CD\(29^{+}\)CD\(24^{+}\) cell to regenerate mammary epithelium 10 (left) and 8.5 (right) weeks after transplantation. This study demonstrated the regenerative capacity of basal epithelial cells isolated from mammary tissue.
Of note, Stingl et al. concluded that this mammary stem cell population harbored multiple features indicating their origin is localized to the basal epithelium (See Figure 1.8.). While these studies were useful in identifying that mammary stem cells are localized to the basal layer in tissue, it was not possible to highlight a distinct population from myoepithelial cells, which populate the majority of the basal compartment.

Work in mammary stem cell populations has been particularly exciting as of recent, with new work by Prater et al. (2014) furthering our understanding of the mammary stem cell and suggesting that it actually possesses myoepithelial cell properties. In this study, purified myoepithelial cells were isolated using flow cytometry through a double sorting protocol. Cells that were dual positive for alpha6 integrin and epithelial cell adhesion molecule (epCAM) were sorted from mouse tissue, yielding a 97% SMA+ population of cells in culture, suggestive of a pure myoepithelial cell isolation. Of the isolated basal single cell populations, 65% were able to repopulate the mammary fat pad when transplanted into mice. Cells expressing smooth muscle myosin heavy chain were also isolated from tissue, and demonstrated mammary repopulating capacity; these cells were also SMA+. The majority of basal cells in adult tissue are known to be myoepithelial in phenotype; hence, there must be some overlap between mammary repopulating units and myoepithelial cells. After performing lineage-tracing experiments, it was confirmed that the mammary myoepithelium can function as lineage-restricted stem cells. This recent work suggests
that isolated single cell populations of cultured myoepithelial cells might be particularly useful in the context of mammary gland tissue regeneration, but future work remains to determine whether this finding can be replicated in other exocrine organs or species.

**Examining Functional Aspects of Mammary Myoepithelial Cells: Contraction**

Myoepithelial cell contractility in mammary and lacrimal glands in response to select agonists has been previously established, but only indirect evidence has been presented in salivary glands. Lacrimal and salivary gland fluid production and expulsion are largely under the control of nervous system input, as nervous tissue is intricately woven throughout the epithelium of each gland. Mammary glands differ, however, in that their contractility is mediated by the endocrine system. Oxytocin receptors are found primarily in the central nervous system, where they mediate a range of behaviors including stress responses, context-dependent forms of social memory, and maternal bonding. Oxytocin receptors are also found in the myometrium and endometrium of the uterine lining, where they facilitate uterine contractility during pregnancy. Downstream cellular responses mediated by oxytocin receptors are similar in the uterine myometrium to that of the mammary myoepithelium, both of which rely on these receptors for initiation of contraction.

Upon agonist binding of oxytocin receptors localized to the mammary myoepithelium, a pronounced contraction of the mammary alveoli has been observed in intact tissue. These receptors belong to the g₄ family of G-protein coupled
receptors, which mediate intracellular calcium-dependent second messenger responses. Receptor activation yields increased activity of phospholipase C, which cleaves a specific phosphatidylinositol (PIP2) into two smaller molecules, diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 then travels through the cytosol, and can bind its associated receptor located on the ER membrane. The IP3 receptor controls a rapid release of Calcium ions (Ca++) from the ER, which can quickly bind cytoplasmic proteins that mediate diverse downstream cellular processes\textsuperscript{68}. In the context of mammary myoepithelial cell contraction, the calcium-binding protein calmodulin becomes active in response to increased cytosolic calcium\textsuperscript{53}.

Activation of calcium-calmodulin complexes is well documented in smooth muscle cells, whose expression and organization of contractile machinery most closely mirrors that of myoepithelial cells. In smooth muscle, calcium-calmodulin binds to and activates a myosin light chain kinase, which in turn phosphorylates select serine and threonine residues (thr18 & ser19) on myosin’s regulatory light chain\textsuperscript{69}. This increases activity in the ATPase domain of myosin heads; ATP hydrolysis in the myosin head generates energy, propelling the sustained force required during acto-myosin crossbridge cycling. As myosin heads bind actin filaments, hydrolyze ATP, release from the filament and cycle this process, actin and myosin filaments slide continuously in opposite directions. This dynamic process of cytoskeletal rearrangement ultimately drives the contractile cell phenotype.
While this initial response occurs very rapidly in the cell, it must be sustained in order for the tissue-specific response of smooth muscle or myoepithelial cells to work properly. There are a few proteins implicated in prolonged ATPase activity of myosin heads; rho kinase (ROCK), for example, is capable of phosphorylating specific myosin light chain phosphatases (MLCP), rendering them less active. Mammary myoepithelial cells treated with ROCK inhibitor were unable to initiate contraction upon oxytocin stimulation, implicating that parallel Calcium-MLCK activity and ROCK-mediated inhibition of MLCP are needed for efficient contraction. In addition, DAG, a product of PIP2 cleavage by PLC, is capable of activating tissue specific PKC isoforms that are thought to play an indirect role in sustained contraction. Calcium flux plays a role in the temporal nature of a contractile response, as pumps in the ER and cell membrane both work actively to remove calcium from the cytosol and prevent sustained activation of calcium-dependent cytosolic proteins.

A similar mechanism to that of smooth muscle likely occurs in mammary myoepithelial cells, given their robust expression of smooth muscle-analogous contractile elements and calcium-dependent contractility. However, intracellular signaling downstream of calcium is less well studied in myoepithelial cells than in smooth muscle. Smooth muscle actin expression, which is commonly used to identify the myoepithelial cell phenotype in tissue and isolated cells, is thought to play an integral role in the contractility of these cells. Work by Haaksma et al. (2011) demonstrated that alveolar contraction and milk ejection were severely impaired in
Figure 1.9. **Myoepithelial Cell Contraction in Mammary Gland Tissue.** Mammary alveoli stained for α-SMA in wild type (B&D) and α-SMA knockout (C&E) lactating dams. Pups were removed either 6hr prior to (B&C) or immediately before (D&E) the animal was sacrificed. Panels D & E clearly show wild type dams’ alveoli have undergone significantly greater contraction than in α-SMA knockout dams, implicating its role in force generation during contraction of lactating mammary alveoli (Haaksma C.J. et al., 2011).
alpha-smooth muscle actin knockout mice\textsuperscript{32} (See figure 1.9.). Pups nursed by alpha-SMA null mice failed to prosper, with 20-day post-natal weights averaging just half that of pups nursed by wild-type mothers. Another interesting aspect of mammary myoepithelial cell contractility that has only recently been elucidated involves extracellular matrix adhesions and subsequent signaling mediated by specific integrin isoform-ECM attachments. Mammary myoepithelial cells in α3β1 integrin (a laminin receptor) knockout mice were able to contract in response to oxytocin, but failed to achieve a post-contractile relaxation required for efficient, continuous milk ejection\textsuperscript{79}. Pups who nursed from a population of mice harboring this mutation failed to thrive. Coupled contraction-relaxation cycling is crucial to efficient fluid secretion, and it now established that fine tuning of multiple signaling pathways working in parallel is required for this process to flow smoothly.

The aforementioned study determined that α3β1 integrin-mediated activation of a FAK/Rac/PAK signaling cascade inhibits myosin light chain kinase (MLCK) activity, a pre-requisite for myoepithelial cell relaxation. Focal adhesion kinase (FAK) phosphorylation was reduced in this model; FAK mediates activity of the Rho-family of GTPases upon formation of integrin-ECM adhesions\textsuperscript{92}, suggesting that decreased FAK phosphorylation in myoepithelial cells disrupts balanced Rho-GTPase signaling associated with contraction-relaxation coupling.
Figure 1.10  Schematic of Contraction/Relaxation in Mammary Gland Myoepithelium. Proposed mechanism of α3β1 integrin-mediated relaxation in mammary myoepithelial cells (Raymond et al., 2011)
In this model, RhoA/ROCK activation in response to oxytocin receptor stimulation works in concert with FAK/Rac/PAK activation through integrin-ECM adhesions to balance MLCK/MLCP activity; this balance regulates proper milk ejection by the mammary myoepithelium\(^7\) (See Figure 1.10.).

**Examining Functional Aspects of Mammary Myoepithelial Cells: Role in Tissue Architecture and Polarity**

Mammary myoepithelial cells are situated between the luminal epithelium on one side and underlying basement membrane and surrounding stroma on the other. Interestingly, because the myoepithelium forms at an epithelial/stromal interface, they become a prime candidate for facilitating communication between different layers in tissue. As such, myoepithelial cells have been associated with a number of important functional roles in adult tissue, ranging from establishment and maintenance of tissue structure to alveolar polarity\(^3\).

The phenomenon of luminal epithelial cells spontaneously forming acini-like spheroid structures when encapsulated in three-dimensional microenvironments is well established\(^4, 29, 55\). For example, human mammary luminal epithelial cells encapsulated in a reconstituted basement membrane gel (Matrigel) are capable of aggregation, spheroid formation, and correct polarization. Interestingly, when the same cells are encapsulated in collagen I gels which represent more of a mesenchymal microenvironment, spheroids form but are incapable of correctly polarizing\(^30\); basement membrane deposition by luminal epithelial cells in collagen I was virtually
non-existent. This led to the hypothesis that in native tissue, myoepithelial cells might provide the necessary signals to drive polarity in the luminal epithelium. Gudjonsson et al. (2002) tested this hypothesis using isolated myoepithelial cells from healthy human mammary glands. By encapsulating mammary luminal epithelial cells in collagen I gels simultaneously with an isolated myoepithelial cell population (See Figure 1.11.), polarized, double layered acini formed in the majority of structures observed.
Figure 1.11. Acinar/Myoepithelial Cell Co-cultures with Mammary Gland Cells (A) When encapsulated in Matrigel, luminal epithelial (acinar) and myoepithelial cells sort independently and aggregate with their own cell-type. Interestingly, acinar cells encapsulated in collagen I gels can form spheroids, but demonstrate inversed polarity (not shown). (B) Acinar cells co-encapsulated with myoepithelial cells in collagen I gels are able to form double-layered secretory units with myoepithelial cells situated on the exterior of the structure. Interestingly, these structures correctly polarize and are representative of secretory units observed in (C) intact mammary tissue (Gudjonsson et al., 2002).
It was proposed that basement membrane synthesis by the myoepithelium might be responsible for inducing spheroid polarity in co-culture experiments. The presence of laminin is robust in the mammary basement membrane; Laminins 1, 5, 10 & 11 are all found, each expressing different alpha chain isoforms that contribute to the diversity of functional roles performed by the mammary basement membrane. Of note, only addition of laminin 1 to luminal epithelial cells cultured in collagen I can reverse and correctly polarize the spheroids to the extent observed in Matrigel. This finding, in addition to the discovery that tumor-derived myoepithelial cells synthesize minimal, often non-functional laminin 1 and cannot correctly polarize luminal epithelial cells, highlights a crucial functional role for myoepithelial cells in mammary tissue: Laminin 1 synthesis by the mammary myoepithelium is a contributor to apicobasal polarity in mature tissue; this finding has yet to be explored in the context of salivary epithelium, but might prove useful in tissue regeneration when employing matrices that promote cell adhesion, migration, and polarization.

In addition to mammary basement membrane synthesis, myoepithelial cells help maintain tissue architecture and facilitate coordinated secretion through specific cell-cell adhesions systems. Unique desmosomal adhesions systems are present between both acinar and myoepithelial cells, as well as myoepithelial cells lying proximal to one another around acinar lobules. Functional inhibition of myoepithelial cell-specific desmosomal components desmocollin 3 and desmoglein 3 using a peptide that blocked their respective cell adhesion recognition sites, prevented formation of double-layered mammary acini, and myoepithelial cells were unable to position
themselves correctly around the luminal epithelium. This suggested that proper desmosomal adhesions systems are crucial for correct cell sorting and positioning during the formation of functional acini.

Studies in transgenic mice have highlighted additional roles for adhesions systems in mammary development and structural maintenance. For example, mice that do not produce P-cadherin, a myoepithelium-specific adhesion protein, undergo superfluous and temporally premature branching. Virgin mutants had mammary tissue resembling that of a pregnant wild-type mouse, indicating a role for adhesions systems in providing inhibitory feedback to regulate branching morphogenesis. Molecular mechanisms underlying the contribution of adhesion systems to feedback inhibition during branching morphogenesis remain to be elucidated. In addition, myoepithelial cells rely on gap junctions and cadherin-mediated attachments for synchronized responses to external stimuli and structural maintenance of tissue, respectively. Taken as a whole, the growing body of literature on mammary gland myoepithelial cells highlights their indispensable role in mammary development, specifically the establishment and maintenance of proper tissue architecture. Exciting new work in the field has also implicated the basal epithelial compartment, rich in myoepithelial cells, as a viable source of cells for repopulating damaged or surgically excised mammary glands. The potential utility of myoepithelial cells in the context of mammary gland tissue regeneration is an exciting topic open to future inquiry.
1.5. The Lacrimal Gland Myoepithelial Cell

Localization and Morphology in Tissue

Myoepithelial cells in lacrimal gland tissue have similar localization and morphology to those of the salivary glands. They are mostly present surrounding the secretory acini, and stain minimally in the ductal network\textsuperscript{46}; lacrimal glands do not contain striated ducts, and there are no reports suggesting a significant myoepithelial presence in lacrimal gland intralobular ducts either. Characteristic of their distinct phenotype, lacrimal gland myoepithelial cells express smooth muscle-specific \(\alpha\)-SMA and epithelial cytokeratin CK-14 in tissue. Some groups have attempted to isolate single cell populations of myoepithelial cells from intact lacrimal gland tissue; Ohtomo et al. (2011) successfully isolated a population of cells which took on a myoepithelial cell phenotype in culture after \(\sim\)28 days\textsuperscript{61}. In addition to expressing their characteristic smooth muscle and epithelial cytoskeletal elements, these cells synthesized significant levels of adenylate cyclase II and \(\alpha\)-actinin; interestingly, immunofluorescence studies in intact tissue revealed cell-type specificity for these proteins, as expression was restricted to the myoepithelium. This study was the first to demonstrate that myoepithelial cells can be isolated from tissue and cultured as a homogeneous population.

In other work, intact acini were isolated from lacrimal gland tissue and used to investigate myoepithelial cells in a more physiologically relevant context. After performing an enzymatic tissue digest, isolated spheroids were approximately 50\(\mu\)m in
diameter, similar to those observed in native tissue; myoepithelial cells remained present, taking on stellate morphologies enveloping the basal surface of acini as evidenced by strong smooth muscle actin staining that was completely absent from luminal acinar cells. Consistent with literature describing myoepithelial cell morphology in salivary gland tissue, Lemullois et al. (1996) noted two distinct morphologies in isolated lacrimal gland acini; one subset had shorter cytoplasmic processes with very thin cell bodies, while the other had much longer cytoplasmic extensions (up to 1mm in length). It has yet to be determined whether the distinct myoepithelial cell subsets described are indeed unique from one another, or just two different physiological states of the same cell. In mammary glands, similar work was performed in isolated acini containing myoepithelial cells, and it was determined that contracted myoepithelial cells undergo morphological changes in response to the hormone oxytocin. This might be one explanation for the observed differences among lacrimal gland myoepithelial cells in isolated acini, although it should be noted that lacrimal gland myoepithelial cells respond to a different array of agonists than those of the mammary gland, namely endocrine signals versus neurotransmitter receptor agonists.

Lacrimal Gland Differentiation and Organogenesis; Acquisition of the Myoepithelial Cell Phenotype

Lacrimal glands begin to develop in the mouse at embryonic day 13.5, through an initial process that mirrors salivary gland organogenesis. First, a primary duct bud
infiltrates from the conjunctival epithelium into surrounding mesenchyme\textsuperscript{23}, with an initial stalk branching into two distinct buds around embryonic day 15.5 to form a major extraorbital and minor intraorbital lobe. Human lacrimal glands are made up of two distinct lobes as well, named the palpebral and orbital lobes, which develop around embryonic week eight. Spatiotemporal release of select fibroblast growth factors and bone morphogenetic protein isoforms are key regulators of proliferative and branching processes in the developing gland\textsuperscript{16}.

While the molecular pathways underlying myoepithelial cell differentiation in the lacrimal gland have yet to be characterized, recent work in salivary gland developmental biology has shed light on FGFR signaling that might mediate expansion and subsequent differentiation of a myoepithelial progenitor\textsuperscript{48}. Postnatal development of the lacrimal gland has been characterized somewhat, however; In one study, Wang et al. (1995) investigated the maturation of myoepithelial cells in the mouse lacrimal gland by characterizing morphological changes in the myoepithelium during postnatal development\textsuperscript{97}.

In newborn mice, early acini did not appear to have clearly defined lumens or secretory granules, and no $\alpha$-SMA staining is apparent in the surrounding basal epithelium. By postnatal day three, a small fraction of acini had begun to develop lumens, and $\alpha$-SMA began to appear in basal cells that had a more rounded morphology. By one week, lumens were present and the majority of acini had polarized, with visible secretory granules and basally localized nuclei; myoepithelial cells had begun to take on distinct morphology, as they flattened
around the secretory lobules and began to project few cytoplasmic processes. By weeks 4-8, acini increased in size and number of secretory granules, while myoepithelial cells had undergone visible proliferation and extended long, thin processes surrounding the secretory unit. The authors commented that growth and expansion of acinar lobules during postnatal development might influence myoepithelial cell density and configuration\textsuperscript{97}.

It is clear that differentiated myoepithelial cells have distinct functional roles, cell-type specific expression profiles, and morphology from other epithelial cell types in exocrine organs. However, there does appear to be some variety within the myoepithelial lineage in tissue, as some have noted distinct morphological characteristics between myoepithelial cells of a single tissue. Interestingly, one group performed a characterization of progenitor cells in the adult rat lacrimal gland, and found they had characteristics of the myoepithelial lineage\textsuperscript{85}. First, myoepithelial cells were distinguished in tissue sections by labeling for $\alpha$-SMA. Surprisingly, it appeared that smooth muscle actin staining co-localized with a number of stem and progenitor cell markers in intact tissue, including nestin, Musashi 1, PAX6, and Sox 2, among others. A protocol was then used to isolate an immature population of myoepithelial cells from tissue.
Figure 1.12.  **Myoepithelial Cell-Specific Staining in Lacrimal Gland Tissue.** Histological section of the rat lacrimal gland stained for α-smooth muscle actin (red). Staining appears to be very specific to the myoepithelial cell population in lacrimal gland tissue.
These cells retained α-SMA expression, and many of the stem and progenitor markers that co-localized with α-SMA in tissue. When cultured in different media, each designed to promote growth and survival of different cell types (neuronal, corneal endothelial, myoepithelial), isolated immature cells began to express markers representative of the cell type for which the media was designed. It appears that myoepithelial cells possess progenitor cell properties when isolated from tissue and cultured as single cells.

**Lacrimal Gland Myoepithelial Cell Functionality: Contraction**

Similar to the salivary glands, lacrimal glands receive dense autonomic nervous input\(^1\); this allows studies performed in lacrimal glands to serve as a template for investigating hypotheses regarding the salivary gland response to neural input. Stimulation of corneal sensory nerves results in vasodilation, an established prerequisite for tear film secretion, while mastication stimulates sensory nerves of the oral cavity to augment fluid secretion by the salivary glands. Lacrimal gland myoepithelial cells express alpha and beta-adrenergic, cholinergic, select neuropeptide and purinergic receptors\(^13, 14\). Addition of carbachol, an acetylcholine analog, resulted in contraction of guinea pig lacrimal gland myoepithelial cells\(^83\). This makes sense in relation to mammary myoepithelial cell contractility, as oxytocin receptors and muscarinic acetylcholine receptors (a target of carbachol), belong to the same family of \(g\)q-coupled GPCRs. Alpha and beta-adrenergic agonists, however, did not initiate a contractile response in lacrimal myoepithelial cells. To date, the function of many
lacrimal gland GPCRs in the context of lacrimal gland fluid production and secretion are poorly understood. While the majority of functional studies in lacrimal glands have been performed using whole tissue, one study highlights an attempt to separate these cells from intact tissue for further investigation in isolated culture. Ohtomo et al. (2011) were able to successfully isolate lacrimal gland myoepithelial cells, confirmed by expression of a number of cell-type specific biomarkers observed in tissue. The primary focus of the study was to investigate purinergic receptor signaling, as ATP among other purines can be released from nerves concomitantly with acetylcholine or norepinephrine\textsuperscript{36}. Isolated lacrimal gland myoepithelial cells expressed a wide range of purinergic receptors, and increased intracellular Calcium in response to a variety of purinergic agonists. Some of these receptors are ionotropic rather than metabotropic, while others do not signal through mechanisms similar to oxytocin and muscarinic acetylcholine receptors. The significance of cytosolic calcium increasing in response to purinergic agonists has yet to be investigated. It is clear however, that cytosolic calcium plays a significant role in both lacrimal and mammary myoepithelial cell contractility.

1.6. The Salivary Gland Myoepithelial Cell

Morphology and Location in Tissue

Salivary gland myoepithelial-like cells have been cultured as isolated populations\textsuperscript{8}, but remain poorly understood. They are thin and spindle-shaped in
morphology, extending multiple, thin cytoplasmic processes around the secretory acini. In salivary gland tissue, myoepithelial cells are found around secretory units and intercalated ducts, but much less around larger ducts, including the excretory ducts. These cells stain positive for α-SMA, SMMHC, calponin, keratin 14, vimentin, and metallothionein.

Like other exocrine organs, the salivary myoepithelium is situated between the acini and basement membrane, forming diverse cell-cell contacts with each. Salivary gland myoepithelial cells have not yet been thoroughly characterized in isolated populations or in tissue, thus the majority of hypothesis regarding their functional role in tissue derive from work in other exocrine organs containing myoepithelial cells.

**Myoepithelial cell differentiation during organogenesis**

Salivary gland development occurs through a complex process known as branching morphogenesis; by embryonic day 13.5, an initial epithelial end bud begins repeated rounds of cleft formation and progression to establish a highly branched structure, maximizing tissue surface area for efficient fluid production. The early embryonic salivary glands are comprised of epithelial progenitor cells, which eventually separate into distinct populations capable of responding differentially to external cues. Pluripotent stem cells give rise to both acinar and ductal progenitor cells in the salivary gland, with acinar progenitors subsequently giving rise to the salivary myoepithelium. Recent work has begun to elucidate a distinct myoepithelial progenitor cell niche in the salivary gland, while previous work in mammary and
lacrimal glands have also highlighted prospective roles for myoepithelial cell progenitors in their respective tissues. Interestingly, mice lacking the transcription factor p63, an in situ marker for myoepithelial cells, fail to develop submandibular glands\textsuperscript{20}; this suggests that basal progenitors, which may serve as myoepithelial cell precursors, are crucial for normal tissue development.

ETS and Sox family transcription factors are both expressed in the embryonic salivary endbuds, controlling early processes ranging from stem cell maintenance to proliferation and differentiation\textsuperscript{40,58}. Lombaert et al. (2012) discuss the appearance of Sox10 expression beginning at embryonic day 13 in the mouse submandibular gland; interestingly it remains present in the myoepithelium of the adult human SMG, leading to speculation about its potential role in myoepithelial cell differentiation and maintenance\textsuperscript{49}. For the most part, transcriptional regulation in the context of salivary myoepithelial cell differentiation is not yet well understood.

In other exocrine organs such as the pancreas, FGF10 produced by the mesenchyme controls expression of ETV family transcription factors that regulate early salivary gland development. Some of these phenomena have recently been tested in the context of salivary gland development; In the embryonic salivary gland, FGF10 addition to isolated submandibular gland end buds increased ETV4, ETV5, and Sox10 expression, suggestive of its early developmental role\textsuperscript{48}. The same study identified unique progenitor populations in the developing gland, characterized by expression of kit in combination with either keratin 5 or keratin 14 for proximal or distal progenitors, respectively. FGF10 signaling through FGFR2b resulted in up-regulation
of an epithelial kit pathway, with combined signaling resulting in progenitor expansion specific to the distal end bud population.

FGF10 secreted by the mesenchyme appeared to selectively influence distal progenitors, suggestive of a potential role in determining the myoepithelial cell fate in tissue. In another study, Nelson et al. (2013) tracked the temporal and spatial localization of specific progenitor and differentiation marker-expressing populations of epithelial cells. This analysis of cell population dynamics in the developing mouse submandibular gland from embryonic day 14 through postnatal day 20 revealed a potential epithelial progenitor origin for what ultimately become smooth muscle actin-positive myoepithelial cells.

Proposed functional roles for salivary gland myoepithelial cells

There is currently minimal literature providing direct experimental evidence for the functional role of myoepithelial cells in salivary gland tissue. Most of the speculation regarding roles for the salivary myoepithelium derives from experimental evidence in other exocrine tissues. Given this broad area of research open to future investigation, I attempted to isolate salivary gland myoepithelial cells from human tissue so their functionality could be investigated, with the ultimate goal of incorporating myoepithelial cells in our bioengineered model for salivary gland tissue regeneration.
Chapter 2

CHARACTERIZATION OF MYOEPITHELIAL CELL BIOMARKERS IN TISSUE AND ISOLATED CELLS

2.1 Introduction

Myoepithelial cells have a characteristically unique phenotype in tissue, making it easy to highlight their localization and morphology using immunohistochemistry. They synthesize contractile machinery present in smooth muscle, in addition to epithelial cytokeratins and adhesion molecules, collectively giving them a name that reflects dual expression of cell-type specific biomarkers. Contractile elements found in the myoepithelium, which are not expressed at significant levels in other epithelial cell types of exocrine tissues, include α-SMA, SMMHC, and calponin. They also synthesize epithelial cytokeratins 5 & 14, and epCAM, a cell-cell adhesion protein. In previous work, myoepithelial cell-specific biomarkers were identified in mammary and lacrimal gland tissue, and populations of single cells derived from rat lacrimal glands were isolated and grown in culture. A panel of biomarkers has been established that allows for the characterization of isolated cells, to distinguish their myoepithelial phenotype. Ohtomo et al. (2011) isolated immature cells from lacrimal gland tissue, and confirmed their myoepithelial phenotype after 4 weeks in culture by highlighting expression of α-SMA, α-actinin,
and adenylate cyclase II. I sought to isolate myoepithelial cells from human salivary gland tissue by adapting this protocol, and determine the phenotype of isolated cells using a panel of myoepithelial cell biomarkers.
2.2. Materials and Methods

Isolation and Culture of Human Salivary Gland Myoepithelial Cells

A protocol for isolating human salivary gland myoepithelial cells was adapted from work by Ohtomo et al (2011), and optimized for our own system. Human salivary gland tissue biopsies were washed for 5 minutes in 1% Betadine solution in DMEM/F12, and rinsed in DMEM/F12 for 2 minutes. Tissue was then minced to a slurry and incubated at 37 °C in 0.1% Collagenase I (Sigma, C9891) for 20 minutes. The minced tissue was then centrifuged at 1400rpm for 3 minutes, and supernatant strained through a 70μm cell strainer. Strained supernatant was spun down at 1700rpm for 4 minutes, and re-suspended in 7mL RPMI + 10%FBS + P/S to wash. This was spun again at 1700rpm for 4 minutes, and remaining cells were suspended in 5mL RPMI + 10%FBS + P/S; cells were then plated in a T-25 cell culture flask. A second digest was performed on the minced tissue as previously described, but this time, a final cell pellet was re-suspended in 1mL RPMI + 10%FBS + P/S; This was added to the same T-25 flask. Cells were grown in incubator for 2 days, and then media was changed. After about one week, a small population of myoepithelial cells could be observed in culture and subsequently expanded.

Cell Culture Conditions for Isolated Myoepithelial Cells

Isolated myoepithelial cells were cultured in RPMI + 10%FBS + P/S in T-25 flasks. Cells were expanded to T-75 flasks when 60% confluent, and retained phenotypic markers through at least seven passages. 0.05% trypsin/EDTA was used to
passage cells, and culture media was used to inactivate the trypsin; media was changed three times per week.

**Immunofluorescence**

Staining of tissue sections was performed as follows: briefly, frozen tissue sections of approximately 8μm were fixed in 4% paraformaldehyde, washed for 15 minutes in 1x PBS, and blocked overnight in 3% milk at 4°C. The next day, tissue sections were incubated with primary antibodies α-SMA (abcam, ab7817), SMMHC (abcam, ab53219), or CK14 (abcam, ab49747) for 45 mins at 37 °C, and then washed for 30 mins with 1x PBS. Secondary antibodies Alexa 488 and Alexa 568 were incubated for 45 mins at 37 °C, and after 30 mins of washing in 1x PBS, Draq5 was used to stain nuclei. Stained tissue sections were stored in Gel Mount to preserve fluorescence.

Staining of cells was performed as follows: Cells to be used for immunofluorescence studies were plated in 8 well NUNC chambers, and cultured at 37 °C for approximately 24 hours. Cells were then fixed in 4%PFA, washed for 15 minutes with 1x PBS, and blocked overnight at 4 °C in 3% milk. Primary antibodies α-SMA (abcam, ab7817), SMMHC (abcam, ab53219), CK14 (abcam, ab49747), and epCAM (abcam, ab71916) were used for 1 hour at 37 °C, and cells were then washed for 30 mins in 1x PBS. Secondary antibodies were used for 50 mins at 37 °C, and then cells were washed for 30 mins in 1x PBS. NucBlue (DAPI) was then added
to cells for 10 mins, after which cells were washed for 10 more minutes in 1x PBS. Gel Mount was then added to the stained cells to preserve fluorescence. Cells were imaged using a Zeiss LSM 710 confocal Microscope.
2.3. Results

While some biomarkers have already been established for highlighting myoepithelial cells in tissue, we sought to confirm them in human biopsies that were then subject to cell isolation procedures. Myoepithelial cell-specific expression of α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SMMHC), and cytokeratin 14 (CK14) were all observed in human tissue samples. This provided a template for characterizing isolated cells in culture, as the concomitant expression of these proteins with epithelial cell markers could be used to confirm a myoepithelial cell phenotype. These cells have never been isolated and characterized from human salivary gland tissue, so a protocol was adapted which had been used to isolate rat lacrimal gland myoepithelial cells. Following an enzymatic digest of tissue (see methods), isolated cells were grown for about three weeks until confluent and characterized by immunofluorescence.
Figure 2.1. **Morphology and Localization of Salivary Gland Myoepithelial Cells in Human Tissue.** Human tissue sections stained for myoepithelial cell biomarkers highlight their location in tissue. Notice that myoepithelial cells are localized to the basal portion of secretory acini in tissue, encircling the spheroid. Myoepithelial cells stained with high cell-type specificity for (A) smooth muscle myosin heavy chain (SMMHC), (B) cytokeratin 14 (CK14) and (C) α-smooth muscle actin (α-SMA).
Figure 2.2. **Phenotypic Characterization of Cells Isolated from Human Tissue.** (A) Isolated salivary gland cells after 28 days in culture shown in brightfield. Scale Bar = 400µm. Isolated cells express myoepithelial cell biomarkers (B) epCAM, (C) α-SMA, (D) SMMHC, & (E) CK-14. (B,C,E) scale bar = 100µm; E, scale bare = 50µm.
2.4. Discussion

I sought to establish a protocol for isolating myoepithelial cells from human salivary gland tissue, with the goal of incorporating these cells in our established three dimensional culture system for *in vitro* salivary gland regeneration. Previous work in the rat lacrimal gland demonstrated that myoepithelial cells could be isolated and maintained in culture for prolonged periods. By adapting this protocol I was able to attempt to perform an analogous isolation with human tissue biopsies. First, human salivary gland tissue sections were stained for established myoepithelial cell biomarkers, to create a panel for cell-type specific phenotypic characterization of isolated cells. After optimization of this new protocol, I was able to isolate homogeneous populations of cells that morphologically resembled myoepithelial cells in native tissue. These cells were then stained for α-SMA, SMMHC, CK14, and epithelial cell adhesion molecule (epCAM); Positive staining for these biomarkers was observed in all isolated samples, confirming that myoepithelial cells had been isolated from tissue. Expression of myoepithelial cell biomarkers was characterized through multiple cell passages, and retention of all biomarkers was observed through several passages. This new protocol for the isolation of myoepithelial cells from human salivary gland tissue provides a template for their repeated isolation further studies to investigate their function.
3.1. Introduction

Smooth muscle contraction is a well-characterized phenomenon, observed across the vertebrate kingdom; smooth muscle cells are non-striated and undergo involuntary contraction to aid processes throughout the body, from the lining of blood vessels and some arteries, gastrointestinal and urinary tracts, to male and female reproductive tracts and the uterus\(^3\). The mechanism of smooth muscle contraction is highly conserved, but contractile initiators vary significantly depending on the organ in which smooth muscle is found. This allows for fine-tuning a tissue-specific response to contractile ligands, either in isolated organs or in multiple organ systems synchronously. Myoepithelial cells express contractile machinery and smooth muscle cytoskeletal networks mirroring that of smooth muscle cells, and may undergo a similar mechanism of contraction.

Calcium ions serve as the primary initiator of contractile events in smooth muscle cells; receptor activation by select agonists stimulates an increase in cytosolic calcium levels by triggering the release of calcium ions from sarcoplasmic storages\(^9\),
as well as pumping ions into the cell from the extracellular space through membrane calcium channels. In the cytoplasm, calcium binds to and activates calmodulin, which in turn activates myosin light chain kinase. Phosphorylation of myosin light chain at select residues, threonine 18 and serine 19, stimulates the ATPase activity of myosin’s head domain, promoting actomyosin crossbridge cycling. Mammary gland myoepithelial cells contract in response to endocrine agonists such as the hormone oxytocin, while lacrimal gland myoepithelial cells contract in response to neurotransmitter agonists. I hypothesized that salivary gland myoepithelial cell contraction occurs in response to ligands similar to those in lacrimal glands, as saliva secretion is a nervous system mediated process. To test this hypothesis, I treated isolated myoepithelial cells with neurotransmitter agonists to see if specific agonists caused the cells to increase cytosolic calcium levels.
3.2. Materials and Methods

**Immunofluorescence**

Staining of tissue sections was performed as follows: briefly, frozen tissue sections of approximately 8μm were fixed in 4% paraformaldehyde, washed for 15 minutes in 1x PBS, and blocked overnight in 3% milk at 4°C. The next day, tissue sections were incubated with primary antibodies for 45 mins at 37 °C, and then washed for 30 mins with 1x PBS. Secondary antibodies were incubated for 45 mins at 37 °C, and after 30 mins of washing in 1x PBS, Draq5 was used to stain nuclei. Stained tissue sections were stored in Gel Mount to preserve fluorescence.

Staining of cells was performed as follows: Cells to be used for immunofluorescence studies were plated in 8 well NUNC chambers, and cultured at 37 °C for approximately 24 hours. Cells were then fixed in 4%PFA, washed for 15 minutes with 1x PBS, and blocked overnight at 4 °C in 3% milk. Primary antibodies M3 mAChR (Santa Cruz, sc9108) and β2-AdrR (abcam, ab13989-50) were used for 1 hour at 37 °C, and cells were then washed for 30 mins in 1x PBS. Secondary antibodies were used for 50 mins at 37 °C, and then cells were washed for 30 mins in 1x PBS. NucBlue (DAPI) was then added to cells for 10 mins, after which cells were washed for 10 more minutes in 1x PBS. Gel Mount was then added to the stained cells to preserve fluorescence. Cells were imaged using a Zeiss LSM 510 confocal microscope.
Calcium Release Studies to Examine Myoepithelial Cell Functionality

Studies that investigated the release of intracellular calcium \([\text{Ca}_i]\) in isolated myoepithelial cells used the calcium indicator dye, Fluo4 (Invitrogen). Cells were plated in NUNC chambers using a “sandwich 3D” model system of encapsulation, described as follows: First, a modified hyaluronic acid (see Pradhan-Bhatt et al, 2013) based hydrogel was mixed 1:1 with 6mg/ml laminin (R&D, 3446-005-01), and 50µL of this mixture was added to NUNC chambers to be incubated for 30 mins at 37 °C, which promoted thermal crosslinking. Then, \(4 \times 10^3\) myoepithelial cells were seeded on top of the hyaluronic acid / laminin hydrogel, and left to adhere and spread overnight. Then, another 50µL hyaluronic acid / laminin hydrogel was poured on top of the myoepithelial cells so that they were sandwiched between two hydrogels. This allowed for the majority of the cells to be visualized on the same focal plane in three-dimensions during functional assays.

Loading Cells with Fluo4-AM Dye

10 µL fluo-4 AM was diluted in 74 µL DMSO to make a 10 µM solution. Cells were then incubated with Fluo4 in HBSS (no Calcium or Magnesium) at 37 degrees Celsius for 20 min. Cells were spun down and dye removed, then RPMI medium was added. After 30 mins, cells were treated with select agonists and imaged in real time using an LSM 510 highspeed confocal microscope.
3.3. Results

In order to examine the functional response of isolated myoepithelial cells, I set up a culture system that permitted a large sample of cells to be assayed simultaneously in three-dimensions. This culture system is called “sandwich 3D”, because cells are encapsulated between two hydrogels all on the same plane, so that all of the plated cells can be observed in focus. After confirming the expression of adrenergic and cholinergic neurotransmitter receptors in isolated myoepithelial cells, the cells were plated in a sandwich 3D system and treated with parasympathetic agonists that activate cholinergic neurotransmitter receptors. A robust increase in cytosolic calcium was observed immediately following treatment, suggesting that myoepithelial cells undergo a functional response to neurotransmitter stimulation.
Figure 3.1. **Neurotransmitter Receptor Expression in Myoepithelial Cells Isolated Myoepithelial.** Isolated cells express adrenergic and cholinergic neurotransmitter receptors. After characterizing the phenotype of isolated cells and determining that they were indeed myoepithelial, expression of adrenergic and cholinergic neurotransmitter receptors was investigated. It was determined that isolated myoepithelial cells express both (A) M3 muscarinic and (B) β2-adrenergic neurotransmitter receptors. The next step was to examine the functional response of myoepithelial cells in response to select receptor agonists.
Figure 3.2. **Increased Cytosolic Calcium in Treated Myoepithelial Cells.** (A) Control treated with HBSS does not respond with increased cytosolic calcium. (B) Cells treated with 100 μM carbachol show a robust increase in cytosolic calcium, with calcium waves propagating across the cell. (C) Graph showing fluorescence intensity (indicates relative cytosolic calcium levels) over time in control and treated samples.
3.4. Discussion

In previous work, isolated human salivary gland acinar cells were encapsulated in HA-based hydrogels, providing a system to explore their functionality. These cells expressed adrenergic and cholinergic neurotransmitter receptors, and increased intracellular calcium in response to select agonists. In these cells, calcium initiates the fluid and protein secretory pathways, but mechanisms of neural communication with the salivary myoepithelium are not as well characterized. Expression of β1 & β2 adrenergic receptors, as well as M3 muscarinic acetylcholine receptors was present in isolated myoepithelial cells.

Stimulation with the cholinomimetic muscarinic receptor agonist carbachol resulted in a pronounced increase of intracellular calcium in myoepithelial cells. Calcium waves could be seen propagating across the cells as imaged with the intracellular calcium chelator Fluo4AM, which fluoresces upon complex formation with calcium ions. At this point, we hypothesized that intracellular calcium release in myoepithelial cells might drive a contractile response; myoepithelial cells express similar contractile machinery to that of smooth muscle cells, whose contractility is regulated by changes in cytosolic calcium levels. Discussed more in the introduction, increased intracellular calcium in smooth muscle cells, and subsequent complex formation with the regulatory protein calmodulin leads to activation of myosin light chain kinase. This protein serves as the primary initiator of actomyosin crossbridge cycling during cell contraction. The next step in my work was to examine whether contractile machinery is activated in response to muscarinic receptor agonists. This
could provide a rationale for designing functional assays to examine cell contraction in isolated myoepithelial cells.
Chapter 4
INVESTIGATING THE EFFECTS OF PARASYMPATHETIC AGONISTS ON MYOEPITHELIAL CELLS BY OBSERVING CHANGES IN THE ACTIVATION OF CONTRACTILE MACHINERY

4.1. Introduction

Smooth muscle contractility is initiated by an increase in cytosolic calcium levels. Complex formation of calcium ions with calmodulin, a regulatory calcium-binding protein, activates a myosin light chain kinase; subsequent phosphorylation at select residues on myosin’s 20 kDA light chain by MLCK increases the ATPase activity of myosin heavy chain’s associated head domains. Phosphorylation at two particular residues, Thr18 and Ser19, is widely implicated in the initiation of contractile activity in smooth muscle. Other proteins such as rhoA and its downstream target ROCK are crucial to maintaining a cell’s calcium sensitization by inhibiting myosin phosphatases that decrease myosin heavy chain’s ATPase activity; ROCK activation is crucial during sustained contractions to suppress the activity of myosin phosphatase.

For my series of experiments, a very short time course was used between treatment and fixation, to highlight the phosphorylation state of myosin light chain immediately following the addition of a neurotransmitter that increases
cytosolic calcium levels. Smooth muscle relaxation can occur rapidly in the presence of myosin phosphatases, and because I did not use a myosin phosphatase inhibitor, the shorter time course should accurately reflect the immediate myoepithelial cell response to muscarinic agonists. Phosphorylation at Ser19 on myosin light chain was investigated in isolated myoepithelial cells for this set of experiments, as an indicator of contractile activity at the molecular level; changes in the phosphorylation state at this specific site in response to neurotransmitter receptor agonists would implicate a nerve-mediated mechanism for increased myoepithelial cell contractility.
4.2. Materials and Methods

Examining the Activation of Contractile Machinery in Isolated Myoepithelial Cells in Response to Parasympathetic Agonists

Isolated myoepithelial cells were plated in 8 well glass NUNC chambers at a density of approximately 4x10^3 cells per well. After allowing one day for the cells to adhere, a treatment of 100μM carbachol was administered, and cells were quickly returned to 37 °C. After one minute, the cells were fixed with 4% PFA and stained (see immunofluorescence) for phosphorylated serine19 on myosin's regulatory light chain.

Immunofluorescence

Immunofluorescence was performed as follows; Cells to be used from p-ser19 studies were fixed in 4% paraformaldehyde in an 8 well NUNC chamber, and stored at 4 °C. Cells were then fixed in 4%PFA, washed for 15 minutes with 1x PBS, and blocked overnight at 4 °C in 3% milk. Primary antibodies were used for 1 hour at 37 °C (phosphorylated serine 19 on regulatory myosin light chain, SIGMA C7740), and cells were then washed for 30 mins in 1x PBS. Secondary antibodies were used for 50 mins at 37 °C (Alexa 488), and then cells were washed for 30 mins in 1x PBS. NucBlue (DAPI) was then added to cells for 10 mins, after which cells were washed for 10 more minutes in 1x PBS. Gel Mount was then added to the stained cells to preserve fluorescence. Cells were imaged using a Zeiss LSM 710 confocal microscope.
4.3. Results

Isolated myoepithelial cells were plated on glass cover slips, and treated with 100μM carbachol to investigate the effect of parasympathetic agonists on the activation of myoepithelial cell contractile machinery. Upon treatment, a robust increase of phosphorylation at serine 19 on myosin’s regulatory light chain was observed, indicative of activated contractile machinery (Phosphorylation at Thr18 and Ser19 stimulates the ATPase activity of the head domain on myosin II). In addition, a ruffling at the edge of the cell membrane was also observed using color depth coding software. I hypothesized that this might be indicative of a contractile response, but these results were inconclusive and required further investigation.
Figure 4.1. **Activation of Contractile Machinery in Myoepithelial Cells.** Isolated myoepithelial cells were treated with 100µM CCh and activation of contractile machinery was observed by staining with antibody for p-ser19 myosin light chain (green). Cells treated with 100µM CCh (C, D) demonstrated greater staining for p-ser19 MLC than control (A, B) treated with PBS. (B, D: scale bar = 10µm. A, C: scale bar = 100µm).
Figure 4.2. **Cell Membrane Dynamics in Control and Treated Samples.** (A) Representative control sample has a thickness of approximately 5.3µm at the edge of the cell membrane in comparison to (B) representative 100µM treated sample has a membrane thickness of approximately 8.5µm. This is presumably due to ruffling at the edge of the cell membrane. (Scale bar for A & B = 10µm)
Figure 4.3. **Depth-Coded 3D Reconstructions of Myoepithelial Cells.** (A) Color depth-coded 3D reconstruction of representative untreated cell and (B) Color depth-coded 3D reconstruction of representative 100µM treated cell.
4.4. Discussion

Stimulation of myoepithelial cells with carbachol resulted in increased phosphorylation at Ser19 relative to control treatment, suggestive of a nerve-mediated activation of myoepithelial cell contractile machinery. The time course of this activation was very short, which seems appropriate and representative of the timescale for downstream intracellular phosphorylation events in response to extracellular ligands.

Interestingly, when observed at high magnification (100x oil apochromat objective), subtle differences in the cell membranes of control and treated samples were visible as well. Using color depth-coding software, I was able to measure the distance of the cells from the glass coverslip on which they were plated; it appeared that the edge of a significant number of cell membranes in treated samples had ruffled, and that the edge of the cell in these samples was often further from the underlying substrate than in control samples. This may have been indicative of a contraction, during which the cell membrane would likely pull inwards and ruffle, but other explanations are also possible. Receptor recycling and membrane turnover is common when cell are exposed to high concentrations of a soluble agonist, as was the case in this experiment. If receptor turnover was occurring rapidly in response to treatment, the cells could have in part lost contact with the coverslip. In order to determine whether the observed increase in myosin light chain phosphorylation coincided with myoepithelial cell contraction, additional assays were required.
Chapter 5
DEMONSTRATING MYOEPITHELIAL CELL CONTRACTILITY USING A COLLAGEN I GEL ASSAY

5.1. Introduction

After observing increased intracellular calcium and phosphorylation of myosin light chain at position ser19 in response to carbachol treatment, it seemed the hypothesis that neurotransmitters stimulate a calcium-mediated contraction in myoepithelial cells was strengthened. From this point, I decided to set up an appropriate assay to investigate the contractility of myoepithelial cells. One challenge with experimental design for this assay was that no one has yet attempted to examine the contractility of isolated myoepithelial cells in culture; myoepithelial cell contraction has only been observed experimentally in intact tissue, and isolated whole acini in which the cells were still adhered to acinar lobules. The strength and duration of myoepithelial cell contraction does not necessarily mirror that of cardiac, skeletal, or smooth muscle cells. Resulting morphological changes to the cells, and the force applied by myoepithelial cells during contraction, were both uncertain. After trying a few different approaches, it was decided that a collagen I gel contraction assay would serve as an adequate approach to investigating myoepithelial cell contraction.
Cells encapsulated within a Collagen I gel are understood to remodel and compact the collagen matrix over time, with a more robust effect observed in contractile cells\(^8\). When seeded in Collagen I matrices, contractile cells can generate an observable shrinkage in gel diameter by remodeling the collagen fibers through forces applied during contraction. Myoepithelial cells were encapsulated in Collagen I gels to investigate their relative contractility in comparison to salivary acinar cells and gels without any cells seeded. In this well-established assay, cells are encapsulated in a three-dimensional collagen I gel, and once polymerized, the gel can be detached from the mold in which it was cast. In this free-floating method, the gel is not adhered to the sides of a plate, and will shrink freely as the collagen matrix is remodeled.
5.2. Materials and Methods

Collagen I Gel Contraction Assays

Collagen I gel contraction assays were prepared as follows: approximately 1.5 x $10^5$ myoepithelial cells or human salivary acinar-like cells (hSACS) were encapsulated in a 50 μL collagen I gel (1.5 mg/mL). Some gels were left unseeded, as an additional negative control. Once cells had been added to the collagen mixture, they were incubated at 37 °C for 20 minutes to allow gelation. Gels were then detached and placed in separate wells within a 24 well plate in RPMI + 10%FBS + P/S. Floating gels were left at 37 °C for one hour, and treated with 100μM carbamoylcholine (carbachol). Gels were then photographed at select time points, and the average gel diameter for each experimental group was calculated.
5.3. Results

After just one hour, myoepithelial cells were able to generate a significant decrease in the diameter of a Collagen I gel, relative to acinar seeded and unseeded gels. This effect became more pronounced over time; after 24 hours, Collagen gels seeded with myoepithelial cells had shrunk to approximately 42% of their initial diameter, while acinar seeded and unseeded gels remained at 99% and 100% of their initial diameters, respectively. Myoepithelial cells seem to demonstrate significantly greater contractility than cell types that are not characterized as having a contractile phenotype, evidenced by their greater capacity for collagen matrix remodeling.
Figure 5.1. **Myoepithelial Cell Contraction in Collagen I Gel Assay.** (A,D) Collagen I gels without cells do not result in gel shrinkage after 24hr. (B,E) HSACs cause no gel shrinkage in response to 100µM carbachol (CCh) after 24hr. (C,F) Encapsulated myoepithelial cells treated with 100µM CCh cause robust shrinkage of collagen I gel after 24hr. (G) Graph representing % of initial diameter of collagen gels (A-F) over time. Distance between blue bars = 1mm.
5.4. Discussion

Importantly, contraction is understood to be a relatively quick process, with multiple cycles of contraction and relaxation occurring over the course of a stimulated secretion. Each cycle of contraction and relaxation would in theory, further compact the collagen gel, explaining the significant shrinkage observed continuously over time.

While myoepithelial cell contraction is a probable explanation for the observed changes in collagen gel diameter, there are some other possible events that could explain what was observed. The relative secretion of different matrix metalloproteinases (MMPs) and in what abundance is not fully characterized in isolated acinar and myoepithelial cells. It is possible that MMP synthesis and secretion is more abundant in myoepithelial cells than in acinar cells, and that myoepithelial cells are capable of degrading a collagen matrix more rapidly than acinar cells. If the integrity of the collagen matrix was weakened by MMP degradation, myoepithelial cell might have had an easier time remodeling and compacting the matrix. However, the rapid time course during which collagen gel compaction was observed is suggestive that myoepithelial cells have a significantly greater capacity to remodel a collagen matrix through more immediate processes, such as contraction.
Chapter 6

BASEMENT MEMBRANE SYNTHESIS BY ISOLATED SALIVARY GLAND MYOEPITHELIAL CELLS

6.1. Introduction

Myoepithelial cells are relatively well characterized in mammary glands, where they play a crucial role in structural maintenance of tissue and establishing polarity. Synthesis of specific laminin isoforms by the myoepithelium in mammary tissue is a determinant of apicobasal polarity, and is shown to be lost in cancers of the salivary gland that affect tissue organization and polarity. Myoepithelial cells that do not synthesize and secrete functional laminin I, as observed in select myoepitheliomas, results in a failure of acinar cell spheroids to correctly form bilayered, polarized structures in co-culture experiments. To this end, it has been observed that basement membrane synthesis by the myoepithelium is an important determinant of proper tissue function in the mammary glands.

In addition, the extracellular matrix component fibronectin, while not found in the adult salivary gland basement membrane, plays a crucial role during organogenesis. Daley et al. (2009) demonstrate that fibronectin accumulation at
the site of initiating clefts is a driver of cell proliferation, with concomitant ROCK-mediated non-muscle myosin II contraction providing the force necessary to drive cleft progression into distinct lobules\textsuperscript{11}. We decided to examine isolated salivary gland myoepithelial cells, to determine if they actively secrete components of the salivary gland basement membrane and extracellular matrix when grown in culture. Abundant basement membrane synthesis by myoepithelial cells in 3D co-culture systems could provide crucial components necessary for re-creating branching morphogenesis and maintaining the integrity of growing structures.
6.2. Materials and Methods

**Immunofluorescence:**

Isolated myoepithelial cells were plated in 8 well NUNC chambers, and left to adhere for 24 hours. After an additional 24 hours in culture, myoepithelial cells were fixed with 4% PFA (see fixation protocol). Cells were then stained with primary antibodies against extracellular matrix proteins laminin 1 (Sigma), collagen IV (abcam, ab6586), perlecan (A76, non-commercial), and fibronectin (abcamab2413). Primary antibodies were incubated at 37°C for one hour, and then washed for 30 minutes (3 x 10 min wash) in PBS. Secondary antibodies Alexa 488 and Alexa 568 were then added to cells and incubated at 37°C for 50 minutes. Gel mount was added to samples following staining to preserve fluorescence. Confocal microscopy was then performed to examine basement membrane synthesis in isolated cells. Cells were imaged using an LSM 710 confocal microscope.
6.3. Results

Immunoflorescence studies suggested that myoepithelial cells likely play an important role in establishing the salivary gland basement membrane. After culturing myoepithelial cells for one day on glass coverslips, robust staining for basement membrane components perlecan, laminin I, and collagen IV was observed. These proteins comprise a significant portion of the basement membrane; their synthesis and secretion by isolated myoepithelial cells implicates the myoepithelium in ECM production for the adult gland and potentially, by basal progenitors during organogenesis.
Figure 6.1 **Extracellular Matrix Synthesis by Myoepithelial Cells.** Isolated myoepithelial cells synthesize extracellular matrix components (A) fibronectin (B) perlecan (C) collagen IV (D) laminin I. DAPI (blue, nuclei). Scale Bar = 50µm.
6.4. Discussion

The myoepithelium appears to play an integral role in synthesis and deposition of salivary basement membrane and some extracellular matrix components. This makes sense given their localization in tissue, sandwiched between the secretory acinar lobules and basement membrane. Isolated myoepithelial cells also secreted abundant fibronectin; while fibronectin is found in the basement membrane of some tissues, it is not known to be a component of the adult salivary gland basement membrane. However, this protein does play an important role throughout organogenesis, as localized fibronectin accumulation during branching morphogenesis is an important regulator of epithelial cleft formation and progression. It was previously determined that fibronectin promotes epithelial cell proliferation and cleft progression in a ROCK-dependent manner during branching morphogenesis. In a separate mechanism, ROCK regulates a negative feedback loop that inhibits epithelial clefting when sufficient branching has taken place. Synthesis of fibronectin by isolated myoepithelial cells might be an artifact of the role of the basal epithelium during salivary gland development.
Chapter 7

RECREATING SALIVARY LOBULE MICROSTRUCTURE USING ACINAR AND MYOEPIHELIAL CELL COCULTURES

7.1. Introduction

In native tissue, acinar cells pack in close proximity to one another and establish tight junctions. The morphology of acinar cell-based structures in native tissue is spheroidal, with a hollow lumen into which salivary fluid and enzymes are secreted. Myoepithelial cells interact directly with acinar cells in tissue, forming cell-cell contacts and gap junctions with acinar spheroids; they are postulated to play a variety of roles in tissue ranging from establishment and maintenance of apicobasal polarity to contraction as a means of aiding fluid propagation from the acinar lumen through the salivary ductal network.

In attempt to re-create acinar-myoeipithelial cell interactions in vitro for subsequent characterization and functional analysis, a co-culture system was developed utilizing polyacrylamide-based microwells (see methods). This allowed for the sequential addition of acinar and myoeipithelial cells to ensure myoeipithelial cells would organize around the outside of acinar cell-based spheroids. This system was devised after repeated, unsuccessful attempts to recreate acinar/myoeipithelial cell
interactions in different three-dimensional matrices by encapsulating both cell types simultaneously.
7.2. Materials and Methods

**Polyacrylamide Microwell Culture System**

Polyacrylamide-based microwells were used to create an environment that facilitated native acinar and myoepithelial cell interactions. Each polyacrylamide mold contained 256 microwells, and could be inserted into one well of a 24 well plate. Subsequently, acinar cells were seeded at varying ratios on top of the polyacrylamide molds, and cells were incubated for one week to allow spheroid formation. Following a one week incubation, myoepithelial cells stained with PKH-26 dye were added at physiologically relevant ratios (~20:1 acinar:myoepithelial) to the polyacrylamide molds where acinar cells had previously been seeded. Co-cultures were incubated for 48 hours, and then the molds were flipped upside down in media so that the co-culture spheroids were floating in solution. Spheroids were centrifuged @ 1200rpm (then supernatant was removed) and encapsulated in Biotyme HA-based hydrogels. After 24 hours, cells were fixed in 4% PFA (see fixation protocol), and confocal microscopy was performed using an LSM 710 to determine localization of each cell type within the spheroids.
7.3. Results

The polyacrylamide microwell system used to promote acinar-myoeplithelial cell interactions appears to be an effective approach to recreating salivary lobule microstructure. Spheroids that remained compact appeared to have myoeplithelial cells localized to the exterior of the spheroid. This was apparent when z-stacks were taken through the spheroid; PKH26-labeled myoeplithelial cells were only observed on the outside of the spheroids in intact spheroids. In some cases, spheroid integrity was disrupted during the encapsulation process, and acinar and myoeplithelial cells appeared to be present in both the interior and periphery of the spheroid. As this system is improved, it should become a useful system for co-culturing acinar and myoeplithelial cells.
Figure 7.1. Cocultures of Salivary Acini-like Spheroids and Myoepithelial Cells. (A) Polyacrylamide mold microwell system used to induce spheroid formation and proper acinar/myoepithelial cell interactions in vitro. (B) In the co-culture system, enclosure of myoepithelial cells (red) around acinar spheroids encapsulated in three-dimensional hydrogels. (C) Multitude of Myoepithelial (red) Acinar Cell spheroid co-cultures in three-dimensional HA-based hydrogels. Nuclei are shown in blue.
7.4. Discussion

In order to differentiate between acinar and myoepithelial cells throughout the co-culture procedure, myoepithelial cells were tagged with a fluorescent membrane dye. By sequentially seeding acinar cells in the polyacrylamide microwells, followed by myoepithelial cells in a physiologically relevant ratio, acinar spheroids were already present and compacted by the time myoepithelial cells were added; this encouraged myoepithelial cell adhesion to the exterior of cultured spheroids. The polyacrylamide-based microwell approach to recreating acinar/myoepithelial cell interactions in cultured spheroids appears to be a promising method. When acinar cells are added to the system first and allowed to form compact spheroids, followed by fluorescently labeled myoepithelial cells, the myoepithelial cells appeared to attach to the exterior of the spheroids. This would need to be confirmed by taking confocal sections of the spheroids: To achieve this, the polyacrylamide molds were flipped and spheroids released into culture media. The spheroids could then be encapsulated within HA-based hydrogels, and either fixed soon after encapsulation or tested for functionality.

The first analysis that was performed on these spheroids was to determine the localization of myoepithelial cells within the encapsulated spheroids. The gels were fixed one day after encapsulation, and confocal z-stacks were taken through the structures. In a significant majority of spheroids where the cells had maintained a compact association through the encapsulation process, myoepithelial cells were
localized to the exterior of the structure. Spheroids that had lost some integrity during encapsulation appeared to have myoepithelial cells incorporated throughout the structure. It was determined that acinar cells should be left in microwell culture for at least one week in future experiments to ensure that all spheroids formed compact, uniform structures.

There are a number of avenues that this new system has made possible. For example: in other work in our lab, during the characterization of isolated acinar-like cells, we discovered that they express a wide array of progenitor markers. Early work exploring their capacity for proliferation and differentiation suggested that beginning as spheroids, these cells can undergo morphological changes and differentially express proteins when treated with FGF7 and FGF10 in a manner reminiscent of the developing salivary gland. This served as proof of principle that 3D spheroids of acinar-like cells can serve as a building block for functionally regenerating tissue. It is possible that the addition of other cells types to these early spheroids, such as isolated myoepithelial cells might aid the branching and differentiation process; signaling between the developing luminal epithelium and myoepithelium is a crucial component of successful branching morphogenesis as evidenced by work in embryonic mouse submandibular glands.
Chapter 8
DISCUSSION AND FUTURE DIRECTIONS

8.1. Global Discussion

The goal of my project was to isolate myoepithelial cells from human salivary gland tissue, characterize these cells, and identify functional roles they might play in the intact gland with the goal of incorporating them in our bioengineered system for salivary gland regeneration. Myoepithelial cells have never been isolated from human salivary gland tissue, and there was no protocol available, so literature reporting isolation protocols in other tissues and species were used for new ideas. A paper was identified that reported the isolation and characterization of myoepithelial cells from rat lacrimal glands, so I used this as a starting point. By adapting the protocol, I was able to create a reliable method for consistently isolating a homogeneous population of cells from tissue that morphologically resembled myoepithelial cells. Isolated cells were stained using immunofluorescence techniques for myoepithelial cell biomarkers α-smooth muscle actin, smooth muscle myosin heavy chain, cytokeratin 14, and epithelial cell adhesion molecule; all of these proteins were expressed in the majority of isolated cells, suggestive of a myoepithelial cell phenotype. These cells are proliferative, and can maintain expression of myoepithelial cell biomarkers through
multiple passages; this is the first time a protocol has been developed permitting the repeatable isolation of myoepithelial cells from human salivary gland tissue.

In salivary gland tissue, myoepithelial cells are situated between the luminal epithelium comprised of acinar cells, and the underlying basement membrane. They form cell-cell contacts with acinar cells, and anchor to the basement membrane through integrins and desmosomal adhesions. Mammary gland literature exploring the functional role of myoepithelial cells in tissue suggests that these cells play a crucial role in maintaining proper tissue architecture and polarity, in part because they are key contributors to basement membrane synthesis and deposition. Part of this work showed that tumor derived myoepithelial cells could not properly polarize acinar cell spheroids in three-dimensional cultures because they synthesized either non-functional or insufficient laminin-1, which is a major component of both the salivary and mammary basement membrane. This suggests that signaling events initiated through epithelial cell adhesions to the underlying basement membrane promote unidirectional fluid flow from secretory units. Currently, the acinar cell spheroids our lab grows in hyaluronic acid-based hydrogels are not polarized, and cannot expel uni-directional secretions into the lumens they develop. I decided to examine basement membrane synthesis in isolated myoepithelial cells; depending on the extracellular matrix proteins they secrete, the utility of myoepithelial cells in different co-culture systems could vary. Immunostaining revealed that isolated myoepithelial cells actively secrete multiple components of the salivary basement membrane, including laminin I, Collagen IV, and perlecan.
In addition to the importance for proper basement membrane/epithelium contacts in maintaining healthy adult salivary gland tissue, the extracellular matrix also plays an important role during organogenesis. Fibronectin, which is found in the extracellular matrix but not salivary basement membrane, aids proliferation and cleft progression during branching morphogenesis. Daley et al. (2009) identified a mechanochemical checkpoint whereby ROCK and myosin II-mediated fibronectin accumulation at immature clefts aids cell proliferation during embryonic salivary gland cleft progression\textsuperscript{11}. I decided it would be useful to look at fibronectin secretion in isolated myoepithelial cells, and immunostaining revealed robust fibronectin synthesis and deposition in two-dimensional cultures. As previously mentioned, the addition of FGF7 and FGF10 have been shown to promote morphological changes in acinar-like spheroids that express progenitor markers, resulting in the formation of what appear to be early clefts (Cannon in prep). By adding isolated myoepithelial cells to these cultures, the fibronectin they produce might aid cleft progression in our \textit{in vitro} system for salivary gland regeneration by promoting further clefting. While future work would require optimizing temporal addition of growth factors and different cell types, myoepithelial cells, through both cell-cell and cell-matrix signaling with acinar spheroids might improve the extent to which salivary spheroids are capable of branching when incorporated \textit{in vitro}.

After confirming the phenotype of the cells I isolated to be myoepithelial, and revealing that they actively secrete components of the salivary gland basement membrane as well as fibronectin, I attempted to identify more functional roles these
cells might play in adult tissue. A contractile response has long been attributed to the myoepithelium of exocrine glands, and has been demonstrated experimentally in mammary and lacrimal gland tissue; however, there is no experimental evidence highlighting a functional role for myoepithelial cells in the salivary gland. I decided to investigate contractility in isolated myoepithelial cells by identifying agonists that might induce myoepithelial cell contraction, as well as the underlying mechanism.

The contractile apparatus of smooth muscle consists primarily of α-smooth muscle actin and smooth muscle myosin, with a number of regulatory proteins ultimately defining the rate of contraction/relaxation cycling. The phosphorylation state of myosin’s regulatory light chain is fine-tuned by select myosin light chain kinases and phosphatases, with the activity of myosin light chain kinase increasing in response to cytosolic calcium release to promote contraction. Myoepithelial cells have long been postulated to be contractile, owed to their expression of smooth muscle-associated contractile proteins. As such, I decided to test neurotransmitter agonists that are understood to promote contraction in smooth muscle, to first determine if a second messenger response would occur that was associated with initiating cell contraction. Isolated myoepithelial express both M3 muscarinic and β1 & β2 adrenergic receptors, so neurotransmitter stimulation seemed like a logical approach to explore molecular mechanisms of contractile initiation.

The addition of 100 μM carbachol, an acetylcholine analog and muscarinic acetylcholine receptor agonist, induced a robust calcium release in a significant percentage of the total population of myoepithelial cells assayed. This experiment was
performed with the intracellular calcium chelator Fluo4 AM using a LSM510 highspeed confocal microscope, which permitted the observation of calcium waves propagating across the cells in response to agonist stimulation. After observing intracellular calcium increase in response to neurotransmitter stimulation, the hypothesis that myoepithelial cells contract in response to the nervous system, through a mechanism mirroring that of smooth muscle cells, was strengthened. The next logical step for this study was to examine changes in the activation state of myoepithelial cell contractile proteins in response to the same neurotransmitter that elicits cytosolic calcium release. For my study, I decided to look at phosphorylation of myosin’s regulatory light chain at position ser19; phosphorylation of the regulatory light chain at positions thr18 and ser19 is associated with increased activity in the ATPase domain of myosin heavy chain’s head, the region of the protein that binds to actin and hydrolyses ATP to generate the force required for actomyosin crossbridge cycling.

For this next study, myoepithelial cells were plated in 2D on glass cover slips. The timescale for extracellular ligand-mediated phosphorylation events inside the cell should be on the order of seconds, so a fixative was prepared prior to beginning the experiment. Isolated myoepithelial cells were treated with 100μM carbachol, and fixed at a time point that had been previously optimized. Initially, I had planned to take protein lysates from treated cells, and perform a Western analysis to look at relative phosphorylation at MLC ser19 in treated and untreated samples; however, after trouble shooting it was determined that the antibody I’d chosen was not ideal for
Western blots. Instead, I performed immunofluorescence studies to look at relative phosphorylation levels by observing fluorescence intensity in a representative population of both treated and untreated cells.

The results from this experiment complimented the calcium study, as a clear increase in phosphorylation at ser19 on myosin light chain was observed in response to stimulation with 100μM carbachol. Indeed, it seems that M3 muscarinic acetylcholine receptor agonists may increase the activity of myoepithelial cell contractile machinery through the following calcium-dependent mechanism: M3 muscarinic acetylcholine receptors are \( \text{g}_{\alpha q} \)-coupled GPCRs, so upon agonist binding, phospholipase C is activated and cleaves PIP2 to produce IP3. IP3 will stochastically bind its associated receptor on the sarcoplasmic membrane, triggering the rapid release of calcium into the cytosol. Calcium activates the cytosolic calcium-dependent regulatory protein calmodulin, which forms a tertiary complex with myosin light chain kinase to stimulate its activity. Enhanced myosin light chain kinase activity is responsible for the observed increase in phosphorylation at ser19 on myosin’s regulatory light chain. The end result in this model would be activation of myoepithelial cell contractile machinery producing a cell contraction.

It should also be noted that interestingly, a ruffling at the edge of the plasma membrane was observed in a number of treated cells. Using color depth-coding software, it was possible to measure the membrane thickness of individual cells; it appeared that a number of treated cells had thicker membranes at the edge of the cell in comparison to untreated cells. The initial interpretation was that this ruffling
occurred as a result of the cells contracting, but there are alternate explanations that bring this hypothesis into question. When cells are treated with high concentrations of a neurotransmitter agonist, rapid receptor recycling commonly takes place as the cells attempt to maintain sensitization to the agonist. In this case, the high rate of vesicular trafficking to, and subsequent fusion with the membrane could temporarily produce a thickened appearance. In order to establish with greater confidence that myoepithelial cells have a contractile phenotype, a number of contractile assays were devised.

The first two assays used to investigate cell contraction were LSM510 live microscopy on 2.5D-seeded cells, in addition to TIRF microscopy. In the former, myoepithelial cells we’re plated on top of different ECM-based matrices composed of laminin I, collagen I, and reconstituted basement membrane (Matrigel). Cells were treated with the parasympathetic agonist carbachol, and recorded in real-time in attempt to capture cell contractions which I hypothesized would be clearly visible if cells were seeded on top of sufficiently compliant substrates. However, there remained a key variable that was difficult to anticipate because the force and extent of myoepithelial cell contraction, if any, is not documented. Upon treatment, no visible changes in cell morphology were observed that could be considered indicative of a contraction. While this does not necessarily mean that the cells were not responding, alternative assays would be required to get a more definitive sense of what may have been occurred.

In a second assay employing TIRF microscopy, myoepithelial cells with fluorescently tagged membranes could be observed with high resolution and
magnification at the edge of the cell to examine more subtle membrane dynamics in real time. When treated with carbachol, a pronounced retraction of the cell membrane was observed using this method; however, the timescale during which the membrane retracted seemed misrepresentative of what would be expected during contraction. A relatively quick response did occur, with retractions observed beginning around 30 seconds after treatment, but this continued for approximately 10 minutes. It was hard to determine if this prolonged response was observed because an initial contraction had caused the cell to lose contacts with its substrate causing it to slowly and continuously retract. In addition, there was no method available for installing an environmental chamber for TIRF experiments, so they were performed at room temperature. The length of time that cells were required to sit at room temperature prior to beginning and during the experiment may not have been favorable for observing physiologically representative cell behavior.

Next, a third assay was devised to investigate myoepithelial cell contraction more comprehensively in larger populations of cells. Collagen I gel contraction assays are well established in the literature, and it is understood that contractile cells can remodel a collagen matrix within which they are encapsulated; during this process, the matrix compacts, resulting in an observable, quantifiable decrease in overall gel diameter or surface area. To perform this assay, myoepithelial cells were encapsulated in collagen I gels, and treated with parasympathetic agonists. In addition, salivary acinar cells, which are not a contractile cell type in tissue, were encapsulated in another set of collagen I gels for comparison. After just one hour, an observable
decrease in gel diameter was observed in collagen I gels seeded with myoepithelial cells. The short timescale required for matrix remodeling by myoepithelial cells was suggestive that they are indeed contractile. The observed changes in gel diameter progressed for 24 hours, with its final diameter measuring less than half that at the start of the experiment. Additionally, no significant changes in gel diameter were observed in collagen I matrices with encapsulated acinar cells, confirming the myoepithelial cells were responsible for the changes observed. Other explanations are possible for the outcome of this study; for example, myoepithelial cell secretion of select MMPs could aid matrix degradation and cause the cells’ influence on gel diameter to appear more pronounced. However, acinar cells also secrete MMPs (although not necessarily the same ones), but the gels in which they were encapsulated decreased only 1% in diameter over the course of 24 hours. Thus, some degree of myoepithelial cell contractility was likely necessary for efficient matrix remodeling and gel compaction. This was the first experimental evidence produced suggesting that salivary gland myoepithelial cells have a contractile phenotype.

Once the isolated myoepithelial cells had been characterized phenotypically and functionally, they were deemed suitable for incorporation in our bioengineered model for salivary gland tissue regeneration. For the initial series of experiments, I attempted to co-culture myoepithelial cells with isolated acinar-like cells in different three-dimensional matrices with the goal of recapitulating salivary tissue microstructure (spheroids composed of acinar cells, surrounded by myoepithelial cells adhered to the outside of the spheroids). Previous work by Gudjonsson et al. (year)
demonstrated that when isolated mammary acinar and myoepithelial cells are co-encapsulated in collagen I gels, they have the ability to sort amongst themselves properly and form polarized spheroids with myoepithelial cells attached to the basal compartment of acinar cells. I attempted this experiment in our own hydrogels, mixing either collagen I or laminin I into hyaluronic acid based hydrogel that were previously used in our lab to facilitate the growth of acinar-like spheroids.
8.2. Future Directions

From the work I have performed thus far, it seems as though we now have a protocol that permits the repeated isolation of myoepithelial cells from both human parotid and submandibular gland tissue. I have characterized these cells phenotypically, and performed a series of functional assays that have helped elucidate myoepithelial cell functionality. Now that these cells have been sufficiently characterized, there are two major future directions that I envision for the continuation of this work. I have begun to work on a co-culture system that promotes native acinar / myoepithelial cell interactions in three-dimensionally cultured spheroids. This could be a promising method for establishing proper polarization of the acinar-like spheroids that have previously been characterized by our lab. If prior work in mammary tissue is a good indicator of salivary gland cell behavior, which it should be given the similarities between the two systems, this would be a promising avenue to pursue. Proper polarization is a crucial component that is still missing from our model for salivary gland regeneration, and the addition of myoepithelial cells to this system could help in achieving this end.

Second, it will be crucial for our acini-like spheroids to progress further both morphologically and functionally, to recreate salivary gland organogenesis in vitro. This would include the induction of branching morphogenesis in these spheroids. Our lab has promising data thus far which highlights the progenitor qualities of acini-like spheroids, and their capacity to initiate branching morphogenesis when treated with
physiologically relevant agonists in a manner that temporally mimics salivary gland development. Recent work in the field of salivary gland developmental biology has highlighted the importance of communication between luminal and basal cells in the developing gland, and how they respond differentially to mesenchymal and neuronal cues in order to undergo proper branching and differentiation. The addition of myoepithelial cells to acini-like structures might prove very useful when trying to induce progression of branched morphologies.

To successfully translate this project to the clinic, I think one particular approach would be most effective. If it turns out that myoepithelial cells, when surrounding an acinar spheroid, can properly polarize the spheroid as is the case in mammary tissue, this would be an excellent proof of principal study demonstrating the importance of the myoepithelium to proper salivary gland function. However, the spheroids produced using this co-culture method are not interconnected, and would ultimately require structures reminiscent of intercalated ducts for secreted fluid to reach a larger duct that feeds into the oral cavity in an in vivo system. I don’t see how this could be achieved with spheroids grown in isolation from one another, and therefore think a progenitor-based approach would be most effective. The acinar-like cells our lab has isolated are capable of undergoing morphological changes and differentiating towards distinct lineages when treated with the appropriate growth factors; these spheroids remain interconnected and appear to have duct-like components when provided with the appropriate cues. If we are able to force a spheroid with progenitor characteristics to commence branching morphogenesis, the
subsequent addition of myoepithelial cells may aid cleft progression and proper polarization if added to the system at an appropriate time point. Although difficult, I think the most effective approach to salivary gland regeneration would be progenitor-based, involving the addition of physiologically relevant growth factors to commence branching, and myoepithelial cells to aid cleft progression and polarization. It will be fascinating to see where this project ultimately leads.
REFERENCES


Appendix A.

PERMISSION LETTERS

Re: permission to use figure in thesis

Małgorzata Gajewska <małgorzata.gajewska@sggw.pl>
to me ✒

12:22 PM (26 hours ago)

Dear Dan,

your message has been forwarded to me by prof. Metyl, because I am the main author of the chapter that includes the figure.

I'm glad that you find our chapter on autophagy in the mammary gland helpful in writing your thesis, and of course I give you a permission to use the mentioned figure. Just remember to include the citation by the end of the figure caption.

I wish you all the best,

good luck with your thesis -

Małgorzata (Margaret) Gajewska

Małgorzata Gajewska PhD, DSc
Department of Physiological Sciences
Faculty of Veterinary Medicine
 Warsaw University of Life Sciences (SGGW)
Nowoursynowska 159
02-776 Warsaw, Poland
tel: (+48) 22 593 82 77
fax: (+48) 22 647 24 52
e-mail: małgorzata.gajewska@sggw.pl
Hi Dr. Gudjónsson,

My name is Dan Zakheim, and I'm a graduate student at the University of Iceland. I'm writing to ask for permission to use Figure 130 in our paper. I understand that it is copyrighted material. Thank you in advance for your consideration.

If you have any questions, please don't hesitate to contact me.

Sincerely,
Daniel Zakheim

Hi Dan,

You are very welcome to do that. I think, however, the right process is ask the journal (Journal of cell science). This should be no problem. Good luck with your thesis.

regards
Thorarin

Thorarin Gudjónsson, professor,
Stem Cell Research Unit,
Biomedical Center,
University of Iceland
Email: thorj00n@hi.is
tel:+354-8658271/354-8615606
Hi Dr. Giukhova, My name is Dan Zakheim, and I'm a graduate student at the Un...

Hi Dan,

If you want to include the figure in the text that will be published - a scientific article, for example, then, I am afraid, you need to get permission of the EMBO J publisher at:


I think, it's rather easy, they always allow to use the images. Otherwise, if it's about your thesis or a report, or a poster, then, certainly, you can use it.

Thank you for your interest to our work.

Kind regards.

Marina.

De : Daniel Zakheim [danzak@udel.edu]
Envoyé : dimanche 29 mars 2015 17:06
À : Giukhova Marina
Hey Dan, I'm in the process of getting approval for using the figures I have...
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**Author:** Mark Shackleton, François Vaillant, Kaylene J. Simpson, John Stingl, Gordon K. Smyth et al.

**Publication:** Nature

**Publisher:** Nature Publishing Group

**Date:** Jan 5, 2006

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Appendix B.

IRB PROTOCOL FOR TISSUE PROCUREMENT

CHRIStIANA CARE
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FWA#00006557

Helen F. Graham Cancer Center & Research Institute
West Pavilion - Suite 2350
4701 Ogletown Stanton Road
Newark, DE 19713

302-623-4983 phone
302-623-4989 phone
302-623-6863 fax

MEMORANDUM

Steven Kostiner, MD
Chairman, IRB #1
Gary Johnson, PhD
Chairman, IRB #2
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IRB Education Specialist
Field Bert, BA, CIP
IRB Regulatory Affairs/Staff
Sara Martinez-Color
Executive Assistant
Annmarie Wise
Administrative Assistant
Wendy Basset
Administrative Assistant

DATE: April 8, 2015

TO: Robert Witt, MD
Oncology Research
Christiana Hospital

FROM: Wendy Bassett

RE: CCC# 26131 - Acquisition and Experimental Use of Head and Neck Tissue for Tissue Engineering and Biomarker Discovery: (DDD# 504560)

This is to officially inform you that the Continuing Review to your protocol which was received on 04/07/2015 was reviewed by Expedited Review and approved by Jerry Castellano, Pharm.D, CIP, Corporate Director of the Christiana Care Health System Institutional Review Board, on 04/07/2015.

Approval was extended for a period of one year, through 04/06/2016.

Our records indicate this study is OPEN.

If you have any questions or concerns, please contact the IRB Office. Thank you.
Informed Consent CHRISTIANA CARE HEALTH SERVICES, HELEN F. GRAHAM CANCER CENTER

STUDY TITLE: Acquisition and Experimental Use of Head and Neck Tissue for Tissue Engineering and Biomarkers Discovery

Christiana Care Health Services Helen F. Graham Cancer Center and Research Institute is conducting research in regeneration (regrowing) of diseased salivary glands, thyroid glands, parathyroid tissue, and vocal folds, preservation of tissue (cryopreservation), along with finding biomarkers (looking for differences in how cells are constructed) of different forms of cancer. To make this research possible, we are collecting tissue samples from many individuals. We will make these specimens available to researchers who want to study causes of cancer. We are asking you to take part in this cancer research. In addition we will link scientific data to your clinical information as part of research proposals.

You should understand why we are asking you to join this tissue collection program and whether there are any risks or benefits. This allows you to decide whether or not you want to join. This form provides information about this program. By signing this form, you give us your informed consent. Informed consent means this: You have the information you need to choose whether or not you want to be in this study.

VOLUNTARY PARTICIPATION: You can choose not to take part in this research. Your decision will not affect your medical care at Christiana Care Health Services Helen F. Graham Cancer Center and Research Institute.
We will give you a copy of this consent form to keep. After you read this form, and after we discuss it with you, you may still have questions. If you have questions now or in the future, please ask us.

THE GOAL OF CANCER RESEARCH: The goal of cancer research is ultimately to regenerate salivary gland tissue, thyroid tissue, parathyroid tissue, and vocal fold tissue, to preserve tissue (cryopreservation), and find biomarkers that provide information to develop new and better ways to prevent, detect and treat cancer. This material will be provided to research scientists.

WHO WILL BE ASKED TO TAKE PART: People who meet one of the criteria listed below.

. (1) Larynx cancer
. (2) Salivary gland tumor (benign or malignant)
. (3) Upper aero-digestive tumor
. (4) Tumor of the lymph nodes of the neck
. (5) Tumor of thyroid or parathyroid gland

THE TISSUE COLLECTION PROCESS: If you agree to take part in this study, we will ask you to do some or all of the things listed below. You can choose to take part or not to take part in any of these things.

We may ask your permission to get copies of your medical records. In order for our research to be valid, we must document the precise diagnosis of cancer and other conditions that might be associated with cancer. If you received medical care at
Christiana Care Health Services, we ask your permission to review these records. We ask you to help us obtain your medical records from other hospitals or doctors by signing a medical record request form and sending a copy to your hospital or doctor. Alternatively, we ask you to give us the names of your doctor(s) and hospital(s) that have your records; we ask your permission to contact them to get your records.

Specimen Procurement: All normal and pathological tissue specimens collected during head and neck surgery will be supplied through the mechanism established in the Helen F. Graham Tissue Procurement Center under the direction of Mary Iacocca, M.D. in the Department of Pathology. Dr. Robert Witt will oversee all aspects of tissue collection from patients undergoing scheduled head and neck surgeries and will ensure that they are submitted to the Department of Pathology in a timely manner. Specimens (vocal fold, salivary gland, thyroid and/or parathyroid gland, lymph node, upper aero-digestive mucosa) will be collected for one of three general purposes (collection for culture, collection for cryopreservation studies, and collection for biomarker analysis) and the treatment of the tissue at the time of collection will be governed by the purpose. Selected tissues samples will be submitted for investigational study and will be in excess of tissue submitted to pathology for analysis. The decision as to whether any of the tissue is collected for the Tissue Procurement Center (TPC) will be the decision of the pathologist as per the TPC protocol.

TESTING TO BE PERFORMED: The tissue you give us will be a source of biomarkers and will be used for cancer research over the coming years. The exact tests that will be performed are not known at this time but are likely to include testing to regenerate salivary gland, thyroid gland and/or parathyroid gland, and vocal
folds, means to preserve tissue (cryopreservation), and identification of biomarkers. By signing this form you agree to let us make your tissues available to future approved research programs. In granting this permission, you also agree to relinquish all rights and ownership of the tissues.

We will not store tissue for future testing for you. We cannot return your tissue to you, or use it for any testing you may want in the future. Research samples may be destroyed without consulting you.

RISK TO YOU There are no physical risks to you from participating in this research.

With any kind of tissue testing, even for research, there are social implications for you to consider. One such potential issue is misuse of information by insurance companies or employers. We will not release any information about you to your insurance company or employer. However, rare instances are known in which information has been obtained by others that has affected research participants’ ability to get or to maintain their health insurance, life insurance, and/or job. Because research findings are not identified by name and are not given to you or entered into your medical record, we believe there are no such privacy risks to you.

BENEFIT TO YOU: Participation in research studies that will use your tissue may or may not be direct benefit to you. This is truly a volunteer study for research purposes, and you will not receive results. However, if the principal investigator or their designated investigator finds information that is clinically significant to health care then participants will be notified of these findings if they so desire.
NOTIFICATION OF FINDINGS: Because the laboratory testing to be done using the samples you provide is not diagnostic testing, it is not appropriate to give you results of this research. However, you may choose at the end of this consent to be notified periodically of advances in the fields of regeneration of salivary glands, thyroid gland and/or parathyroid gland, and vocal folds, as well as preservation (cryopreservation) of tissue, and biomarkers. It is your responsibility to inform us in writing if you move or change your address if you wish to continue to receive information.

IF THIS RESEARCH PROGRAM IS ENDED, the information you provided will be stored in accordance with applicable research regulations. Tissue samples will be destroyed.

PRIVACY AND CONFIDENTIALITY: The records, which reveal your identity, will be kept in locked files. Information that could identify you as an individual will not be released to any research scientist using your tissue samples. Your identity will only be available to the data collection research team and to those people responsible for research program oversight. These are the Christiana Care Health Services Helen F. Graham Cancer Center, Institutional Review Board (the IRB is a committee that reviews research projects to help insure that the rights of the research subjects are protected); the Office for Human Research Protection (OHRP) of the United States Department of Health and Human Services and the Food and Drug Administration (FDA).

Confidentiality and privacy of Information about personal and medical history is maintained even between members of the same family.
USE OF RESEARCH RESULTS: The results of research using the tissue in this program may be presented at scientific meetings or published in the medical literature. In such presentations or publications, it will not be possible for others to identify you as an individual.

QUESTIONS ABOUT THIS DATA, BLOOD AND TISSUE COLLECTION PROGRAM should be directed to Robert L. Witt, M.D. (302) 888-1980.

QUESTIONS ABOUT YOUR RIGHTS AS A RESEARCH SUBJECT should be directed to the Christiana Care Institutional Review Board (a committee that reviews all research to be certain the rights and welfare of research participants are protected) at (302) 623-4983.

If the Principal Investigator or their Designated investigator finds information that is clinically significant for your health care, would you like to be notified of these results:

_________________________  _______________________

Please initial appropriate box and date: