Spring 5-2022

The Role of Angiocrine Factors in the Progression of Salivary Disease

Owen Zon

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The Role of Angiocrine Factors in the Progression of Salivary Disease

An honors thesis presented to the
Department of Biology,
University at Albany, State University of New York
in partial fulfillment of the requirements
for graduation with honors in Biology
and
graduation from The Honors College

Owen Zon

Research mentor: Amber Altrieth, B.S.
Research Advisor: Melinda Larsen, Ph.D.

May 2022
Abstract

In our blood vessels, cells called endothelial cells line the inside of veins and arteries. Due to the discovery that endothelial cells actually organogenesis during embryogenesis, our interest in these cells has increased to a new understanding of their functions in tissue repair and regeneration. These cells are no longer only considered passive modes of transport for bodily fluids and nutrients, but, with new research in the fibrosis of liver cells, are also considered to be key players in the repair of damaged organs and tissue. These cells release signals known as angiocrine factors that act in damaged areas of the body to induce regeneration, fibrosis, or inflammation. The angiocrine factors help to guide many steps of the process of self-renewal and differentiation of stem cells into functioning cells for the damaged tissue. These factors play a key role in the health and healing of organisms, but the question remains as to what factors are expressed from salivary gland endothelial cells, and how the expression of these factors changes due to injury. A good source of insight into this problem could be identifying the specific factors that contribute to the fibrotic phase in salivary gland injury and disease. First, we use a Non-Obese Diabetes (NOD) model mouse, which has phenotypes similar to Sjogren’s Syndrome, to examine the levels of expression of different genes in endothelial cells derived from NOD vs control mice. Second, to induce injury to the glands we performed a ductal ligation surgery, where a clip was applied to the salivary gland ducts, inducing a reversible fibrosis. We compared multiple protocols for releasing cells from fibrotic tissue to determine an optimized cell isolation protocol to use for single cell RNA sequencing. We found that utilizing the enzyme, Liberase, to dissociate cells in adult salivary glands, in combination with magnetic activated cell sorting to deplete EpCAM+ epithelial cells and red blood cells, produced the highest yield and cell viability with a yield of 4.175 x 106 cells and viability of 98.11% as compared to our other trials and our original collagenase/hyaluronidase cell dissociation protocol. We also released larger numbers of PDGFRβ+ fibroblasts and CD31+ endothelial cells with the optimized protocol. Compared to the mock sample, the ligated sample had a decrease in PDGFRβ+ cells. We then used the optimized cell dissociation protocol to release cells from a ligated salivary gland and a mock surgical gland and performed scRNASeq. Seurat-based clustering methods revealed a large number of endothelial cells released from both glands. This work paves the way for identification of differential gene expression by endothelial cells of the salivary gland following injury to inform future therapeutic strategies.

Keywords: Angiocrine, Endothelial, Fibrosis
Acknowledgements

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I thank the friends I have made throughout my college career who gave me respite during this process, especially those friends who wrote theses themselves, for sharing their topics and helping to inspire me.

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Introduction

Located near the mouth of many vertebrates are a group of glands called salivary glands (SG). These glands secrete saliva into our mouths, which helps in taste and digestion of food. There are three major pairs of SG: parotid, submandibular, and sublingual, each with similar structure. SG have branching, ductal structures, which are made up of different cell types. The epithelial cells that make up the gland include ductal cells, which are responsible for transporting the saliva, acinar cells, which are responsible for producing the saliva, and myoepithelial cells, which are thought to squeeze the acinar cells to assist in the secretion of saliva. The ducts lead to the oral cavity, in which it deposits the saliva created (Holmberg & Hoffman, 2014). The saliva created from the gland is an important contributor to several processes that take place in the mouth, with over 70% of it coming from the submandibular SG. It is an essential tool in taste and digestion, but it also helps to lubricate the mouth for swallowing and talking, as well as playing an important role in dental health (Holmberg & Hoffman, 2014). The gland also includes other cell types like endothelial cells, stromal cells, and nerves which surround the epithelial portion of the gland, aiding in its function. The glands, however, are not immune to disease and various ailments can cause a change in saliva production such as Sjögren’s Syndrome, cancer, and drug use.

In our capillaries, the cells that line the inside surface are typically known as endothelial cells. These cells were originally thought to simply be passive conduits for delivering oxygen, regulating the transportation of inflammatory cells and gatekeeping cellular metabolism (Rafii et al., 2016). The location of the endothelial cells allows for such varied function, as the cells reside on the barrier between blood and tissue. This causes these cells to have not only jurisdiction on what moves between the barrier, but it also gives them access to the rest of the body via the
circulatory system. Endothelium structure varies, depending on the needs of the underlying cells, with some parts being continuous and others having a large degree of disjunction. Along with varied structure, the endothelial cells display great heterogeneity in function. This further gives the cells flexibility in the extent of their permeability for nutrients to enter the tissues. As a whole, endothelial cells play an important role in metabolism (Aird, 2012).

While this function is widely known, there has been a recent development in the knowledge pertaining to endothelial cells, showing that endothelial cells play an important role in tissue repair. One mechanism that allows for this interaction are called angiocrine factors. These factors are composed of secreted, and membrane-bound inhibitory and stimulatory growth factors, cytokines, chemokines, and other cellular products that help to regulate regenerative processes. The specific type of angiocrine factor is determined by the endothelial cell, as differing ECs (for example, ECs can be isolated from the brain, heart, fetal tissue, etc.) yield different factors (Rafii et al., 2016).

The activation of angiocrine factors is caused by the activation of signaling pathways caused by stress due to tissue damage and inflammation. The resulting angiocrine factors travel to a local site of tissue damage. They then activate the processes of stem cell differentiation to make replacement cells for damaged tissue. This process has been found in the regeneration of organs such as the liver, which already has a history of being a very regenerative organ, as well as development in fetuses. In the liver, the angiocrine factors enable the repopulation of liver cells by enabling the proliferation of Axin2- and T-box transcription factor 3-positive cells (Wang et al., 2015). A similar process occurs in other instances of regenerating organs; however, the specific angiocrine factor(s) and the resulting differentiation are different. Considering endothelial cell position in the body, it’s possible that endothelial cells have a role in releasing
angiocrine factors through the blood vessels that they line. This makes them a prime target for research.

To better understand the contribution of endothelial cell signaling to fibrosis, I will use two approaches. First, I will examine gene expression differences in the endothelial cells derived from a Non-Obese Diabetes (NOD) model mouse, which has phenotypes similar to the autoimmune disease, Sjogren’s Syndrome, in comparison with control mice to examine the levels of expression that different genes have during this disease. Second, as single-cell RNA-sequencing (scRNASeq) is an ideal method for identifying gene differences between endothelial cell types in a diseased or injured state vs cells in a normal state, I will optimize conditions for isolation of endothelial cells from injured vs normal salivary glands. To induce injury to the glands we performed a ductal ligation surgery, where a clip was applied to the salivary gland ducts, inducing a reversible fibrosis. Using methods previously identified in studies of other organs, I tested multiple parameters for cell isolation to identify a suitable protocol to use in combination with scRNASeq. We then performed scRNASeq and evaluated the success of the cell isolation procedures in capturing endothelial cells.

Methods

A. Animal Maintenance

All animal husbandry, surgical procedures, and tissue collection were performed in accordance with protocols approved by the University at Albany, SUNY IACUC committee. Mice were housed in 12-hour light/dark cycle with access to water and dry food. C57BL/6-Tg(Cdh5-cre/ERT2)1Rha mice were ordered from Taconic Biosciences and crossed to the reporter strain (CAG)ROSA26<sup>tdTomato</sup> (tdT) from Jackson Laboratories (JAX#007909). C57BL/6-Tg(Cdh5-cre/ERT2)1Rha;(CAG)ROSA26<sup>tdTomato</sup> (Cdh5tdTom) mice were assigned a unique
identifier between postnatal day 7 and 10 and were genotyped with PCR to detect Cre and only a wildtype sample was used for RNA Extraction.

**B. RNA Extraction**

For qPCR optimization, the left salivary gland was harvested with no sublingual and weighed. A mortar and pestle were then used to disrupt the tissue in 600 $\mu l$ of Buffer RLT, and then homogenized using a pipette in the same Buffer. The Lysate was then centrifuged for 3 minutes at full speed, removing the supernatant afterwards via pipette and transferred to a new microcentrifuge tube. One volume of 70% ethanol was added to the cleared lysate and mixed immediately through a pipette. 700 $\mu l$ of the sample was then transferred, including any precipitate, to an RNeasy spin column placed in a 2 mL collection tube. The sample was then centrifuged for 15 seconds at over 10,000 rpm. The flow through was discarded. After this, 700 $\mu l$ of Buffer RW1 was added to the same spin column and the collection tube was centrifuged again for 15 seconds at over 10,000 rpm. This flow through was also discarded. After that, 500 $\mu l$ of Buffer RPE was added to the spin column and centrifuged for 15 seconds at over 10,000 rpm, and the flow through was discarded. Another 500 $\mu l$ of Buffer RPE was added, but this time, it was centrifuged for 2 minutes at over 10,000 rpm. The Spin Column was then placed in a new 2 mL collection tube and centrifuged for 1 minute at full speed. The flow through and old tube were discarded. The Spin Column was then placed in a new 1.5 mL collection tube and 50 $\mu l$ of RNAse-free water was added. The sample was then centrifuged for 1 minute at over 10,000 rpm. This step was then repeated, this time with 50 $\mu l$ of RNAse-free water for a total of 80 $\mu l$.

**C. cDNA Synthesis**

In a sterile RNAse-free microfuge tube, 7 $\mu l$ of the RNA sample, 2 $\mu l$ of 50 $\mu M$ dT oligo primer, and 1 $\mu l$ of 10 $\mu M$ dNTP are mixed to make a total of 10 $\mu l$. The sample was then
denatured for 5 minutes at 65°C, Spun briefly, and then immediately put on ice. In a separate PCR tube, 2 \( \mu l \) of 10X M-MuL V buffer, 1 \( \mu l \) M-MuL V RT (200 U/\( \mu l \)), 0.2 \( \mu l \) of RNase inhibitor (40 U/\( \mu l \)), and 6.8 \( \mu l \) of nuclease-free water were mixed together. The 10 \( \mu l \) RNA sample/primer solution was then added to the PCR tube. The mixture was then incubated at 42°C for 1 hour, and then the enzyme was inactivated at 65°C for 20 minutes. The product was stored at -20°C until used in Real-Time PCR.

D. Real-Time PCR

Enough of each component was collected for all samples as well as one extra sample (8 primers and 1 extra) and enough for 4 wells is made. The cDNA sample was diluted to be 1 \( \mu M \) in preparation for the PCR. Two master mixes are made. The Master mix of for the sample group consisted of 360 \( \mu l \) of Sybr Green, 396 \( \mu l \) of Nuclease-free water, and 36 \( \mu l \) of cDNA. In the master mix for the non-template control (NTC), 360 \( \mu l \) of Sybr Green and 432 \( \mu l \) of Nuclease-free water were mixed. In labeled microfuge tubes, 88 \( \mu l \) of the Master Mix was added to half of tubes and 88 \( \mu l \) of the NTC Master Mix was added to the remaining half. Next, 6 \( \mu l \) of a 10 \( \mu M \) Forward Primer and 6 \( \mu l \) of a \( \mu M \) Reverse primer were added to a microfuge tube labeled with their corresponding primers. These primer/sample solutions were then pipetted into 4 wells each on a PCR plate. The amount pipetted was 20 \( \mu l \) for each well. The identity of the primer in each well was carefully recorded for future reference. The PCR Plate was centrifuged for 5 minutes at 1465 rpm, and then put in the thermocycler for the following protocol: 96°C for 3 minutes, 96°C for 15 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and then this process is repeated 39 times. After 39 cycles the plate is heated! to 65°C for 5 seconds and then heated to 95°C in a stepwise manner (5°C every 30 seconds), during which the readings are done.
E. RNA Extraction (Micro)

First, Wash Solution 2/3 is warmed to room temperature, and 50 µL of Elution Solution are heated to 75°C on a heat block. Cultured Cells are obtained from salivary gland tissue and counted. The cells are centrifuged and pelleted with the supernatant removed afterwards. The cell pellet is then resuspended in 100 µL of Lysis solution by vortexing for about 10 seconds. 50 µL of 100% ethanol is then added to the mixture and vortexed briefly but thoroughly. The Ethanol/Lysate mixture is then loaded onto a Micro Filter Cartridge Assembly. The assembly is centrifuged for 10 seconds on the highest speed. After centrifugation, 180 µL of Wash Solution 1 is added and the assembly is centrifuged again for 10 seconds. Another 180 µL is added, this time of Wash Solution 2/3, and the centrifugation is repeated. Afterwards, the filter cartridge is removed from the collection tube and the flow through is discarded. The filter cartridge is put back in the collection tube and centrifuged again for 1 minute to remove any residual fluids. Next, 10 µL preheated Elution Solution is added to the filter and allowed to sit at room temperature for 1 minute. The tube is centrifuged for 30 seconds to elute the RNA. This is repeated with 5 µL of Elution solution for a total of 15 µL.

F. DNase Treatment

In the collected RNA, 1.5 µL of 10X DNase Buffer and 1 µL of DNase 1 were added. The DNase reaction is incubated at 37 °C for 20 minutes, as the DNase Inactivation Reagent is allowed to thaw at room temperature. After vortexing the DNase Inactivation Reagent to ensure it is properly mixed, 2 µL of DNase Inactivation Reagent is added to the RNA. The reaction is then stored at room temperature for 2 min, vortexing once during this time frame. The Mixture is then centrifuged at full speed for 1.5 minutes to pellet the Inactivation Reagent. The RNA is pipetted out and transferred to a new RNase free tube and stored at -20 °C.
NOD Models

A. Background

The salivary gland, like many other organs in the human body, is susceptible to various diseases. Sjogren’s Syndrome (SS) is an inflammatory disease that causes the impairment of exocrine glands, such as SGs. The specific cause of the disease is currently unknown, but it has been attributed to a combination of genetic, environmental, and hormonal causes. SS affects 9 females to every 1 male, and is usually hard to detect, as its primary symptoms (dry mouth and eyes) are usually found in other diseases as well and is sometimes a common aging effect in elderly patients (Tincani et al., 2013). The disease has also shown structural changes in epithelial cells in salivary glands, with said glands showing alterations in cell-cell adhesion and shape. In some cases, the disease causes apoptosis in the ECs, usually caused by activated T or B cells (Tincani et al., 2012). The impairment of the SG can lead to health risks in the mouth such as bad breath, pH imbalance, increased chance of oral infection and cavities, as well as trouble eating due to a lack of lubrication (Iorgulescu, 2009). There are methods to alleviate these symptoms, as well as improved criteria in the diagnosis of the disease, but there remains a lack of a method that can directly repair damage done to the SG in such diseases (Shiboski et al., 2017). Current Treatments include over-the-counter remedies such as sugar-free gum, and Biotene rinses, which may not be able to fully alleviate symptoms, and sialagogues, which may have side effects and require a commitment to the regimen.

To better study this disease, we have obtained a mouse strain from the Jackson Laboratories in which the conditions of SS, as well as other similar diseases, are replicated. This non-obese diabetic (NOD) mouse model will allow us to perform experiments on the expression of angiocrine factors in the diseased condition, giving us an informative view into how certain
factors are changed when disease is present. Female mice have SGs that more closely resemble the human SG and thus female mice were used in the study. The NOD mice develop autoimmune exocrinepathy within 8 to 12 weeks of age. This condition shows significant similarities to SS in human patients as well as characteristic autoantibodies for the SS disease. The phenotype presents itself earlier in the submandibular glands of female mice (Gervais et al., 2015). We first plan to test the primers we will use to ensure that they are functional. After, we will use qPCR to determine the fold change in expression between an NOD mouse and a healthy CD1 mouse.

B. Results

1. RNA isolation purity

The goal of this first experiment was to determine if the primers we had on hand were acceptable for use in future experiments. We used a Cdh5CreERT2;ROSA26tdtom mouse (mouse T22) (Cdh5tdttom) and harvested the left salivary gland, which weighed .0272 g without the sublingual. In this experiment we isolated RNA using the RNA micro extraction kit from the tissue sample using the RNA micro kit and got a product with a concentration of 137.8 ng/μL. The product was eluded in 80 μL of RNAs-free water, giving us a total of 11.024 μg of extracted RNA. To determine the purity of the extracted RNA, we look to the 260/280 ratio and the 260/230 ratio, which will be discussed later. For the 260/280 ratio, we got a measurement of 2.12 and for the 260/230 ratio, we obtained a measurement of 1.64 (Figure 1). Afterwards, we converted the RNA into cDNA and it underwent qPCR.
2. qPCR Amplification Curves and Melt Curves

**Figure 1: Nano drop Spectrophotometer**
This is a nanodrop spectrophotometer reading of RNA that was collected from the left submandibular salivary gland (no sublingual) from a Cdh5tdtom mouse, identified as T22.

K5

**Figure 2: Krt5 qPCR Amplification Curve**
The qPCR amplification curve for cytokeratin (Krt5) in Cdh5tdtom T22 mouse (left) and a non-template control (NTC) (right). This specific graph shows a good example of an expected amplification curve. There are 4 replicates denoted by red line graphs for each in the graph.
**Figure 3: PDGFRα Amplification Curve**

The qPCR amplification curve for Pdgfra gene in a Cdh5tdtom T22 mouse (left) and NTC (right). This specific graph shows an example of an unexpected amplification curve. There are 4 replicates denoted by red line graphs for each in the graph.

**Figure 4: Krt5 Melt Curves**

Melt curves for the Krt5 cDNA from Cdh5tdtom T22 mouse (top) and NTC (bottom). These specific graphs show an expected melt curve for a single qPCR product displaying only a single peak.
Figure 5: PDGFRα Melt Curves
Melt curves for the Pdgfrα gene for Cdh5tdtom T22 mouse (top) and NTC (bottom). These specific graphs show unexpected melt curves for a single qPCR product with more than one peak.
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Note. A chart showing the C\textsubscript{T} values and their distribution obtained from the qPCR experiment for different genes on a Cdh5tdtom mouse. For each gene, four replicates were tested and the C\textsubscript{T} value for each is shown along with the average and the standard deviation (St Dev).

After the qPCR was completed, we found the C\textsubscript{T} values of each primer from four replicates for Cdh5;tdtom (mouse T22) or non-template control (NTC) samples. Two different genes, Krt5 and Pdgfra, were highlighted in the figures section, along with their amplification curves and melt curves. The amplification curves show at which cycle expression began to accelerate, essentially giving us an idea of how high the expression is for each gene (Figures 2 and 3). For K5, there were four replicates whose values were close together with the NTC values being close to zero (Figure 2). For Pdgfra, the replicates had values that were more varied and NTC values that were greater than zero (Figure 3). The melt curves, on the other hand, show the purity of the product generated (Figures 4 and 5). The melt curve for Krt5 has four replicates with each sharing a similar peak, while the NTC has no peaks (Figure 4). The melt curve for Pdgfra has multiple peaks with peaks present in the NTC replicates. For the Krt5 gene, after setting a threshold, we obtained Ct values of 25.58, 25.85, 25.67, and 25.65. These values give an average of 25.6875 and a standard deviation of 0.115 (Table 1). The Pdgfra gene had Ct values of 27.8, 25.19, 27.36, and 28.1, with an average of 27.1125 and a standard deviation of 1.317203 (Table 1). The remainder of the primers’ Ct values are recorded in Table 1, along with their averages and standard deviations to show how similar their values are.
3. Fold Change Graphs

**Figure 6: Fold Change Graph Set 1**
Fold Change between CD1 and NOD Mouse strains for Acta 2 (encodes aSMA) (a), Spno2 (b), Pdpn (c), Col6a6 (d), CD31 (e), and Krt5 (f).
Figure 7: Fold Change Graph Set 2
Fold Change between CD1 and NOD Mouse Strains for Igfbp2 (a), Vegfa (b), CycB (c), and CD31 (d).
4. Ct Value Graphs:

**Figure 8: Fold Change Graph Set 3**

Average Ct Values for 4 technical replicates between CD1 and NOD mouse strains for CD31 (a) and Acta 2 (encoding SMA) (b)
We wanted to determine the difference in the expression of certain genes in a healthy mouse’s glands and an NOD strain, which mimics SS. We first isolated the RNA using Arcturus PicoPure RNA Isolation Kit. We used qPCR to quantify the fold changes between the two strains. In figures 6a – f, qPCR was run for two mouse strains: A healthy strain (CD1) and a diseased NOD strain, which has a similar genetic background to the CD1 strain. In Figure 6a, the fold change between those two strains for the SMA gene, a filament that is expressed by myoepithelial cells that aids in saliva secretion, was .07. According to Figure 6b, the fold change for the Spno2 gene, a regulator of a pro-inflammatory cascade which is used to induce an inflammatory response in diseased tissue, was 0. The fold change was found to be .68 for the Pdpn gene, a lymphatic endothelial cell marker, (Figure 6c), and .69 for the Col6a6 gene which anchors the basement membrane to the extracellular matrix as well as providing mechanical structure to the cell (Figure 6d). In Figure 1e, the fold change for the CD31 gene, a marker that is commonly found in endothelial cells, which also has been known to have anti-inflammatory functions was .28, and according to Figure 1f, the fold change for the K5 gene, which is another structural protein that helps with the structure of certain cells, was 9.95. SMA, Spno2, Pdpn, Col6a6, and CD31 decreased in expression in the NOD model except for Krt5.

In figures 7a – 7d, another separate qPCR was run for the same mouse strains to examine additional genes. The fold change for the Igfbp2 (Insulin Growth Factor Binding Protein 2), an inhibitor of IGF-mediated growth factors, gene was 6.58 (Figure 7a), and the fold change for the Vegfa gene, which codes for a growth factor specific to endothelial cells, was found to be 4.59 (Figure 7b). According to Figure 7c, the fold change for the CycB gene, a primary protein used in the cell cycle, specifically the G2/M gateway before mitosis, was 2.67. Finally, in Figure 2d,
the fold change for the CD31 gene was .49. We found that Igfbp2, Vegfa, and CycB all showed an increase in expression, while CD31 decreased.

To determine how reliable the pipetting was in each experiment, one primer from each qPCR experiment had its average $C_T$ value and standard deviation displayed in a graph (Figures 8a – b). According to Figure 8a, the average $C_T$ value for the primer CD31 was 29.02 with a standard deviation of .84 for the CD1 model mouse, and 31 with a standard deviation of 2.8 for the NOD mouse. Finally, the average $C_T$ value for the aSMA primer in the CD1 mouse was 32.26 with a standard deviation of .98, as compared to the average $C_T$ value of the same primer in the NOD mouse, which was 37 with a standard deviation of 3.2 (Figure 8b). We found that the $C_T$ values were close together in both SMA and CD31, suggesting robust pipetting technique.

C. Discussion

The experiment first had the purpose of testing the primers on hand, and then compare the expression of certain genes between a diseased and control mouse strain. Earlier, we obtained 11.024 µg of RNA from our RNA Extraction on Cdh5tdtom T22. To validate the purity of our RNA, we used a nanodrop spectrophotometer to measure 260/280 and 260/230 values (Figure 1). The 260/280 ratio is used to determine if there is any protein contamination evident in our product. The ideal value for this is usually expected to be around 2.0. With our measured value being 2.12, we can safely assume there is likely no protein contamination occurred during our extraction. There are other organic contaminants, however that this ratio does not account for, so the 260/230 ratio must also be used to verify purity. The ideal value for this ratio is from 2.0 to 2.2. Our measurement, however, was 1.64. With such a different value from the expected, we can assume that some organic material may have contaminated our extraction. This may have led to some of the discrepancies we can see in our Ct values. In the future, this could be avoided by
taking more precautions to keep the workspace sterile, like cleaning more thoroughly or keeping all tubes closed until they need to be open. We could also include some additional washing steps to further remove the buffers that were used in the extraction. From our nanodrop graph, we found that there was very little protein contamination, but there was organic contamination that might have affected downstream analysis.

After the isolation, the RNA was then used in qPCR to generate amplification curves and melt curves for each primer. We obtained this data from amplification curves measured after the synthesis of cDNA was complete. Each primer had its own graph which showed all four replicates’ curves. As an example, we can see the amplification curve for the Krt5 and the Pdgfra genes. In figure 2, The Krt5 amplification curve, we can see that the curves for each sample are hardly distinguishable without the help of the program used to obtain the curve. If each sample had the same primer for the same gene, then it would make sense for each sample to begin rapid expression at the same time. Since each sample measured out to have similar C_T values along with an average close to the C_T values and small standard deviation, and because the NTC seems to have had no expression as indicated by its flat amplification curve, we can conclude that the K5 primer worked well in our tissue and that it is expressed.

The PDGFRα curve, however, tells a different story. In the graph we can see that some samples have radically different curves, along with curves appearing in the NTC version of the amplification Curve. In the NTC, there is no cDNA available to cause any expression at all. We can see in the NTC version of the Krt5 Amplification Curve, there are no curves similar to the ones found in the T22 version. In fact, the NTC version is relatively flat, indicating no expression. The curves appearing in the NTC Curve, may indicate there was some contamination in the affected samples, possible the organic contaminant indicated by the 260/230 ratio in figure
1. Either that, or the primer might have been non-specific. In the T22 Curve for Pdgfr, the fact that the curves are not similar, along with the less than ideal standard deviation and average, show that the Pdgfr Curve is a good example of a less-than-ideal Amplification curve. As we know that there is PDGFRα and PDGFRβ RNA in salivary glands of adult mice based on protein detection by immunohistochemistry, these primers likely require resdesign.

The melt curves also help to determine the purity of the product of the experiment. In a pure substance, there should only be one melting point, as only one qPCR product is generated. If there is more than one melting point in a substance, that means that the substance is impure due to other contaminants, such as primer dimers, or other products caused by non-specific amplification of the primer itself, or that there are different isoforms of the gene expressed in the tissue. In the melt curves shown in figures 4 and 5, we can see this principle. In the Krt5 melt curve, we can only see a peak at one temperature on the graph. This means that all four replicates had the same melting point, meaning that the product of the qPCR reaction was the same in each case, thus leading to the conclusion that the product was relatively pure. In the Pdgfr melt curve, however, we can see multiple peaks at various temperatures. This means that the product of the qPCR reaction for this primer differed in purity in each replicate. This also shows that the Pdgfr primer requires further investigation.

We used the same criteria for the 18 other primers tested. For the rest of these primers, very few have a standard deviation above 1, which would be similar to the Pdgfr samples, which we already established as the example for an unexpected result. Judging from this and the amplification curves and melt curves obtained from each. Most of the primers seemed to have worked as intended, even with one primer (Ubc) being used twice and both times having good results, although the second time yielding even better results from the first. Using our generated
amplification and melt curves, we were able to verify the functionality of the primers. We found that the primers for Cdh5, CD31, Krt5, Krt7, Krt19, Krt14, Vim, Ubc, Acta2, Spdef, Spno2, Podn, Pdpn, Col6a6, and Col6a3 passed this quality control test while primers for Pdgfra, Cnn1, Thbs4, and Gdf6 were considered less than ideal and require reassessment or redesign (Table 1).

We wanted to determine the changes in CD31, Cdh5, Krt5, Krt7, Krt14, Krt19, Vim, Ubc (Housekeeping primer), aSMA, spno2, Pdpn, Col6a6, Igfbp2, Vegfa, and CycB, in a healthy control mouse and a diseased mouse. To do this, we utilized qPCR to measure changes in expression (Figure 6a-f and 7a-d). For most of the primers tested, we noticed a decreasing trend between the two strains. The aSMA gene codes for alpha Smooth Muscle Actin, which, in addition to being a structural protein, is also expressed in pericytes which surround endothelial cells as well as in myofibroblasts. A decrease in this gene may cause a change in the salivary gland’s structure, possibly leading to changes in function as caused by the disease. Since it is a corepressor of the inflammatory response, a decrease in Spno2 expression would cause a greater inflammatory response, which is typical of a diseased tissue. Past studies have shown that chronic inflammation is a component in human, diseased salivary glands (Kiripolsky et al., 2017). The decrease in Pdpn is unexpected, because lymphatic endothelial cells are involved in the inflammatory process, so a decrease in expression may lessen an inflammatory response. The decrease in Col6a6 shown can lead to a decrease in extracellular matrix stability which suggests that the structure of the SG may be altered in the diseased state. We determined that there was a drastic decrease in the expression of CD31 in the diseased model, which indicates a decrease in endothelial cells or loss of their differentiation. This is in agreement with previous studies showing changes in endothelium in SS patients (Tektonidou et al., 1999). This can also indicate an increase in inflammation. We see that there was an increase in the expression of Krt5 when
diseased, which may means that there is an increase in structural support in the diseased mouse; however, more likely indicates in increase in the number of Krt5+ basal cells in the NOD mouse that were captured in our assay, which is consistent with results of our prior work (Gervais et al., 2015).

A second qPCR experiment was performed with the same CD1 vs NOD model, but different primers were used in order to see the fold change in expression (Figure 7a-d). The first primer, Igfbp2 showed an increase in expression in the diseased model, meaning an increase of the expression could lead to less growth of the cell. This is possibly due to a cellular response to the disease, in which the cell is trying to inhibit itself to lessen the effects of the disease. We see the opposite effect in the next primer, Vegfa, which shows an increase in this growth factor, suggesting a possible increase in endothelial cell proliferation. Although Vegfa increases, its possible that endothelial cell proliferation does not actually increase because it may be regulated at the protein level. In the next primer, CycB, we can see that there was a large increase in Cyclin B expression, meaning that there is more proliferation. The CD31 primer was used again in this experiment and yielded the same results as before.

Overall, we wanted to validate our primers using melt curves and amplification curves generated from them. Due to the characteristics displayed from melt and amplification curves, we found that majority of the primers tested were validated. Afterwards, we wanted to find the changes in expression between predetermined genes in a NOD diseased mouse and a CD1 healthy mouse. We found that SMA, Spno2, Pdpn, Col6a6, and CD31 all decreased in the NOD model while Krt5, Igfbp2, Vegfa, and CycB all increased. It seems that myofibroblasts and vascular smooth muscle cells (SMA), Endothelial cells (Spno2 and CD31), lymphatic endothelial cells (Pdpn) and some extracellular matrix genes (Col6a6) may be decreasing in the diseased
model. Ductal cells (Krt5), cells transitioning from G2 to M phase (CycB), and a subset of growth factors (Igfbp2 and Vegfa) seem to be increasing in the NOD Diseased model. Identifying genes that change with disease will contribute to future therapies.

**Single Cell Isolation for Single-Cell RNA-sequencing in Surgical Samples**

**A. Single Cell Sequencing**

When working with biological functions that may affect genetic expression in an organism, an important piece of data is the actual gene expression of an organism’s individual cells. Single-cell RNA-sequencing (scRNASeq) is a technique that sequences mRNAs produced by each individual cell, allowing us to look at differences in cell make up in an organ as well as gene expression in the same types of cells in different conditions. This can allow us to study the distribution of certain expressed genes in a genome at the single cell level. In doing this, we can determine not only how a cell in a sample expresses parts of the genome, but also predict the possible functions of specific cells via what genes are expressed. scRNASeq can also be used to see the effects of treatments on cell populations. The procedure begins by harvesting tissue from an organism and using specific protocols to break the tissue down into single cells and enriching them for the type of cells wanted. Afterwards the cells undergo a process that reads the entire transcriptome, and sequences it through software. The data is analyzed and presented in a graph that groups cells together based on how similar their expression levels are (Abreu, 2021; Stuart et al., 2019).

To do scRNASeq, we need to be able to separate the cells from the extracellular matrix (ECM) and from one another to prepare them for sequencing. There are many processes with which you can separate them, but most follow a similar process. First the harvested tissue undergoes a digestive process to remove the ECM, while also breaking connections between
cells to make single cells. However, the enzymes used for digestions can vary. For example, some protocols call for a mixture of enzymes including collagenase, deoxyribonuclease I (DNase I), and elastase (Adams et al., 2020; Habermann et al., 2020; Kalucka et al., 2020; Sasagawa et al., 2018). Collagenase is used to break down ECM and comes in different varieties including collagenase I and II, where II yields a higher degree of digestion of collagens. DNAse breaks down the DNA in cells to make it easier for RNA extraction by removing long strings of DNA released by dead cells, and elastase is used in the digestion of lungs and other organs which are high in elastin, giving the tissue a stretchy characteristic. Other protocols only call for Liberase, an enzyme which consists of a mix of collagenase I and II. Once the cells are separated from the ECM and other extraneous structures, it is time to sort the cells through a cell capture or depletion method. The cells are sorted due to the needs of the experiment.

The remaining cells are then washed and filtered through cell strainers of varying size to remove any more extraneous material. Finally, the cells may be stained and or enriched for specific types of cells depending on the purpose of the isolation. For isolating specific cells, some protocols call for erythrocyte lysis buffer to be added. This is done to remove red blood cells. Others use positive selection techniques, such as magnetic activated cell sorting (MACS) or fluorescent activated cell sorting (FACS). For MACS, microbeads identify and attach to specific proteins in cell types of interest using an antibody coat. These beads are magnetic, which allows you to separate attached cells using magnetic separation techniques. Staining involves associating cells with an antibody that includes a fluorescent molecule as a tag in FACS. An instrument can then differentiate the cells based on whether they have the fluorescent molecule attached to their surface or not.
Once the cells are successfully isolated, they undergo a PCR reaction which creates cDNA of expressed genes in each cell. The cDNA is then pooled into libraries containing barcodes to identify which cell it originated from (10X Genomics, 2017). We used Illumina sequencing to sequence the cDNA from our single cells by employing a “reversible terminator–based method” that detects specific bases as they are inserted into the DNA templates. This creates a base-by-base sequencing that eliminates context specific errors (Illumina, 2017). The sequencing data is then analyzed using downstream analysis software, including Seurat to cluster cells based on the similarities in their gene expression profiles (Stuart et al., 2019).

In our experiments, we used a mouse that underwent ligation surgery on the submandibular salivary glands. Ligation is a common experimental process in which a tool clamps a duct or vessel to cause controlled damage to said vessel or organ. In terms of animal models, this practice has helped contribute to understanding of inflammation, especially when used in terms of salivary glands (Cotroneo et al., 2008). It has also been shown that salivary glands that undergo ligation are still able to repair themselves following removal of the ligature (Cotroneo et al., 2008). Due to inflammation being a common symptom of many salivary diseases, the ligation gives us a way to simulate damage to the glands by clamping the excretory ducts of the gland, inducing inflammation and ECM deposition and giving us a sample to use single cell sequencing. The results can then be compared to a mock surgery to see the changes in expression of certain repair genes. This would allow us to understand possible physical and genotype changes possibly caused by damage to the salivary gland.
B. Methods

1. Single Cell Isolation

First, a pair of submandibular glands (SMG) was harvested from an adult female ROSA26tdTom<sup>+</sup> mouse with an age of 11 to 12 months old. The excess fat and tissue were removed from the glands and the glands were put in a new 60 mm dish containing 2.5 mL of 1X Phosphate-Buffered Saline (PBS), 0.5 mL of collagenase/hyaluronidase, 30 µL of DNase I and 1 mL of Dispase. The glands were then microdissected at room temperature for 30 minutes, and then triturated 100 times. The cells were incubated at 37°C for 15 minutes, then removed from the incubator and triturated another 100 times. The cells were incubated again for 5 minutes and triturated 100 times twice, then the cell suspension was transferred to a 15 mL conical tube on ice, and gravity sedimentation was performed for 10 minutes. After 10 minutes, the supernatant was isolated into a fresh 15mL conical tube, and the pellet was discarded. The enzyme activity was quenched using 3 mL of Dulbecco’s Modified Eagle Medium/F12/10% Fetal Bovine Serum and then centrifuged at 1490 RPM for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 2 mL of isolation buffer (Ca<sup>2+</sup>, Mg<sup>2+</sup> free PBS, 0.1% Bovine Serum Albumin (BSA), and 2 mM Ethylenediamine tetra-acetic acid pH = 7.4). Then 5 µg of EpCAM-A647 (10 µL) was added and triturated. The mixture was incubated at 4°C for 10 minutes, and then 4 mL of isolation buffer was added to wash the cells. The cells were centrifuged again at 1490 RPM for 5 minutes and the supernatant was discarded. The cells were resuspended in 1 mL of isolation buffer and transferred to a microcentrifuge tube. Prior to use we washed the Dynabeads, by pipetting 50 µL of beads and 1000 µL of isolation buffer into a microcentrifuge tube. The tube was then put on a magnet for 2 minutes and then the supernatant is removed, and the beads are resuspended in 50 µL. Next, 25 µL of sheep anti-rat Dynabeads was added to the microcentrifuge
tube with the 1 mL of isolation buffer with cell suspension, and the tube was incubated on ice on a rocker, moving back and forth, set to 4 (VWR Rocking Platform), for 20 minutes. The tube was then placed in a magnaRack Magnetic Separation Rack for 2 minutes and the supernatant was transferred to a fresh tube. The incubation and removal of supernatant was repeated for a total of 2 EpCAM depletions. The cells were then centrifuged again at 1490 RPM for 5 minutes. The cells were resuspended in 1.5 mL of Dulbecco’s Modified Eagle Medium/F12/10% Fetal Bovine Serum. 5 uL of the cell suspension was then diluted 1:1 in trypan blue. The mixture of cells and trypan blue were mixed and loaded on the hemocytometer and counted.

*Fixed Staining using Single Cell Isolation:* We used the same source of cells as well as the same protocol, however the cells were not imaged live, and were instead fixed in Matrigel.

*E16 Comparison for Single Cell Isolation:* This experiment used PDGFRβ instead of PDGFRα and was used as a positive control to compare the effectiveness of the PDGFRα stain to the effectiveness of PDGFRβ, which was used in the following experiments.

*Ter119 comparison experiment:* after the enzyme activity was quenched with 3 mL of the DMEM solution, the sample was split in half. One half proceeds as normal, aside from adding an additional 5 µg of EpCAM-A647, and in the other half, in addition to the 5 µg of EpCAM-A647, 5 µg of Ter119 antibody is added.

*Liberase with Red Blood Cell Depletion:* 35 mm dishes that contained different digestive enzymes, namely 1.134 mL 1X PBS, 0.2 mL liberase (TL Research Grade low Thermolysin), stored in 2.5 mg/mL aliquots, used at 0.25 mg/ml as recommended by the manufacturer, 30uL of 1 g/100 mL DNase I, and 0.666 mL of 10 mg/mL dispase.
2. Salivary Gland Ligation

C57Bl/6 female mice that were 12-weeks old were used for surgeries. The mice were first anesthetized using 0.1mL/10g ketamine/xylazine via intraperitoneal injection and were given 100 µL of buprenorphine at a concentration of 0.015mg/mL by subcutaneous injection as an analgesic for post-operative pain management. An incision was made to locate the main ducts of the submandibular and sublingual glands. A vascular clamp (Vitalitec/Peter’s Surgical) was then applied to the main ducts, and the incision was closed using two to four interrupted sutures. Mice were continuously monitored under anesthesia for pain, distress, and changes in weight following surgery. The ducts were ligated for 14 days, and successful ligation was determined as having gland weight between 30 to 70% of the control gland weight. Any samples outside of this range were excluded from future analyses. Mock surgery control mice received an incision, and the ducts were located, but not ligated, and then euthanized after 14-days post-surgery. The submandibular and sublingual glands were immediately weighed upon removal.

3. Cell Staining

Two 35 mm dishes had two non-sterile coverslips placed within each. Two of these coverslips would be used as no primary control samples and the other would be stained as normal. The isolated cells were diluted to a final cell number of 2 x 10^5 in 20 µL and 20 µL of the cell dilution was mixed with 20 µL of Matrigel. 10 µL of the cell-Matrigel mixture was plated on each cover glass. The cells were fixed using 1 mL of 4% paraformaldehyde (PFA) in 1X PBS at room temperature for 20 minutes on a rocker. They were then washed using 1 mL of 1X PBS at room temperature for 10 minutes. Then, the cells were permeabilized using 180 µL of 0.4% Triton X-100 in 1X PBS on top of the samples at room temperature for 20 minutes on the rocker. The samples were washed again using 180 µL 1X PBS at room temperature for 10
minutes, and then blocked using 100 μL of blocking solution (3800 μL 3% BSA in 1X PBS Tween + 200 μL Donkey Serum (5%) at room temperature for 1 hour on a rocker. Next, 100 μL of the Primary Antibody solution was added and incubated at 4C overnight. For experiments prior to the E16 experiment, the following antibodies were used: 2μL PDGFRα goat + 198 μL BSA in phosphate buffered saline with tween-20 (PBST) for the samples and 200 μL PBST in the no primary controls. For experiments after E16, the following antibodies were used: 2μL PDGFRβ goat + 198 μL BSA in PBST for the samples and 200 μL PBST in the no primary controls. Next, the samples are washed four times, each time using 180 μL 1xPBST at room temperature for ten minutes on the rocker. 100 μL of secondary antibody solution (187.9 μL 3% Bovine Serum Albumin in 1x phosphate buffered saline with 0.5% Tween 20 + 10 μL (5%) donkey serum + 0.4 μL anti-goat A488 (1:500) + 1μL CD31-PE (1:200) + 0.5 μL EPCAM-A647 (1:400) + 0.2 μL DAPI (1:1000)) is added to the samples and incubated for 2 hours and 15 minutes on the rocker at room temperature. Afterwards, the samples are washed four times using 180 μL of 1x PBST at room temperature for 10 minutes. The samples are dipped in DI water 3 times, and then mounted to a coverslip with the cells on the slide using glycerol-based mounting media.

4. Imaging

After staining and mounting were completed, the samples were then placed under a microscope (Zeiss Z1 Cell Observer widefield with an Axio712 mono camera (Carl Zeiss, LLC)) and imaged using the Zeiss Pro Imaging program. For each stain, an appropriate exposure is determined, taking each sample into account. We then made 10 CZI images; nine single tile images in a grid-like pattern, and one 2x2 tile image. These images were exported into original data TIFF files for quantification.
5. Quantification

Images taken of the samples were uploaded to the Fiji image processing software (Schindelin et al., 2012). First, any background was removed using a 25 pixel rolling ball. Next, a threshold was determined to ensure all visible stain was accounted for in the calculation, while minimizing background. Then, all images for a specific stain were run through the quantification program at their respective thresholds. The area of the stain in each image was then normalized to the area of its matching DAPI image and then averaged for each sample taken.

C. Results

1. Cell Count results

To determine if we could use frozen cell vials in our cell isolation experiments, we thawed cells of a known cell number and concentration and measured cell yield and viability after thawing. Before freezing, we had $1.1 \times 10^6$ primary SMG cells, with a 99% viability in our sample. The cells were then taken out of storage, thawed, and then counted. According to Table 1, in square one of the counting slide, there were 10 living cells and 23 dead cells. In Square 2, there was 1 living cell and 50 dead cells. In square 3, 15 live cells and 16 dead cells were counted, while in Square 4, there were 6 living and 24 dead. This gives us an estimated number of 80,000 living cells after thawing with a viability of 22%. In this experiment, we found that freeze thaw decreased yield and viability.

<table>
<thead>
<tr>
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<th>Square 2</th>
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<td>6</td>
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<tr>
<td>Dead</td>
<td>23</td>
<td>50</td>
<td>16</td>
<td>24</td>
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</tbody>
</table>

*Note. The amount of live and dead cells was recorded after removal of stoma cells from a 48-week-old C57 mouse from liquid nitrogen storage.*
For this series of experiments, we wanted to develop a method that provides the highest cell yield, while enriching for stomal and endothelial cells using a MACS-bead-based isolation method to capture PDGFRα/β-expressing cells and CD31-expressing cells, respectively.

2. Live Cell Staining using Single Cell Isolation

Table 3: Live Cell Staining Cell Yield and Vitality

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</tr>
<tr>
<td>Square 3</td>
<td>224</td>
<td>17</td>
</tr>
</tbody>
</table>

Note. A chart that displays the number of live and dead cells counted for each square counted on a hemocytometer.

Figure 9: Live Staining Images

Immunocytochemistry images of Celltracker, which binds to proteins in cells, and other stains. The first composite image on the left shows cells stained for CD31 and the second on the right shows cells stained for EpCAM.

Freeze and subsequent thawing of cells decreased cell viability, so we wanted to test live cell staining following isolation to measure yield and viability, and to determine what proportions of cells were endothelial. For this experiment, we used a collagenase, hyaluronidase dissociation with EpCAM depletion with live cells harvested from an adult female salivary gland. From the hemocytometer, we got an average number of 243.67 live cells, which gives us a
concentration of $4.773 \times 10^6$ cells/mL, for a total number of $7.31 \times 10^6$ live cells. We got a viability of 94.20% live and 5.80% dead (Table 3) and subjected them to immunocytochemistry (ICC) for Celltracker, CD31, and EpCAM (Figure 9). We found that the average area normalized to DAPI detected in the sample for the stains CD31 (endothelial cells), and EpCAM (Epithelial cells) were 10.04 and .091, respectively. Using Live cell staining with C/H, we counted $7.31 \times 10^6$ live cells, and we were able to detect CD31 and EpCAM in our sample; however, the number of CD31 cells was higher than EpCAM+ cells, revealing an enrichment for endothelial cells and a low level of epithelial cell contamination. Use of live cell staining resulted in a higher yield and viability than the freeze thaw, however not all antigens were able to be detected using this method.

3. Fixed Staining Using Single Cell Isolation

With live cell staining we are unable to stain for sensitive markers, so we wanted to test a fixation method. As we had difficulty in detecting PDGFRα staining in live cells, another experiment was done using the same protocol on fixed cells in order to determine if the fixation increases the expression of our cell markers by comparing the fixation to the live staining.
b.

Figure 10. Examination of PDGFRα protein detection in fixed primary stromal cell isolates
(a) Fixed Staining Images. Composites of DAPI-stained cells for two replicates and a no primary control sample. The first set shows DAPI (cell nuclei) and PDGFRα (stromal cells), the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) Fixed Staining Average Areas. Graphs show the average area of the PDGFRα, CD31, and EpCAM stains, normalized to DAPI area across all samples.

In order to test whether staining live cells yielded more signal than fixed cells, we used a collagenase, hyaluronidase dissociation of adult salivary glands with EpCAM depletion on fixed cells for comparison. Because we used the same source of cells as the previous experiment, the total number of cells should be comparable to the last experiment. We subjected them to ICC. We found that the average area normalized to DAPI detected in sample one for the stains PDGFRα, CD31, and EpCAM were 1.28 x 10⁻¹, .194, and .001, respectively. For replicate 2, the normalized average areas were 4.86 x 10⁻², .013, and .002. For the No primary sample, the normalized average areas were 2.59 x 10⁻⁵, .004, and .002 (Figure 10a-b). We were able to find PDGFRα, CD31 and EpCAM signals in our sample indicating that fixation allows for higher signal than live staining. Using fixed samples we were able to detect more sensitive markers, specifically PDGFRα.

4. E16 comparison

In our fixed sample from an adult mouse, we detected a low yield of PDGFRα stromal cells, so we wanted to use a positive control, for which we used mesenchyme from an embryonic
day 16 (E16) gland, which have previously been shown to express PDGFRα (Moskwa et al., 2022), to verify the antibody. We also wanted to compare PDGFRα and β to determine whether one will yield stronger signals. We plated and fixed a total number of 2 x10^5 cells and subjected them to ICC.

a.

<table>
<thead>
<tr>
<th>DAPI/PDGFRα</th>
<th>DAPI/CD31</th>
<th>DAPI/EpCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Replicate 1" /></td>
<td><img src="image2" alt="Replicate 1" /></td>
<td><img src="image3" alt="Replicate 1" /></td>
</tr>
<tr>
<td><img src="image1" alt="Replicate 2" /></td>
<td><img src="image2" alt="Replicate 2" /></td>
<td><img src="image3" alt="Replicate 2" /></td>
</tr>
<tr>
<td>No Primary</td>
<td>No Primary</td>
<td>No Primary</td>
</tr>
</tbody>
</table>
b.

**Figure 11: Immunostaining for PDGFRα in salivary gland E16 stromal cells**

(a) E16 Staining Images. Composite images of PDGFRα, CD31 and EpCAM-stained cells for two E16 replicates and a no primary control sample. The first set shows DAPI and PDGFRα, the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) E16 Average Areas. Graphs show the average area of the PDGFRα, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

We found that the average area normalized to DAPI detected in the sample for the stains PDGFRα, CD31, and EpCAM were $1.30 \times 10^{-5}$, $.003$, and $1.87 \times 10^{-4}$, respectively. For the replicate, the normalized average areas were $6.33 \times 10^{-4}$, $.002$, and $5.59 \times 10^{-5}$, respectively. For the no primary sample, the normalized average areas were $5.15 \times 10^{-4}$, $.001$, and $3.20 \times 10^{-2}$, respectively (Figure 11a-b). As expected for the E16 positive control, we were able to detect CD31 and EpCAM signals in our sample in fixed (or not fixed) cells; however, we were not able to detect PDGFRα.

5. Detection of PDGFRβ in E16 Cell Preparations

Due inconsistent PDGFR signal in the E16 gland in the previous experiment, we repeated the E16 positive control experiment to see if the PDGFRβ antibody would label cells more strongly than the PDGFRα antibody.
Figure 12. Immunostaining for PDGFRβ in salivary gland 16 stromal cells
(a) E16 Staining Images using PDGFRβ. Compositions of DAPI stained cells for 1 sample and a No Primary control sample. The first set shows DAPI and PDGFRβ, the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) E16 Average Areas using PDGFRβ. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.
For this experiment, we performed ICC to detect PDGFRβ in salivary gland stromal cells due to low PDGFRα signal in the first experiment. We had a total of 2 x 10^5 cells. We found that the average area normalized to DAPI detected in replicate one for the stains PDGFRβ, CD31, and EpCAM were .005, .001, and .027. For the No primary sample, the normalized average areas were 9.94 x 10^{-5}, 8.96 x 10^{-5}, and .018 (Figure 12a-b). We were able to detect Pdgfrβ, CD31 and EpCAM in our sample, demonstrating that the PDGFRβ antibody is effective for identifying stromal cells in primary SMG cells that were fixed with 4% PFA.

6. Depletion of red blood cells using the Terr119 antibody in combination with epithelial depletion

Now that a baseline for cell yield a viability was established and that we had a way to detect endothelial and stromal cells, we tested using C/H with EpCAM depletion compared to using C/H with EpCAM and red blood cell depletion (RBC).

<table>
<thead>
<tr>
<th>Cell counts</th>
<th>Live</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square 1</td>
<td>325</td>
<td>12</td>
</tr>
<tr>
<td>Square 2</td>
<td>364</td>
<td>5</td>
</tr>
<tr>
<td>Square 3</td>
<td>337</td>
<td>14</td>
</tr>
</tbody>
</table>

*Note:* A chart that displays the number of live and dead cells counted for each section of the hemocytometer.
a. DAPI/PDGFRβ  DAPI/CD31  DAPI/EpCAM

Replicate

Replicate 2

No Primary

40
b.

**Figure 13. Depletion of epithelial cells without RBC depletion**

(a) EpCAM Depletion Only Staining Images. Compositions of DAPI stained cells for two replicates and a No Primary control sample with EpCAM depletion only. The first set shows DAPI and PDGFRβ, the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) EpCAM Staining Only Average Areas. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

In order to test whether a red blood cell (RBC) depletion in tandem with the EpCAM depletion to remove epithelial cells would lead to higher cell viability and purity, we created two trials, one in which we did an EpCAM depletion by itself, and one where we did an EpCAM depletion along with the red blood cell depletion. This data reflects the EpCAM only trial. From the hemocytometer, we got an average number of 342 live cells, which gives us a concentration of $6.84 \times 10^6$ cells/mL. This gives a total number of $6.84 \times 10^6$ live cells. We got a viability of 97.1% live and 2.9% dead (Table 4) and we subjected them to ICC.

We found that the average area normalized to DAPI detected in replicate one for the stains PDGFRβ, CD31, and EpCAM were .122, $3.12 \times 10^{-3}$, and $4.61 \times 10^{-4}$. For replicate 2, the normalized average areas were .339, $1.79 \times 10^{-2}$, and $7.75 \times 10^{-4}$. For the No primary sample, the normalized average areas were .027, $1.03 \times 10^{-3}$, and $4.15 \times 10^{-4}$ (Figure 13a-b). We were able to detect Pdgfrβ, CD31 and EpCAM signals, indicating that we were able to detect stromal, epithelial, and endothelial cells.
7. Terr119 Comparison (Terr119)

### Table 5: RBC and EpCAM Depletion Cell Yield and Viability

<table>
<thead>
<tr>
<th>Cell counts</th>
<th>Live</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square 1</td>
<td>114</td>
<td>5</td>
</tr>
<tr>
<td>Square 2</td>
<td>94</td>
<td>7</td>
</tr>
<tr>
<td>Square 3</td>
<td>70</td>
<td>3</td>
</tr>
<tr>
<td>Square 4</td>
<td>114</td>
<td>5</td>
</tr>
</tbody>
</table>

*Note.* A chart that displays the number of live and dead cells counted for each section of the hemocytometer.

a. 

![Images of cell counts](image1.png)
b.

**Figure 14. Depletion of epithelial cells with RBC depletion**

(a) RBC and EpCAM Depletion Staining Images. Compositions of DAPI stained cells for two replicates and a No Primary control sample with EpCAM depletion and RBC depletion. The first set shows DAPI and PDGFRβ, the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) RBC and EpCAM Depletion Average Areas. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

Continuing from the last experiment, we used the second of two trials, one in which we did an EpCAM depletion by itself, and one where we did an EpCAM depletion along with the red blood cell depletion. This data reflects the EpCAM with the RBC depletion. From the hemocytometer, we got an average number of 98 live cells, which gives us a concentration of 1.96 x 10^6 cells/mL (Table 4). This gives a total number of 1.96 x 10^6 live cells. We got a viability of 95.14% live and 4.85% dead (Table 5). We subjected them to ICC.

We found that the average area normalized to DAPI detected in replicate one for the stains PDGFRβ, CD31, and EpCAM were .045, .002, and 2.12 x 10^-4. For replicate two, the normalized average areas were .057, .003, and 1.52 x 10^-3. For the No primary sample, the normalized average areas were .021, .010, and 1.67 x 10^-4 (Figure 14a-b). We were able to detect signals from Pdgfrβ, CD31 and EpCAM, indicating that we were able to isolate stromal, endothelial, and epithelial cell populations.

From this data we found that the EpCAM depletion with RBC depletion yielded similar areas to the EpCAM only, however they were more consistent across the replicates. Because we
did not want to sequence RBC, and the RBC depletion worked similarly to the EpCAM only, we decided to utilize RBC depletion in conjunction with EpCAM depletion in future trials.

8. Liberase with Red Blood Cell Depletion

Although C/H isolation with EpCAM and RBC depletion gave favorable results so far, we wanted to determine if use of a different enzyme, liberase, would result in a further increase of endothelial cell yield.

Table 6: Liberase Isolation Cell Yield and Vitality

<table>
<thead>
<tr>
<th>Cell counts</th>
<th>Live</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square 1</td>
<td>209</td>
<td>2</td>
</tr>
<tr>
<td>Square 2</td>
<td>203</td>
<td>8</td>
</tr>
<tr>
<td>Square 3</td>
<td>193</td>
<td>2</td>
</tr>
<tr>
<td>Square 4</td>
<td>230</td>
<td>4</td>
</tr>
</tbody>
</table>

*Note.* A chart that displays the number of live and dead cells counted for each section of the hemocytometer.
a.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Replicate 2</th>
<th>No Primary</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI/PDGFRβ</td>
<td>DAPI/CD31</td>
<td>DAPI/EpCAM</td>
</tr>
</tbody>
</table>
b.

![Graph showing Liberase Isolation Average Areas](image)

**Figure 15. Use of liberase to release stromal and endothelial cells**

(a) Liberase Isolation Staining Images. Compositions of DAPI stained cells for two replicates and a No Primary control sample. The first set shows DAPI and PDGFRβ, the second set shows DAPI and CD31, and the last set shows DAPI and EpCAM. (b) Liberase Isolation Average Areas. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

For this final experiment, we used Liberase with red blood cell depletion in the isolation. From the hemocytometer, we got an average number of 208.75 live cells, which gives us a concentration of $1.475 \times 10^6$ cells/mL (Table 5). For a total number of $4.175 \times 10^6$ live cells. We got a viability of 98.11% live and 1.88% dead and subjected them to ICC. We counted $4.18 \times 10^6$ live cells (Table 6).

We found that the average area normalized to DAPI detected in replicate one for the stains PDGFRβ, CD31, and EpCAM were 0.053, 0.078, and $8.05 \times 10^{-4}$. For replicate two, the normalized average areas were 0.076, 0.098, and $9.49 \times 10^{-4}$. For the No primary sample, the normalized average areas were 0.011, 0.024, and $6.28 \times 10^{-5}$ (Figure 15a-b). We were able to find signals of Pdgfrβ, CD31 and EpCAM. Digesting our samples with liberase resulted in an increased endothelial and stromal cell yield with an increase in viability.
### Table 7: Alterations of Isolation Protocol

<table>
<thead>
<tr>
<th></th>
<th>RBC Depletion?</th>
<th>Cell Yield</th>
<th>Cell Yield Normalized to Gland Weight</th>
<th>Viability</th>
<th>PDGFRα area</th>
<th>PDGFRβ area</th>
<th>CD31 area</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/H live staining</td>
<td>No</td>
<td>7.31 x 10⁶</td>
<td>6.26 x 10⁷</td>
<td>94.2% Live</td>
<td>NA</td>
<td>NA</td>
<td>10.04</td>
</tr>
<tr>
<td>C/H Fixed staining</td>
<td>No</td>
<td>Around 7.31 x 10⁶</td>
<td>6.26 x 10⁷</td>
<td>94.2% live</td>
<td>.0883</td>
<td>NA</td>
<td>.1035</td>
</tr>
<tr>
<td>C/H E16</td>
<td>No</td>
<td>2 x 10⁵</td>
<td>NA</td>
<td>NA</td>
<td>3.23 x 10⁻⁴</td>
<td>NA</td>
<td>.0025</td>
</tr>
<tr>
<td>C/H E16 repeat</td>
<td>No</td>
<td>2 x 10⁵</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>.005</td>
<td>.001</td>
</tr>
<tr>
<td>C/H EpCAM only</td>
<td>No</td>
<td>6.84 x 10⁶</td>
<td>6.65 x 10⁷</td>
<td>97.1% Live</td>
<td>NA</td>
<td>.2305</td>
<td>.01051</td>
</tr>
<tr>
<td>C/H Terr119</td>
<td>Yes</td>
<td>1.96 x 10⁶</td>
<td>1.90 x 10⁷</td>
<td>95.14% Live</td>
<td>NA</td>
<td>.051</td>
<td>.0025</td>
</tr>
<tr>
<td>Liberase</td>
<td>Yes</td>
<td>4.175 x 10⁶</td>
<td>3.74 x 10⁷</td>
<td>98.11% Live</td>
<td>NA</td>
<td>.0645</td>
<td>.088</td>
</tr>
</tbody>
</table>

*Note. A graph that displays the cell yield, viability, Pdgfrα/β signals area, and CD31 signals area.*

9. Ligated model staining with optimized protocol

Our previous experiments found that using liberase with EpCAM and RBC depletion had the highest viability and increased endothelial cell yield, so we used this method on surgical...
samples to determine yield, viability, and estimate cell proportions upstream of single-cell RNA-sequencing.

a. 

<table>
<thead>
<tr>
<th>DAPI/PDGFRβ</th>
<th>DAPI/CD31</th>
<th>DAPI/EpCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>
b.

**Figure 16: Release of cells from ligated salivary glands**

(a) Ligated Model Staining Images. Composite images of PDGFRβ, CD31 and EpCAM-stained cells for two replicates and a No Primary control sample. The set on the left shows DAPI and PDGFRβ, the set in the middle shows DAPI and CD31, and the set on the right shows DAPI and EpCAM. (b) Ligated Model Average Areas. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

For this experiment, we ligated the main ducts feeding into the sublingual and submandibular salivary gland of an adult female C57BL/6 mouse, and we used our optimized protocol with liberase followed by EpCAM and red blood cell depletion to isolate cells and stain to validate this effectiveness of this method in our surgical tissue. From the hemocytometer, we got an average number of 268.5 live cells, which gives us a concentration of 5.37 x10^6 cells/mL. For a total number of 1.34 x 10^6 live cells. We got a viability of 87.7% live and 12.3% dead and subjected them to ICC. We counted 7.095 x10^3 live cells.

We found that the average area normalized to DAPI detected in the first replicate for the stains PDGFRβ, CD31, and EpCAM were .231, .275, and .002. For the second replicate, the normalized averages were .146, .148, and .011. For the first no primary sample, the normalized average areas were 3.9 x 10^{-4}, 3.9 x 10^{-3}, and 3.77 x 10^{-4}. Finally, for the second no primary sample, the normalized averages were 6.32 x 10^{-5}, .005, and 4.0 x 10^{-4} (Figure 16a-b). We were able to detect signals for our cell markers. We found that this method was effective for breaking
down the fibrotic surgical sample and allowed for isolation of both endothelial and stromal cells and low epithelial cell contamination.

Figure 17: Ligated UMAP plot and Violin Plots
A UMAP plot showing the clusters of cells organized from RNA single cell sequencing of a ligated sample (a.) and 3 violin plots showing the distribution of cells expressing CD31 (b.), Pdgfrβ (c.), and EpCAM (d.).

Using the optimized cell dissociation protocol with liberase followed by MACS-based depletion of epithelial cells (EpCAM) and RBC removal, we wanted to see if we could find stromal and endothelial cells with single cell sequencing in adult salivary gland samples. Amber Altrieth performed a ligation surgery on a C57BL/6 12 old female mouse. After two weeks, the gland was harvested and the optimized liberase protocol was performed to release cells. Seurat
was used do identify cell clusters. In Figure 17, we have a UMAP plot with various clusters of cells. Through the RNA single cell sequencing we have identified cell clusters 1, 2, 5, 10, 11, 15, 17, 18, and 25 as endothelial cells that express CD31. With a total number of 11,366 cells sequenced, that gives us 35.5% of our cells in these clusters. Clusters 6, 8, 12, 15, and 24 are Pdgfrβ expressing cells, giving us a percentage of 15.3% of our cells in these clusters. Finally, Clusters 14, 21, and 23 express EpCAM, giving only 3.32% of our cells in these clusters (Figure 17a-d). We were able to show that our cell dissociation protocol was effective in releasing stromal fibroblast and endothelial cells for single cell sequencing while minimizing the number of epithelial cells, which was consistent with staining results.

10. Mock sample staining with optimized protocol

<table>
<thead>
<tr>
<th></th>
<th>Live</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Square 1</strong></td>
<td>105+103+98+79= 385</td>
<td>33</td>
</tr>
<tr>
<td><strong>Square 2</strong></td>
<td>91+106+70+83= 350</td>
<td>25</td>
</tr>
<tr>
<td><strong>Square 3</strong></td>
<td>78+89+100+85= 352</td>
<td>30</td>
</tr>
<tr>
<td><strong>Square 4</strong></td>
<td>75+99+81+77= 332</td>
<td>39</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>354.75</td>
<td>31.75</td>
</tr>
</tbody>
</table>

*Note.* A chart that displays the number of live and dead cells counted for each section of the hemocytometer.
a. DAPI/PDGFRβ  DAPI/CD31  DAPI/EpCAM

Replicate

Replicate 2

No Primary
Figure 18: Salivary gland subjected to mock surgery and cell dissociation
(a) Mock Surgical Model Staining Images. Composite images of PDGFRβ, CD31 and EpCAM-stained cells for two replicates and a No Primary control sample. The set on the left shows DAPI and PDGFRβ, the set in the middle shows DAPI and CD31, and the set on the right shows DAPI and EpCAM. (b) Mock Surgical Model Average Values. Graphs show the average area of the PDGFRβ, CD31, and EpCAM stains, normalized to DAPI area across all replicates.

As a control for the ligated surgical sample, we also performed a Liberase digestion with EpCAM and RBC depletion on a mock surgery gland, where the ducts are located, but are not clipped to measure changes in cell yield, viability, and marker signals. From the hemocytometer, we got an average number of 354.75 live cells, which gives us a concentration of 7.095 x10^6 cells/mL. For a total number of 1.773750x10^6 live cells. We got a viability of 91.79% live and 8.21% dead and subjected them to ICC. We counted 7.095 x10^3 live cells (Table 8).

We found that the average area normalized to DAPI detected in the first replicate for the stains PDGFRβ, CD31, and EpCAM were 8.27 x 10^-2, .795, and 6.86 x 10^-4. For the second replicate, the normalized averages were 8.43 x 10^-2, .698, 1.88 x 10^-3. For the No primary sample, the normalized average areas were 2.95 x 10^-3, 2.06 x 10^-4, and 2.04 x 10^-5 (Figure 18a-b). We were able to isolate endothelial and stromal cells with limited epithelial cell contamination.
Figure 19: Mock Ligated UMAP plot and Violin Plots

A UMAP plot showing the clusters of cells organized from RNA single cell sequencing of the mock ligated sample (a.) and 3 violin plots showing the distribution of cells expressing CD31 (b.), Pdgfrβ (c.), and EpCAM (d.).
We wanted to see if we could find cells that expressed our markers using single cell sequencing in the mock ligated sample. In Figure 20, we have a UMAP plot with various clusters of cells. Through the single cell RNA sequencing we have identified cell clusters 0, 1, 2, 4, 5, 8, 10, and 12 as endothelial cells that express CD31. With a total number of 4,334 cells sequenced, that gives us 59.9% of our cells in these clusters. Clusters 9 and 11 are Pdgfrβ expressing cells, giving us a percentage of 8.68% of our cells in these clusters. Finally, Cluster 16 express EpCAM, giving only 1.52% of our cells in these clusters (Figure 19a-d). We were able to show that endothelial and stromal fibroblast cells were released and were detectable using single cell RNA sequencing. The relative abundance of each cell population was consistent with our staining results with CD31 yielding the most signal area and being the most abundant cell population, and the epithelial cells having the lowest signal area and least abundant.

D. Discussion

This set of experiments had the goal of comparing different protocol alterations to determine the change in cell signal and cell count/viability. Between the first two experiments, we can see a great decrease in EpCAM signal, showing that we greatly reduced the interfering cells in our sample when we changed to a fixed cell sample. Between the fixed C/H experiment and the E16 positive control experiment, we found that the PDGFRα signal is very weak compared to the PDGFRβ signal. We can conclude that the β version of the stain yielded more of a signal and was used for subsequent experiments to stain the stromal cell populations. Next, between the two trials of the Terr119, although we had a larger cell yield and viability, the red blood cell depletion demonstrated a decrease in EpCAM signal as well as an increase in PDGFRβ and CD31 signal area, thus it was used in the next experiments. Finally, in the Liberase
experiment, we found that the viability and cell count was greatly increased, counteracting the decrease shown in both values from the RBC depletion experiment.

Looking at each of our experiments, the Liberase with red blood cell depletion protocol was the most successful. With a cell viability of 98.11% live and 1.88% dead, it had the highest ratio of live cells to dead cells out of all our trials. Along with this, the Liberase experiment showed an increase of both PDGFRβ and CD31 signals, showing an increase of stromal and endothelial cells tagged in the sample when compared with and normalized to the number of cells detected.

Another change to the protocol that seemed to be helpful in our data collection was the change from using an antibody to detect PDGFRα rather than PDGFRβ. In past studies, we have found that the PDGFRA epitope is lost following trypsinization and can be internalized during cell isolation. We used the first E16 experiment as a positive control to compare the methods for detecting PDGFRα and β with immunostaining in live cells. After switching to an antibody to detect PDGFRβ, we found it was possible to detect and quantify cells more effectively. The cells, however, are known to be sensitive to surgical removal. This causes the cells to internalize the receptors we stain for, leading to underrepresentation of those receptors. This is a major limitation as it can greatly affect our data.

We next wanted to see if we could get similar amounts of signals in our ligated glands and our mock surgical glands from our new protocol. For our ligation/mock surgical models, we saw that there was a substantial amount of endothelial and stromal cells in both models. There was an increase in PDGFRβ signal from the mock to the ligated model, while the CD31 signals decreased.
Our main goal was to optimize the cell isolation protocol in terms of cell count and viability and to ensure that these optimizations would yield CD31 and PDGFRα or β cell signals. We also wanted to minimize the EpCAM signals in the sample via our alterations. We first determined whether previously frozen cells would give a good cell yield. After thawing cells frozen in liquid nitrogen, we found that the cell count was drastically decreased along with a very small cell vitality. We decided to use fresh cells in our following experiments. After testing many versions of the protocol, we found that a protocol that used Liberase as the digestive enzyme and also incorporated a RBC depletion with the EpCAM depletion gave us both a large yield and vitality, while also showing larger amounts of cell signaling.

After confirming our new protocol, we used it to isolate cells from both a salivary gland that went through a mock surgery. Both the mock and ligated samples yielded a smaller than expected cell count, but the cell vitalities had expected outcomes, with the ligated sample having lower vitality due to damage from ligation. We then used RNA single cell sequencing to determine whether the sequencing would show that high levels of Pdgfrβ+ and CD31+ cells were detectable with the sequencing and low levels of EpCAM-expressing cells. We found that both the cell imaging and the sequencing showed agreeing results. The work in this thesis can allow us to have more accurate cell sequencing data of stromal and endothelial cells, which are largely absent from published salivary gland scRNASeq datasets to help develop future therapies for salivary gland disease.
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