Pdgfra AND b COPOSITIVE FIBROBLASTS DRIVE FIBROSIS IN MOUSE SALIVARY GLANDS THROUGH TGFβ SIGNALING

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Pdgfra AND b COPOSITIVE FIBROBLASTS DRIVE FIBROSIS IN MOUSE SALIVARY GLANDS THROUGH TGFβ SIGNALING

By
Renae Williams Atkinson

A Thesis
Submitted to the University at Albany, State University of New York
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Master of Science

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ABSTRACT

Fibrosis is of significant concern to the medical community as numerous disease processes are characterized by progressive fibrosis leading to organ damage. We begin the process of examining the mechanism of fibrosis to salivary gland hypofunction and briefly consider Sjögren’s Disease (SjD). Method: We employed single-cell RNA sequencing data from a reversible mouse salivary gland injury model and from NOD/ShiLtJ mice, a model of secondary SjD. We performed treatment comparisons with the help of Seurat dotplots and UMAPS. Using differential gene expression analysis and the publicly available R packages: clusterProfiler, WikiPathways and Cytoscape, we identified the processes and pathways increased in the fibroblast population and the Pdgfra/b copositive population and visualized the hits from our dataset in a published fibrosis pathway network. Results: We determined that fibrosis of mouse salivary glands is orchestrated by a subtype of fibroblasts identified as Pdgfra/b copositive fibroblasts. This subtype expands in response to ligation injury likely through TGFβ signaling. We highlight that fibrosis is a characteristic process of the fibroblasts in a Sjögren’s Disease mouse model. We show the molecules involved in the fibrosis process in the Pdgfra/b copositive fibroblasts. The reversible fibrosis mouse model is a relevant model showing concordance with the human ductal obstructive injury. Conclusions: Pdgfra/b copositive fibroblasts are a disease subtype that rise upon injury and are proliferative and fibrotic. Bioinformatics revealed five processes are increased in fibroblasts in response to injury: blood circulation, leukocyte migration, cell chemotaxis and extracellular matrix organization. G-coupled protein receptor signaling is the most significant pathway increased through Wnt signaling which give rise to the TGFβ mediated fibrosis. This work paves the way for further targeted in-vivo and in-vitro analyses for the contribution of TGFβ to this fibroblast disease sub-type.
ACKNOWLEDGMENTS

I would also like to acknowledge the support of the Presidential Doctoral Fellowship for Research Training in Health Disparities with funding from the National Institute on Minority Health and Health Disparities (#MD003373) through the Center for the Elimination of Minority Health Disparities (CEMHD) at the University at Albany, The State University of New York (SUNY). I would also like to acknowledge the following grants from the National Institute of Dental and Craniofacial Research: NIH R01-DE027953 and R01-DE030626-01 to Dr. Melinda Larsen which allowed for the lab experiments used in this study.

I would also like to thank my dear husband, Dr. Travis Atkinson, for his loving and constant support on this journey. My heavenly Advisor was a constant source of inspiration and power.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii

ACKNOWLEDGMENTS .................................................................................................................. iii

LIST OF FIGURES .......................................................................................................................... v

LIST OF APPENDIX FIGURES ....................................................................................................... vi

LIST OF TABLES ............................................................................................................................ vii

Chapter 1: Introduction .................................................................................................................. 1

Scope of thesis ................................................................................................................................. 10

Chapter 2: Pdgfra and b Copositive Fibroblasts Overexpress ECM, Proliferate in response to
Ligation and is Related to TGFβ Signaling ................................................................................... 12

Acknowledgement ......................................................................................................................... 12

Methodology .................................................................................................................................. 13

Results ........................................................................................................................................... 19

Chapter 3: Biological Processes and Pathways in Pdgfra/b copositive Fibroblasts Driving Fibrosis
Through TGFβ signaling in Mouse Salivary Gland .......................................................................... 33

Acknowledgement ......................................................................................................................... 33

Methodology .................................................................................................................................. 34

Results ........................................................................................................................................... 37

Chapter 4: Discussion and Conclusion .......................................................................................... 54

Limitations of Study .......................................................................................................................... 60

Appendix A: Supplemental Figures .............................................................................................. 62

Appendix B: Permission for figure use .......................................................................................... 68

Appendix C: Approved Institutional Review Board form ............................................................... 69

References ....................................................................................................................................... 79
LIST OF FIGURES

Figure 1: Recent findings from Larsen Lab regarding Pdgfra/b copositive fibroblasts .......... 6
Figure 2: Schematic Overview of the Project ................................................................. 8
Figure 3: Schematic showing Reversible Fibrosis Model .................................................. 15
Figure 4: Fibroblast population shows overexpression of ECM 14 days post injury ............ 20
Figure 5: Extracellular matrix production in fibroblast clusters ...................................... 21
Figure 6: UMAPS of control and 7-day ligated SMG cells ................................................ 22
Figure 7: ECM Gene Expression in Fibroblasts of control and 7-day ligated SMGs .............. 24
Figure 8: Association between Tgfbr2 and Pdgfra expression in fibroblasts ...................... 27
Figure 9: UMAP and dotplot of NOD/ShiLtJ mice .......................................................... 30
Figure 10: Fibroblast subset in NOD/ShiLtJ and CD-1 mice ............................................ 32
Figure 11: Schematic of pathway analysis workflow ......................................................... 38
Figure 12: Gene Ontology Biological Processes Increased in Fibroblasts of the dataset ...... 41
Figure 13: Fibroblast subset in control-3-7-14 - day Ligated dataset .................................. 42
Figure 14: WikiPathways upregulated in fibroblasts ........................................................ 44
Figure 15: Enriched GO Biological Processes in Cluster 0 and Cluster 11 ......................... 45
Figure 16: Upregulated WikiPathways in Clusters 0 and 11 combined ............................. 46
Figure 17: WikiPathways upregulated in Cluster 0 (Pdgfra/b-copositive fibroblast cluster) .... 48
Figure 18: Enriched GO biological pathways in cluster 0 of fibroblasts in control - 3-7-14-day
ligated dataset ............................................................... 50
Figure 19: Lung Fibrosis WikiPathway .............................................................. 52
Figure 20: Human submandibular gland sections of a patient with chronic salivary gland
obstruction ................................................................. 53
Figure 21: Summary of the molecules involved in the fibrosis pathway in Pdgfra/b copositive
fibroblasts ........................................................................................................ 61
LIST OF APPENDIX FIGURES

Appendix Figure 1: Control, 3-, 7-, and 14-day ligated with homeostatic controls dataset ..........62
Appendix Figure 2: Feature Plot to determine which cluster is the fibroblast ..................................63
Appendix Figure 3: Heatmap of the gene expression level of fibroblast markers ..........................64
Appendix Figure 4: Fibroblast subset using automated animation .................................................65
Appendix Figure 5: Salivary Gland Fibroblast Expression Data Mapped on the Chondrocyte differentiation pathway. .................................................................66
LIST OF TABLES

Table 1: Anti-fibrotic Agents........................................................................................................................................9
Table 2: Top 3 upregulated WikiPathways in clusters 0 and 11........................................................................67
Chapter 1: Introduction

Xerostomia, or oral dryness, is the most common symptom in patients presenting with salivary gland disease. Saliva is a complex biological fluid important to maintaining oral, dental, and general health. Hyposalivation can impact quality of life and increase the risk of oral diseases such as dental caries, dental erosion, and fungal infections. Diseases that can affect the salivary gland, like tuberculosis and sarcoidosis, may produce sialadenitis, leading to salivary hypofunction (Bowers et al., 2021). In addition, autoimmune conditions can lead to Sjögren’s Disease, which typically affects the salivary glands and/or lacrimal glands. Sjögren’s Disease (SjD) is characterized by dryness of the oral and ocular regions, lymphatic infiltration, dysfunction, and destruction of the exocrine glands driven by an autoimmune epithelitis (Bowers et al., 2021). SjD is the second leading autoimmune disease behind rheumatoid arthritis (ibid. 2021). The disease may occur in the absence of another autoimmune disease (primary SS/pSjD) or with another autoimmune disease (secondary SS/sSjD) or may be induced by a new class of cancer chemotherapeutic drugs-immune checkpoint inhibitors (iciSS) (ibid, 2021). There is no approved drug for the disease, and management is palliative, creating a pressing need for a pharmacologic solution (Carsons & Patel, 2023). All these salivary gland diseases are associated with both an inflammatory and fibrotic response that leads to progressive loss of organ function.

Saliva in humans and mice is produced by three paired major salivary glands: parotid gland, submandibular and sublingual gland along with minor salivary glands (Maruyama et al., 2019). Saliva is produced by two types of acinar cells: mucous acinar cells, which produce a more viscous fluid consisting of glycoproteins and mucins, and serous acinar cells, which produce a more watery fluid containing proteins (Ghannam & Singh, 2024). Myoepithelial cells
surround the acinar units to help then to expel saliva into a branching ductal network transitioning from intercalated to striated to excretory ducts; which modify and transport the saliva into the oral cavity (Rocchi et al., 2021). Saliva is made when water from serum is transported to the terminal portion of the acinar cell followed by selective reabsorption of sodium and chloride and the selective release of calcium and carbonate (Bowers et al., 2021). Proteins are also released. These proteins can be intrinsic to salivary glands or extrinsic from the blood plasma (Saitou et al., 2020). A complete salivary proteome database can be accessed from the Human Salivary Proteome Wiki (HSP-Wiki: https://salivaryproteome.nidcr.nih.gov/). These proteins play various functions in maintaining mouth and gut health.

The fibrosis of the glands leads to the loss of these health rendering functions and is a source of significant morbidity and mortality. Extracellular matrix (ECM) remodeling is a natural response to injury, but prolonged injury leads to excessive ECM deposition, a hallmark of fibrosis. In the salivary gland, obstructive disease and the autoimmune disease, Sjögren’s Disease, are associated with a fibrotic response. Fibroblasts are known to be contributors to fibrosis, but the regulation of ECM production is incompletely understood in the salivary gland. Understanding this is critical to the development of a treatment approach. It was previously shown that Pdgfra/b copositive fibroblasts are primarily responsible for ECM overproduction in the submandibular glands after induced ductal ligation injury in mice (Altrieth, O’Keefe, et al., 2023).

Salivary gland fibrosis is defined as the hardening and scarring due to excessive deposition of ECM components including collagen (Wynn, 2008). Fibroblasts produce ECM proteins as a normal part of their biological process to support the connective tissue of the salivary gland. Because fibroblasts are not terminally differentiated, they are able to be activated
to form myofibroblasts which have a higher rate of matrix production (Kendall & Feghali-Bostwick, 2014). Their activation usually results in proliferation and cellular differentiation as a part of biological wound healing, growth development, inflammation and tissue repair (Bagalad et al., 2017; Kendall & Feghali-Bostwick, 2014). It is the uncontrolled activation of these fibroblasts (and other stromal cells that give rise to myofibroblasts) that results in pathological fibrosis. When differentiated into myofibroblasts, these cells become contractile, exaggerate ECM production and constitutively secrete cytokines, chemokines and growth factors (Kendall & Feghali-Bostwick, 2014). These features will be seen in our results. In our results, we also see indications of this positive feedback mechanism especially with respect to the production of TGFβ1 which is strongly chemotactic for fibroblasts and favors their differentiation in myofibroblasts (Bagalad et al., 2017; Moskwa et al., 2022).

The work of salivary gland fibrosis research has been hampered by the lack of a salivary gland fibroblast atlas of both normal and diseased fibroblasts. There have been attempts to identify cross-tissue fibroblast identities (Buechler et al., 2021). While others have identified multiple fibroblast sub-types in other organs, our group identified a possible pathologic fibroblast sub-type in mouse salivary glands (Altrieth, O’Keefe, et al., 2023a; Moskwa et al., 2022). The results suggest that the Pdgfra/b+ stromal fibroblast population is what is responsible for the laying down of ECM in the injury model (Altrieth, O’Keefe, et al., 2023). The PDGFRα and β expressing stromal fibroblasts promote proacinar and ductal differentiation of surrounding progenitor cells through FGF2 stimulation (Moskwa et al., 2022). Our group later built on this to examine adult mouse salivary gland response to injury and reported that Pdgfra/b expressing fibroblasts also expressed latent TGF-β binding protein-2, osteopontin/ sialoprotein 1 (Spp1) and periostin (Postn) all pointing towards fibrosis (Altrieth, O’Keefe, et al., 2023) (Figure 1).
Fibrosis is a disease of the TGFβ-system (Massagué & Sheppard, 2023). TGFβ signaling is known to drive fibrosis in other tissues, but no specific fibroblast subtype has been implicated in the salivary gland disease process. TGFβ1, which is known to induce fibrosis, has been shown to play a role in SjD (Sisto et al., 2020) and in experimental ductal ligation injury (Woods et al., 2015). Signal transduction involves the activation of TGFBR1/2 heterotetramer, which is then autophosphorylated and in the canonical pathway phosphorylates SMAD2/3. This is then complexed with SMAD4 that is then trafficked to the nucleus which acts as a transcription factor (Zhang et al., 2021). The pathway can be activated by integrins on the cell surface like integrin αvβ6 by shear force produced by actin cytoskeletal forces which releases TGFβ from its latent TGFβ-binding protein straitjacket (e.g. the protein that keeps the TGFβ protein ligand confined and ‘inactive’) to be diffused into the extra-cellular compartment to activate TGFBR2 (Campbell et al., 2020). TGFβ demonstrates its potent biological activity when released from its Latent TGFβ binding protein (LTBP)(Antar et al., 2023). However, recently it was demonstrated that immune cells, particularly T cells, bear integrin 8 (αvβ8) which specifically binds to the prodomain of the latency lasso and allows for the twisting of the prodomain to expose the mature TGFβ without releasing it (Campbell et al., 2020). This makes the ligand accessible for binding to TGFBR2 on neighboring cells and may also auto activate the TGFβ cascade within that same immune cell (ibid, 2020). The implication of this is that T cells, myeloid cells and endothelial cells bearing integrin β8 would be able to activate ‘latent’ TGFβ on fibroblasts thus increasing the likelihood of activation. Therefore, freely diffused TGFβ is not a prerequisite for receptor activation. Hence, drugs that target the TGFβ ligand would not have the desired effect because this mechanism would be able to circumvent free TGFβ.
Fibroblasts play a central role in the progression of fibrosis in Sjögren’s Disease in the salivary glands (Xiang et al., 2023), therefore, investigating this cell type might prove an unexplored therapeutic strategy. Sjögren’s Disease is characterized by periductal and perivascular lymphocytic filtration and interstitial fibrosis, leading to a clinical reduction in saliva production (Gao et al., 2020; Liao et al., 2022). However, numerous fibroblast subtypes exist across tissue contexts. One publication (Korsunsky et al., 2021) identified cross-tissue stromal inflammatory phenotypes using robust statistical, transcriptomic and spatial transcriptomic techniques. In this study, they looked at fibroblasts from diseases consisting of fibrosis and inflammation namely SjD, rheumatoid arthritis, interstitial lung disease and inflammatory bowel disease. They integrated scRNA sequencing data and compared the transcriptomic profiles across tissues in humans and in some diseases in mice. They identified SPARC\(^+\) COL3a1\(^+\) fibroblasts as an inflammatory subtype that is associated with angiogenesis and CCL19\(^+\) and CXCL10\(^+\) fibroblast subtypes as being associated with T-cell signaling and migration into the tissue.

Because fibroblasts are not terminally differentiated, the source of pathological fibroblast subtypes like *Pdgfra/b* is unclear and is an ongoing search. This study does not suggest the source of *Pdgfra/b* fibroblast cells only that they are there and expanding. One group suggested EMT where epithelial cells are transitioning into a more mesenchymal phenotype and contributing to fibrotic disease. This TGF-\(\beta\) (Hall et al., 2010; Mason et al., 2003; Woods et al., 2015), IL-6, IL-17, 1L-22-mediated EMT results in salivary gland fibrosis (Sisto et al., 2019, 2020). This loss of epithelial markers arises due to the TGF-\(\beta\)/SMAD/SNAIL signaling pathway (Sisto et al., 2022). This is consistent with previously published organoid work that demonstrated that TGF-\(\beta\) promotes the differentiation of salivary gland fibroblasts into myofibroblast
(Moskwa et al., 2022). Lisi’s group graded the inflammation associated with SjD and found that increased inflammation correlated with increased SNAIL, vimentin and collagen type 1 (Sisto et al., 2019). IL-6, which was found at very high levels in SjD salivary glands, was correlated with reduced E-cadherin levels while vimentin and collagen type 1 increased in a graded fashion (Sisto et al., 2020). This EMT-mediated fibrosis is often systemic, involving the heart, liver, lung and kidneys (Sisto et al., 2022). There are no time course analyses that confirms the lineage transition from epithelium to mesenchymal transition in the salivary gland; however, current understanding from the literature sets a strong foundation for further investigation. EMT in acute inflammation would result in tissue repair; however, when the EMT becomes chronic, the differentiated fibroblasts become progressively fibrotic.

**Figure 1: Recent findings from Larsen Lab regarding \textit{Pdgfra/b} copositive fibroblasts**

In any chronic fibrotic process, TGFβ plays a central role. The first three ligands of the family: TGF-β1, TGF-β2, TGF-β3, are expressed in humans. The first two have a profibrotic effect and the last generally has an antifibrotic effect (Zhang et al., 2020). TGFB3/Betaglycan is a negative regulator of TGFB1/2 heterotetramer formation except when it is modified by glycosaminoglycan (Eickelberg et al., 2002). Ligand activation occurs when ligand binds to
TGFBR2, which causes positive cooperativity of the TGFBR1/2 heterotetramer and autophosphorylation of the intracellular TGFBR1 in the complex. This then leads to the downstream activation of SMAD 2/3. Collagen Triple Helix Repeat-Containing-1 (CTHRC1) is produced by pathologic fibrotic tissue in response to TGF-β1 (ibid. 2020). This protein has been proposed as the best marker for profibrotic fibroblasts and has been used as an identifying marker in one study (Tsukui et al., 2024). Investigating the role of TGFβ signaling in Pdgfra/b copositive fibroblasts is therefore a reasonable first step in determining the mechanism of fibrosis of this fibroblast subtype.

We must also consider confounding pathways like the non-canonical pathways that are activated by TGFβ signaling. In normal growth and development, myofibroblasts activate the PDGFR pathway through either a mitogen-activated protein kinase (MAPK) path or phosphatidylinositol 3-kinase path (PI3K) for cell activation, proliferation, and migration (Bagalad et al., 2017). Normal wound repair utilizes myofibroblasts for wound contraction and tissue remodeling through the secretion of proteins like matrix metalloproteinases (MMP1-3), chondroitin sulfates and fibronectins (ibid, 2017). In both fibrosis and cancer, TGFβ is known to activate PI3K, extracellular signal-regulated kinase 1/2 (ERK)/MAPK and MMPs which can be activated by other growth factors like platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and Notch signaling, which contribute to the disease process (Bagalad et al., 2017; S. Liu et al., 2018) (Figure 2). ERK signaling in particular is known to increase smooth muscle alpha actin production, ECM production and connective tissue growth factor which lead to fibrosis (Shi et al., 2020). This demonstrates the redundancy that occurs when TGFβ is activated in fibroblasts.
Interestingly, one recently study approached abrogating the TGFβ-driven pathway in lungs and found that fibrosis was inhibited but inflammation was increased (Tsukui et al., 2024). The study highlights the change that takes place in the fibroblast population and traces the lineage of CTHRC1+ fibroblasts to the alveolar fibroblasts which normally maintains tissue homeostasis. They note the rise of first an inflammatory phenotype which then leads to the fibrotic fibroblast phenotype. Our results suggest a similar trajectory, but lineage tracing is necessary to confirm this. In their study, they also demonstrate concordance between the mouse model and the human disease which we suspect is also the case with the salivary glands. This is promising for unraveling the mechanisms leading to fibrosis and inflammation for the future treatment of fibrotic diseases.

**Figure 2: Schematic Overview of the Project.**
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With the poor response of SjD to immunosuppressants like methotrexate (Bowers et al., 2021), the severe clinical course and poor prognosis, there is an active search for better therapeutics. SjD is categorized as connective tissue disease (CTD), and being systemic, is often associated with interstitial lung disease (CTD-ILD) (Erre et al., 2021). Because of the similarities between idiopathic pulmonary fibrosis and CTD-ILDs and the effectiveness of anti-fibrotic drugs in slowing progression of IPF (Erre et al., 2021; Gurujeyalakshmi et al., 1999; Ruwanpura et al., 2020), it is expected that the use of anti-fibrotic drugs would likely be effective in progressive fibrotic Sjögren’s Disease. Pirfenidone is an anti-inflammatory and antifibrotic drug, which despite the anti-fibrotic properties being known since the 1990’s, the mechanism of action remains to be worked out (ibid, 2021). However, it is known to inhibit the fibrogenic cascade triggered by TGF-β1, PDGF isoforms and inflammatory mediators important to SjD (Enomoto et al., 2017; Gurujeyalakshmi et al., 1999; Ruwanpura et al., 2020) (Table 1). Nintedanib, is an antifibrotic tyrosine kinase inhibitor approved for the treatment of idiopathic pulmonary fibrosis (Gole & Bankole, 2023). The drug targets FGFR, VEGFR, PDGFR, CSF1R, and FLT3 (Wollin et al., 2015) (Table 1).

Table 1: Anti-fibrotic Agents

<table>
<thead>
<tr>
<th>Targets</th>
<th>Nintedanib</th>
<th>Perfenidone</th>
<th>Asperulosidic acid</th>
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<tbody>
<tr>
<td>PDGFR</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VegFR</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF1R</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLT3</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGFR</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
<tr>
<td>TNFα</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>PDGF isoforms</td>
<td>Yes</td>
<td></td>
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</tbody>
</table>

Nintedanib was also considered for use in companion CTD-ILDs to SjD but has not yet been considered for the treatment of salivary gland fibrosis (Erre et al., 2021). Both Nintedanib and Pirfenidone have been on the market for a while and are approved therapies for idiopathic pulmonary fibrosis as they slow the progression of the disease (Erre et al., 2021; Gurujeyalakshmi et al., 1999; Ruwanpura et al., 2020; Wollin et al., 2015). Asperulosidic acid, an iridoid glycoside, is an anti-inflammatory that was investigated for its effectiveness in the treatment of fibrotic renal disease (Xianyuan et al., 2019). Their results showed that this drug was effective in improving renal function and reducing protein in the urine and mRNA levels of TNF-α, IL-1β, IL-6. They also noticed an alleviation of inflammation through the inhibition of nuclear factor-kappa B (NF-κB) pathway and a reduction in fibronectin, smooth muscle alpha actin and collagen III. A reduction in TGFβ signaling was also noted (ibid, 2019). These drugs are candidates that could be considered as therapeutics for SjD and other fibrotic salivary glands diseases.

**Scope of thesis:** Fibrosis is a disease of the TGFβ-system (Massagué & Sheppard, 2023) and thus TGFβ is being actively explored as a therapeutic target for fibrosis and with some success. Here, we investigate how TGFβ signaling is involved in fibrosis of the salivary glands using bioinformatics analyses of a reversible fibrosis mouse model and a SjD mouse model. This work explores the Pdgfra/b-copositive fibroblast phenotype, its characteristics and the potential mechanism of action of TGFβ signaling in these cells of the salivary gland. We propose
biological processes and pathways unique to this relatively new sub-type of fibroblasts. We venture to show a possible concordance between the reversible fibrotic mouse injury model and human submandibular glands with ductal obstruction.
Chapter 2: *Pdgfra* and *b* Copositive Fibroblasts Overexpress ECM, Proliferate in response to Ligation and is Related to TGFβ Signaling

Acknowledgement

Credits go to Dr. Sergo Gabunia for performing the surgeries for the 3-day and 7-day ligated mice. Single-cell RNA sequencing (scRNASeq) was performed by Joey Tavarez as well as data cleaning processes with Cellbender and other R-based programs. Dr. Amber Altrieth performed the surgeries on the 14-day ligated mice and the scRNA sequencing preparation. Cleaning of the 14-day ligation data was done with Cellbender and was performed by Dr. James Kenney. The 14-day ligation data is publicly available (Altrieth et al., 2024). Animal husbandry was performed by Joey Tavarez, Dr. Amber Altrieth, and Dr. Sergo Gabunia. NOD/ShiLtJ and CD-1 scRNA sequencing preparation was done by Dr. Nicholas Moskwa and Dr. Amber Altrieth and husbandry of the mice was done by Jennifer Morrissey.
Methodology

Animals

All animal husbandry, surgical procedures, and tissue collection were performed in accordance with Larsen lab protocols approved by the University at Albany, SUNY IACUC review board. Mice were housed in ventilated cages with a 12-hour light/dark cycle with access to water and standard chow. B6.129S-Pdgfra<sup>tm1.1(cre/ERT2)B1Bl/J;Rosa26<sup>tdT</sup> mice line were used for the ligation model. The Pdgfra-CreER<sup>T2</sup> (JAX #032770) were obtained from the Jackson Laboratory. B6.Cg-Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hzc/J</sup> (JAX #007914) homozygous females were crossed with the males of the Pdgfrα-CreER<sup>T2</sup> to get female Pdgfrα-CreER<sup>T2</sup>;Rosa26<sup>tdT</sup>. The mouse line used in the 14-day ligated study shared the same C57Bl/6J background (Altrieth et al., 2024). A total of 11 mice were used in the control-3-day-7-day-14-day ligated datasets: 2 mice for the control, 2 for the 3-day, 2 for the 7-day and 5 for the 14-day.

Mice were genotyped using Polymerase Chain Reaction (PCR) to detect Cre and tdTomato. NOD/ShiLtJ mouse line was used in the SjD Nintedanib investigation. Animal husbandry for NOD/ShiLtJ mice was done as previously published by our lab (Gervais et al., 2015). Three mice were used for the NOD dataset and three for the CD-1 dataset.

Reversible Fibrosis Model

To investigate this cell type, we employed an injury model that has been successfully used previously in our lab (Altrieth, O’Keefe, et al., 2023b; Altrieth, Suarez, et al., 2023). The model involves the unilateral ligation of the Wharton’s and Bartholin’s ducts in 12-week-old mice to induce the fibrotic response in the submandibular and sublingual glands, respectively. To avoid sexual dimorphisms in the male mice that do not reflect human anatomy, only female mice
were used (Maruyama et al., 2019). Each mouse received a unique identifier either 7 to 10 days after birth or at the time of surgery. For general anesthesia, ketamine (100 mg/kg) and xylazine (10 mg/kg) were administered intraperitoneally. These were titrated from a stock concentration of 10 mg/mL ketamine and 1 mg/mL xylazine, respectively. Analgesia was also administered in the form of 100 μL of buprenorphine at a concentration of 0.015 mg/mL subcutaneously for postoperative pain management. A vascular clamp (Vitalitec/Peter’s Surgical) was used to ligate the ducts after which sutures were used to maintain primary wound closure. Mice were carefully monitored under anesthesia for pain and distress using a toe pinch. Post-operatively, changes in weight was monitored for a minimum of 48 hours following surgery. The clamp was allowed to stay in place for 3, 7 or 14, days and then the glands were harvested, and the mice euthanized. Successful ligation was determined by a 30 to 70% gland weight reduction of the glands.
compared to the control. The C57BL/6J homeostatic control mice were euthanized at 12-weeks old and had no surgical manipulation.

**Reversible Fibrosis Model**

![Diagram showing Reversible Fibrosis Model]

**Figure 3: Schematic showing Reversible Fibrosis Model**
Both the Bartholin’s (left) and Wharton’s duct (Right) are clamped for either 3-, 7-, or 14-days on the mouse’s right. SLG, sublingual gland and SMG submandibular gland. Created using Biorender.com

**Single Cell Isolation Protocol**

The homeostatic control, 3 and 7-day ligation were performed on 12 week old female Pdgfα-CreER$^{T2}$;Rosa26$^{tdT}$ mice by Dr. Sergo Gabunia, as described previously (Altrieth et al., 2024) and preparation of the single-cell RNA-Seq samples was done by Joey Tavarez. After harvesting the SMG/SL salivary glands from adult mice, excess fat and tissue were removed. 0.4 mL liberase TL Research Grade low Thermolysin was added to maximize fibroblast yield. To
achieve isolated cells, a series of microdissections and centrifugations were done. Epcam antibody was used to deplete red blood cells, and magnetic activated cell sorting was used to remove dead cells. A similar protocol was used for the 14-day ligated samples, the details of which were published previously (Altrieth et al., 2024).

**Single Cell RNA Sequencing and Analysis using Seurat**

The 14-day ligated sample was processed according to what was published in the Altrieth et al paper (Altrieth et al., 2024). Joey Tavarez sent the homeostatic, 3-day, and 7-day samples for sequencing and initial processing of the datasets was performed by the Center for Functional Genomics at the University at Albany. These steps included generating FASTQ files, aligning to the V2 mouse genome containing elongated transcripts of tdTomato and generating count files using CellRanger. Joey Tavarez further performed cleanup of the data using Cellbender (Fleming et al., 2023).

All subsequent analyses were performed by the author for both ligation datasets and NOD/ShiLtJ datasets: Data files were imported using Seurat v5 in R v4.3.2 (Hao et al., 2024; *R: The R Project for Statistical Computing*, n.d.). Referring to the ligation dataset: Two technical replicates of homeostatic samples were used along with two biological replicates for 3-day and 7-day ligation. One large dataset for the 14-day ligated was used. For the NOD/ShiLtJ-CD-1 dataset: one sample for each was used. In both datasets, the samples were merged, normalized and then scaled, as previously reported (Hao et al., 2024; Satija et al., 2015). Clusters were calculated using the default pipeline (Seurat - Guided Clustering Tutorial 2023) and dead or apoptotic cells were removed by mapping at least 25% of unique molecular identifiers (UMIs) to mitochondrial genes. Any cells with <200 or >9000 genes were excluded to select for single cells. The function ‘RunPCA’ was used to determine the principal components (PC) of the
dataset, which and was followed by the ‘FindNeighbors’ function which determined which cells
were nearest in identity based on their mRNA expressions. The number of dimensions used was
selected based on the statistically significant PC from the Jackstraw plot, as previously reported
(Chung & Storey, 2015).

The NOD/ShiLtJ-CD-1 samples used 18 dimensions while the Control-3-7-14-day
Ligation Dataset required 25 dimensions. These dimensions were then used to generate the
Uniform Manifold Approximation and Projection (UMAP) using the PCA as the reduction
method (McInnes et al., 2018). The new Seurat v5 function JoinLayers was used to join the
sample counts into one in order to generate the plot for the UMAP. The Seurat function
‘Findcluster’ was applied to the dataset; asking it to produce resolutions in increments of 0.5
between 0 and 1. The results were then displayed using the ‘clustree’ function to generate a
branching resolution tree. The branch before the cells start to divide into numerous lines was
selected as the ideal cluster (Zappia & Oshlack, 2018). The best resolutions were selected
according to each dataset and the clustree plot generated. This initial processing was repeated
after subsetting. Dotplots and annotations were then able to be performed after initial processing.

**Pearson Correlation and Scatterplot Generation in R**

Initial processing was done of one homeostatic sample and one 7-day sample, as
described above, and the fibroblasts were subsetted. Fibroblast genes cited in the Moskwa et al.
paper (Moskwa et al., 2022) were assigned and the numerical values of each expression level
was retrieved with the “fetch” function in R Seurat. These values were then merged and the
Pearson correlation values were obtained using base R. The R package, corrplot, was then used
to visualize the data (Wei & Simko, 2021).
To determine the relationship between $Tgfbr2$ and $Pdgfra$ gene expression in fibroblasts, the expression of both genes was fetched. Then, using the `ggplot` package, they were mapped against each other in a scatterplot and overlayed with a fitted line from a basic ordinary least squares (OLS) regression model of the two variables to emphasize the direction of their relationship. This process was then repeated but disaggregated by clusters and color-coded by treatment (ligated = red vs control = blue) (Figure 8B). The disaggregation was done using the `facet_wrap()` function in `ggplot` based on the “Cluster_ID” variable. While the color coding was done by setting the “color” aesthetic to the “Treatment” variable.
Results

In a previous publication, the lab showed that after 14-days, the fibroblasts that overexpressed extracellular matrix were copositive for *Pdgfra* and *Pdgfrb* (Altrieth, O’Keefe, et al., 2023a) (*Figure 4*). In their Universal Manifold Approximation (UMAP) annotation after subsetting for fibroblasts, it was noted that cluster 0 and cluster 4 were co-positive for *Pdgfra* and *Pdgfrb* (*Figure 4A*). They then showed that these clusters were primarily responsible for the expression of ECM genes (*Figure 4B*). We, therefore, hypothesized that this cell type was responsible for driving fibrosis in the salivary gland injury model. To test this hypothesis, we employed an even more robust dataset to test whether the results would be consistent with what was previously found and to identify genes potentially driving the progression of cells to a fibrotic state. We focused on earlier time points, including the 3-day and 7-day ligation data, to help us to understand the temporal progression of the disease process with respect to these cells at 3 and 7-day because at 14-days ligation the disease is considered to be established (*Figure 5*).

Using a 7-day ligated and a homeostatic control, we compared the UMAPs by treatment type and did an overlap to highlight the comparison (*Figure 6*). By this, we saw that there is an expansion of clusters 2 and 9 representing the fibroblasts in the dataset. A total of 14,531 cells were in the control dataset and 6,274 cells were in the ligated dataset. Statistically, these sample sizes were large enough to allay any concerns about skewness of the data. There were 222 fibroblasts present in the control dataset, making up 1.5% of the control sample. In the ligated dataset, there were 2,418 fibroblasts making up 38.5% of the ligated dataset. This demonstrates the rapid increase in fibroblast cells in the population as well as the relatively rapid increase of the population from control to ligated at 7-day ligation. This is approximately a 25-fold increase in percentage terms.
A. UMAP of fibroblast subset in Mock vs 14-day ligated dataset. Clusters 0 and 4 are copositive for *Pdgfra/b*.  

B. Dotplot of clusters by treatment with upregulation of ECM expression in clusters 0 and 4 after injury.

**Figure 4:** Fibroblast population shows overexpression of ECM 14 days post injury (Altrieth et al., 2023) (Permission granted—Appendix B) A. UMAP of fibroblast subset in Mock vs 14-day ligated dataset. Clusters 0 and 4 are copositive for *Pdgfra/b*. B. Dotplot of clusters by treatment with upregulation of ECM expression in clusters 0 and 4 after injury.
Figure 5: Extracellular matrix production in fibroblast clusters

In this dot plot showing control homeostatic SMG (C), 3-, 7- and 14-day ligation data, the heat map shows levels of gene expression (fold change) with the size of the dot representing the number of cells responding in a cluster with genes indicated on the X-axis. Consistently, *Pdgfra/b* copositive fibroblasts (clusters 0-black box and 11-purple box) overexpress extracellular matrix starting at 3-days ligation, which continues up to 14-days. This fibroblast subtype is distinct from pericytes (distinguished by their expression of *Myl9, Acta2* and *Myh11*) represented in Clusters 8 and 10. *Tgfb2* is also upregulated from early timepoints (3-days). This copositive subtype also increases its *Sparc* average expression in both clusters and in cluster 11 increases the average expression of just *Sparc* in Cluster 0. The average expression of *Col3a1* is noted to be increased only in Cluster 11.
Figure 6: UMAPS of control and 7-day ligated SMG cells
Treatment comparisons show an increase in fibroblast cell types. Top left. UMAP of the 12 clusters for 7-day and homeostatic control. Top right. Overlap of the 7-day treatment groups using the ‘Groupby’ function in Seurat showing an overlay of the two treatment groups. Bottom: Cells separated by ligated (L, left) and homeostatic control (M, right) using the ‘Splitby’ Seurat function in R. The fibroblast population identified as 2 and 9 is sparse in the Control group but increases in the 7-day ligated treatment group.
We next hypothesized that it is the \textit{Pdgfra/b} copositive fibroblasts that are the expanding subpopulation. To test this, we had to validate that the fibroblasts were indeed fibroblasts. Using the three gold standard methods for annotation in the field, we employed manual, semi-automatic and automated annotation which all resulted in the same clusters (2 & 9) being identified as fibroblasts (Appendix Figure 2 to Appendix Figure 4). Our subsetting found that indeed, there is a group of fibroblast subsets that expand upon ligation specifically clusters 0, 1, 5, 6, 7 and 12 (Figure 7B) shown by an increase in the density of the fibroblast population. A dotplot of the fibroblast clusters and their ECM expression, surprisingly showed that these clusters all identify as \textit{Pdgfra/b} copositive cells (Figure 7C). The overlay in Figure 7B effectively shows the expansion of the fibroblast population in the ligated group in comparison to the control group. This indicates that the \textit{Pdgfra & b} copositive markers are associated with ligated and expanding fibroblast populations in response to injury.
Figure 7: ECM Gene Expression in Fibroblasts of control and 7-day ligated SMGs
Subsetting for just the fibroblasts (clusters 2 and 9) from 7-day ligated and homeostatic controls (same as Figure 6). A. UMAP of 12 fibroblast subclusters. Grouping these clusters by treatment type revealed that clusters 0, 1, 5, 6, 7 and 12 were the main ECM-expressing clusters that arose from ligation. These clusters all proved to be \textit{Pdgfra/b} copositive but with varying levels of expression of each receptor.
Since TGFβ is a known regulator of fibrosis and Pdgfra+/b+ cells were previously shown to be related to fibrosis in salivary glands, we wanted to investigate the relationship between these two genes in our dataset using Pearson’s correlations. Using a myofibroblast gene set, we investigated the Pearson correlation of their expressions with our control and 7-day dataset. A myofibroblast gene set was chosen because when activated fibroblasts can differentiate into myofibroblasts that can play a critical role in establishing fibrosis. Interestingly, we found a moderate and positive Pearson correlation between the expression of Tgfr2 and Pdgfra of 0.55 (Figure 8A-B). Plotting these two genes against each other we saw that as the expression of Tgfr2 increased, the expression of Pdgfra also increased. Generating a dotplot of all the TGFβ family ligands and receptors showed an increase in the Tgfb1 ligand and receptor 1 and 2 expression with ligation, especially receptor 2 that corresponded with the expanding clusters (Clusters 0, 1, 5, 6, 7 and 12) (Figure 8C). Disaggregating the scatterplot by fibroblast clusters, we saw the weighted contribution of each cluster’s gene association to the overall Tgfr2 and Pdgfra scatterplot (Figure 8B right). The same clusters that expand (Clusters 0, 1, 5, 6, 7 in Figure 7A-B) in response to injury (red) are the same clusters that are showing significant positive correlations of Tgfr2 and Pdgfra expression (Figure 8C). This analysis predicts a positive relationship between TGFβ signaling, PDGFRα signaling and a proliferative response in specific cell identities in response to injury. Establishing a relationship between these two signaling pathway is significant because of the known non-canonical relationship between them in producing fibrosis (S. Liu et al., 2018). This is our first indication that TGFβ signaling plays an important role in Pdgfra/b copositive fibroblasts.

To determine whether the pattern of fibrosis is the same in Sjögren’s Disease, we employed a SjD model: Non-Obese Diabetic (NOD/ ShiLtJ) mouse, as in our prior work
(Gervais et al., 2015; Lodde et al., 2006). We performed scRNASeq on the NOD/ ShiLtJ mouse SMGs vs SMGs from CD-1 control female mice at 20 weeks. Consistent with the autoimmune profile of the mouse, we see an expansion of the T-cell population (Figure 9A-C). Figure 9D shows that fibroblasts are primarily responsible for the deposition of ECM, as in our fibrosis model, seen by the high expression of ECM genes in almost exclusively the fibroblasts. We also saw a significantly higher expression of ECM proteins in the NOD/ShiLtJ model when compared to the CD-1 mouse model. We see a greater percentage of cells expressing ECM, and in some gene cases, higher average expression. Subsetting for the fibroblasts alone we got a significantly lower yield of fibroblasts than in the ligation models described in Figure 7, most likely because of a less favorable single cell isolation protocol. In Figure 10B, we see that clusters not employed in generating ECM in CD-1 control are overexpressing ECM in the NOD/ShiLtJ.

Taking one anti-fibrotic drug, Nintedanib, we assessed for the targets in the salivary gland and then in the fibroblast population (Figure 9 and Figure 10). Nintedanib primarily affects the fibroblast population, although it can also affect the dendritic cells and the macrophages (Figure 9D). Interestingly, Fgfr2 and Pdgfrb, targets of Nintedanib (Table 1), had significantly higher average expressions in the NOD/ShiLtJ fibroblasts than the CD-1 (Figure 10D). Whether this limited number of targets being affected by the drug is sufficient to produce a meaningful effect is yet to be determined.
Figure 8: Association between *Tgfbr2* and *Pdgfra* expression in fibroblasts

Significant Association between *Tgfbr2* and *Pdgfra* expression in response to salivary gland injury in *Pdgfra/b* copositive fibroblasts. A. The Pearson correlation plot between genes mentioned in the Moskwa (2022) paper. The Pearson correlation between *Tgfbr2* and *Pdgfra* is 0.55 which indicates a moderate and significantly positive correlation between the expression of the two genes in the fibroblast sub-type. B.
left. Scatterplot of the two genes in question, shows the positive linear relationship that the correlation implies. right. Scatterplots by cluster showing the weighted contribution of each cluster gene relationship to the whole scatter plot of B. C. Dotplot of TGFβ genes by clusters of Figure 7.
Figure 9: UMAP and dotplot of NOD/ShiLtJ mice
scRNA seq clusters of CD-1 control and NOD/ *ShiLtJ* mice showing increased ECM production in the fibroblasts of the NOD/ShiLtJ mice. A. UMAP of the merged dataset. B. UMAP split by mouse model: C, control; N, NOD. C. Cell population representation by mouse model: C, control (gray); N, NOD (red). D. Dotplot of ECM genes expressed in all the clusters.
Figure 10: Fibroblast subset in NOD/ShiLtJ and CD-1 mice
A. UMAP of fibroblast subset B. Fibroblast subtypes showing a marginal increase in ECM gene expression in clusters 1 and 2, while some genes show a decrease in expression in the control group. C. Nintedanib markers present in the whole dataset D. Nintedanib target genes present in the fibroblast subclusters.
Chapter 3: Biological Processes and Pathways in Pdgfra/b copositive Fibroblasts Driving Fibrosis Through TGFβ signaling in Mouse Salivary Gland

Acknowledgement

Credits to Dr. Sergo Gabunia for performing the surgeries for the 3 and 7-day ligated mice. Single cell RNA sequencing was performed by Joey Tavarez, as were data cleaning processes with Cellbender. Dr. Amber Altrieth performed the surgeries on the 14-day ligated mice and the scRNA sequencing preparation. The cleaning of the 14-day ligation data was done with Cellbender and was performed by Dr. James Kenney. The 14-day ligation data is publicly available (Altrieth et al., 2024). NOD/ShiLtJ and CD-1 scRNA sequencing preparation was done by Dr. Nicholas Moskwa and Dr. Amber Altrieth.

We also acknowledge Dr. Neil Gildener-Leapman, MD, Ear, Nose, and Throat Surgeon at Albany Medical College for the human salivary gland samples provided by the Albany Medical Center Institutional Review Board (Appendix C). Credits go to Kennedi Weston for the transportation of the specimens.
Methodology

WikiPathway Analysis with Cytoscape Visualization

WikiPathway Analysis was done according to the published work (Agrawal et al., 2024). The 2024 rWikiPathways 1.24.0 tutorial was followed in R v4.3.3. It utilizes the ‘clusterProfiler::enrichWP’ function which takes the up regulated genes and compares them against the background genes to obtain the enriched up regulated WikiPathways. ‘Dose’ is then used to add the gene symbols to these enriched up regulated pathways. These are then plotted by the barplot and dotplot functions. To study the contents of the pathway hits, we use the ‘findPathwayNamesByText’ function. We then applied the organism filter to the pathway and visualized the pathway in Cytoscape.

Our enrichment analyses were based on Gene Ontology and Pathway Analyses with visualization. Cytoscape visualization was done according to published work (Gustavsen et al., 2019; Reimand et al., 2019; Shannon et al., 2003). We worked with three Differential Expression (DE) datasets: one of fibroblasts, the other of the fibroblast clusters that were copositive for Pdgfra/b and one of cluster 0 only. These DEs had the log2 fold change, adjusted p-value and gene symbols for each gene. The ‘clusterProfiler’ package was used to add the EntrezID to our various data frames (Wu et al., 2021; Yu et al., 2012). We found that 4% of the upregulated genes in the copositive datasets failed to map to Entrez IDs, 12.92% in the downregulated genes and 4.86% in the background genes. This is likely a result of one-to-many mapping. ‘ClusterProfiler’ was also useful in generating the enriched gene ontology plot using the ‘enrichGO’ function. The enriched GO is obtained by comparing the upregulated genes against the background genes. We were also able to generate enrichment maps using Cytoscape. WikiPathways were generated
from the up and downregulated dataframes. Using the WikiPathway IDs we generated pathway networks in Cytoscape. Both in the WikiPathway generation and the enrichment GO plots the false discovery rate and an adjusted p-value cutoff of 0.05 was applied except for the fibroblast DE dataframe which used an adjusted p-value of 0.1. This exception did not affect the significance of the chart shown as all the pathways displayed were very significant.

**Cryosectioning**

Submandibular gland sections were transported from the Albany Medical Center immediately after surgical resection in transportation medium. Upon arrival in the lab the sections were further dissected and transferred to tissue freezing medium cryopreserved over liquid nitrogen. After cryopreserving the sections with isopentane and liquid nitrogen, I sectioned the samples in 10 micrometer sections using a Leica CM 1860 cryostat and placed them on Superfrost Plus glass slides (Electron Microscopy Sciences) which were stored at -80°C. Before staining, the slides were returned to room temperature.

**Hematoxylin and Eosin Staining**

Frozen tissue sections were thawed at room temperature and rehydrated in 1X phosphate buffered saline (PBS) for 5 minutes. They were then briefly rinsed under running tap water for approximately one minute. Staining was done using Hematoxylin 7211 (Richard-Allan Scientific) for 10 minutes and rinsed under tap water for 1.5 minutes (Altrieth, O’Keefe, et al., 2023a). Subsequently, Eosin-Y (Richard-Allan Scientific) alcohol was applied for 3 minutes and rinsed under tap water for 1 minute. Sections were then allowed to stand in 100% ethanol for 3 minutes and then air dried briefly before mounting coverslips. Rehydration and staining steps were performed in Coplin jars. Coverslips were mounted using Permount mounting media.
**Picrosirius Red Staining**

Human sections were treated with Bouin’s Fluid at room temperature overnight and then at 60 degrees for 1 hour. After washing the sections were dipped in the Picrosirius Red Solution for 15 minutes and then washed under running tap water. They were then dipped in Picric acid for 40 minutes and then washed in isopropanol for 5 minutes, followed by 100% ethanol. The sections were then dipped in a mixture of xylene and ethanol (1:1) and subsequently washed with xylene and then mounted in non-aqueous mounting media (Permount).
Results

We were aware that other authors (as described in the introduction) have reported a relationship between TGF\(\beta\) signaling and Pdgfra signaling, particularly in cancer (Bagalad et al., 2017; S. Liu et al., 2018) (Figure 2). To investigate whether a similar pathway occurs in fibrotic response in salivary glands, we employed our most robust dataset of control, 3-day, 7-day and 14-day ligated scRNA sequencing data. After performing a similar subsetting of the fibroblasts as in Chapter 2, we performed differential gene expression analyses of the fibroblasts and generated 3 dataframes: one of the fibroblasts, another of Pdgfra/b expressing fibroblasts i.e. cluster 0 and 11 and another of cluster 0 only (Figure 11). An over representational analysis was performed to determine the Gene Ontology Biological Processes that are increased in the fibroblast, the Pdgfra/b expressing fibroblasts and cluster 0 which was the larger of the two clusters expressing Pdgfra/b and positive the fibrosis process (Figure 12, Figure 15, Figure 18). A pathway analysis was used to determine the biological pathways increased (Figure 14, Figure 16, Figure 17). Network analyses using WikiPathway and Cytoscape visualization (Agrawal et al., 2024; Reimand et al., 2019; Shannon et al., 2003) were useful in determining what is already known and how it relates to the specific molecules and their pathways implicated in our dataset.
The biological processes upregulated in the fibroblast population supported what we expected to find, which was increased signaling to immune cells leading to ‘leukocyte migration’, increased ‘ECM organization’, ‘blood circulation’ and ‘cell chemotaxis’ (Figure 12). The role of blood circulation has been noted in previous work (Altrieth et al., 2024) but this shows that the fibroblasts have a regulatory impact on the endothelial cells. In SjD, fibroblasts are known to induce transendothelial migration of immune cells into the tissue (Xiang et al., 2023). The findings of blood circulation regulation support the predominant form of inflammatory fibroblasts found in our dataset, SPARC⁺Col3a1⁺ fibroblasts, which perform angiogenesis through Notch signaling (Korsunsky et al., 2021) (Figure 13B). Angiogenesis also arose as a significant pathway (Figure 12B). An examination of the Gene Ontology Plot highlights the 4 cardinal processes that have been upregulated in the fibroblast population: blood circulation, leukocyte migration, cell chemotaxis, and extracellular matrix organization (Figure 12A).
Surprisingly, adaptive immune response showed up as the most significant biological process in Pdgfra/b copositive fibroblasts (Figure 15). The remaining processes also seemed to align with fibroblast immune cell communication. This might indicate the initiating processes of fibrosis. The pathways found also showed a proclivity to immune cell functioning or an adoption of the inflammatory phenotype with ‘cytokine and inflammatory response’ and ‘complement activation’ (Figure 14A.). The most significant pathway increased in Pdgfra/b copositive fibroblasts was G-coupled protein receptors (GPCRs) (Figure 14A.). G-coupled protein receptors is a large group of receptors that includes frizzled receptor proteins (Kendall & Feghali-Bostwick, 2014). Many other receptors in this group (like the angiotensin type I receptor and CXC chemokine receptors) are known to regulate myofibroblast differentiation and fibrosis (Akhmetshina et al., 2012). It is likely that this GPCR occurrence is due to Wnt signaling because of the frizzled 1 and 2 receptors which were shown to be increased in the GPCR pathways in cluster 0 pathways (Figure 17).
B.

**Figure 12: Gene Ontology Biological Processes Increased in Fibroblasts of the dataset**

Gene Ontology Biological Processes (GOBP) increased in Fibroblasts of the dataset. A. The GO Plot shows that four major biological activities are taking place in the fibroblasts: extracellular matrix organization, cell chemotaxis, leukocyte migration and blood circulation. The plot is composed of nodes-circles and edges-lines. **Nodes** represent GOBPs increased. The **edges** show the relationship between two processes B. GO Biological Pathways increased in fibroblasts that are most statistically significant. Blood circulation regulation to leukocyte proliferation are the most significant processes increased. **Adjusted p-value** is based on bonferroni correction using all genes in the dataset.
Figure 13: Fibroblast subset in control-3-7-14 - day Ligated dataset
A. UMAP for the fibroblast subset. B. Dotplot showing SPARC and Col3a1 and other ECM and fibroblast markers. C. Dotplot of expression of Notch receptor among the clusters.
Further investigation of the WikiPathways that are associated with these increased processes showed focal adhesion through the PI3K, Akt, mTOR signaling pathway has the strongest match to the Wikipathway dataset suggesting motility or autophagy (Figure 14B.). The PI3K pathway is also one pathway that is shared between cancer and fibrosis (Bagalad et al., 2017). Notably, ‘oxidative stress and the redox pathway’ is increased (Figure 14B.). An inflammatory fibroblast phenotype is suggested by the following processes being increased in fibroblasts: inflammatory response pathway, prostaglandin synthesis and regulation, cytokines and inflammatory response and complement activation - the classical pathway (Figure 12).

Two clusters of fibroblasts expressed both Pdgfra and b: cluster 0 and cluster 11. We therefore isolated the DE for these clusters for pathway analysis. Initially, we combined both clusters and then we examined cluster 0 as it had fibrosis as one of the significant pathways enriched. When we selected specifically for Pdgfra and b copositive fibroblasts (clusters 0 and 11) and we found GO biological processes related to fibroblast activation as well as immune cell activation (Figure 15). Specifically, pathways activated in Pdgfra/b copositive fibroblasts seem to be involved in pathogen response; namely ‘microglial pathogen phagocytosis’ and ‘chemokine signaling’ (Figure 16).
Figure 14: WikiPathways upregulated in fibroblasts
A. Barchart of Upregulated WikiPathways by Count. Count in clusterProfiler is the number of genes that belong to a given gene set. B. ClusterProfiler dotplot of upregulated WikiPathways by GeneRatio. The GeneRatio in clusterProfiler dotplot= count/setSize, where count is the number of genes in our dataset that matches with the genes in the WikiPathway dataset for that pathway and setSize is the total number of genes in the WikiPathway dataset for that pathway.
Figure 15: Enriched GO Biological Processes in Cluster 0 and Cluster 11.
The differential expressions of clusters 0 and 11 were combined into one dataset and analysed as outlined in the methods. *Pdgfra/b*-copositive fibroblasts appear to be significantly involved with immune compartment activation. *Count* in ‘clusterProfiler’ is the number of genes that belong to a given gene set.
Figure 16: Upregulated WikiPathways in Clusters 0 and 11 combined.
A. The bar chart plots the most significant processes by count. Count in ‘clusterProfiler’ is the number of genes that belong to a given gene set. A gene set here is one that belongs to a specific WikiPathway. B. The dotplot represents the relative number of genes responsible for each function. The GeneRatio in ‘clusterProfiler’ dotplot is calculated as count/setSize, where count is the number of genes in our dataset that match with the genes in the WikiPathway dataset for that pathway and setSize is the total number of genes in the WikiPathway dataset for that pathway (refer to Table 2).
Cluster 0 alone had five statistically significant pathways including fibrosis (Figure 17). The most significant molecule upregulated is NDUFAe4L2 in the oxidative phosphorylation pathway. This molecule has been found in mice to be induced in response to hypoxic conditions likely caused by ligation. It is a counteractive measure in fibroblasts to inhibit the production of mitochondrial reactive oxygen species in response to hypoxia (Chaillou et al., 2016). However, seeing as there is a separate pathway for oxidative stress it is also likely that the oxidative phosphorylation pathway is due to increased metabolic function. The cytochrome b on the mitochondria is the most significant molecule expressed in the ‘electron transport chain pathway’ (Figure 17). This is followed by cyclooxygenases and NADH dehydrogenases. Together these suggest an effort of the mitochondria to deal with oxidative stress. The activation of the electron transport chain could also likely be related to cellular proliferation, which we have seen in Chapter 2, which is a significant characteristic of this cell-types response to stress. The upregulation of the G-Protein coupled receptor (non-traditional) pathway communicates a reversion to an earlier state of differentiation to respond to the stress stimulus. Specifically, the pathway utilizes frizzled class receptors 1 & 2 for Wnt signaling. Wnt signaling has been shown to be activated by TGFβ signaling to promote fibrosis (Akhmetshina et al., 2012). In fact, this developmental pathway is required for TGFβ-mediated fibrosis and has been implicated in another fibrotic disease and in fibroblast to myofibroblast differentiation in human skin (Akhmetshina et al., 2012; Kendall & Feghali-Bostwick, 2014). Parathyroid hormone receptor 1 was found to be implicated in hepatic fibrosis regulating collagen production (Hong et al., 2023).
Figure 17: WikiPathways upregulated in Cluster 0 (*Pdgfra/b*-copositive fibroblast cluster)

The differential gene expression of Cluster 0 alone was filtered out and analyzed processed according to the WikiPathway Methods described in the Methodology section above.

**WP1248 - Oxidative Phosphorylation:**
Ndufa4l2/ATP6/ND4/ND1/ND2/ND3/Atp5pf/Atp5f1a/Atp5f1c/Atp5pd/ND5/Atp5mg/Atp5f1b/Atp5mc2/Atp5pb/Atp5f1d/Atp5mf/Atp5mc1/Atp5po/Atp5mc3/ND4L

**WP295 - Electron Transport Chain:**
CYTB/COX3/ATP6/COX1/COX2/ND4/ND1/ND2/ND3/Atp5pf/Atp5f1a/Atp5f1c/Atp5pd/ND5/Atp5mg/Atp5f1d/Atp5mc2/Atp5pb/Atp5mf/Atp5f1c/Atp5po/Atp5mc3/ND4L

**WP3632 - Lung Fibrosis:**
Spp1/Timp1/Ccn2/Tgfb1/Fgf2/Pdgfa/Hmox1

**WP1396 - GPCRs non odorant:**
Fzd1/F2r/Ednra/Pth1r/Adgrl3/Fzd2/Calcrl

**WP1270 - Endochondral Ossification:**
Spp1/Mgp/Enpp1/Tgfb1/Pth1r/Runx2/Mef2c/Fgf2/Chst11
The WikiPathway represented as ‘lung fibrosis’ denotes the system in which that WikiPathway was studied which is understood simply as fibrosis. Osteopontin (Spp1) is the most abundant gene expressed in the fibrosis pathway. Osteopontin (Spp1) is known to promote TGFβ processing in fibroblasts through MMPs, making the ligands more accessible (Kramerova et al., 2019). From the receptor side Ccn2 promotes Tgfr2 expression (Tejera-Muñoz et al., 2022) which would increase TGFβ signaling as well. An exploration of the TGFβ ligand and receptor expressions in the whole dataset showed that clusters identified as fibroblasts (clusters 3 and 8) had the highest expression of Tgfr2 (Appendix Figure 1B.). Figure 13 and Figure 17 indicate that Pdgfra/b copositive fibroblasts are mostly metabolically active, perhaps explaining the expansion of the fibroblast population that we saw in Chapter 2 (Figure 6 and Figure 7B). A review of the possible roles being performed by each molecule in the fibrosis pathway is summarized in Figure 21.
Figure 18: Enriched GO biological pathways in cluster 0 of fibroblasts in control - 3-7-14-day ligated dataset.
The processes shown here indicate that this cluster is more metabolically active.
Taking the ‘Lung Fibrosis’ pathway and running a Cytoscape visualization, showed that the most significant signaling in this network of pathways in \( Pdgfra/b \) co-positive fibroblasts was TGF\( \beta \) signaling (Figure 19). Osteopontin (\( Spp1 \)) was the most significant molecule contributing to this TGF\( \beta \) signaling, the ‘key events’ being effected being T-helper 2 and M2 Macrophage activation/secretion of interleukins and growth factors (Figure 19). TGF\( \beta \) 1 ligand, when produced by this fibroblast subtype, affects the same ‘key event’(KE) along with fibroblast and myofibroblast proliferation, suggesting that it is TGF\( \beta \) signaling that is driving the proliferation of this population. This corroborates our correlational results found in Chapter 2. In a positive feedback loop driving its own dysregulation, the ligand has been shown to impact connective tissue growth factor (CTGF) (gene name: \( Ccn2 \)) through a signaling pathway known in ‘chondrocyte differentiation’ (Figure 19KE3/4). Associated with this ‘chondrocyte differentiation’ is stress fiber assembly for which tropoelastin (\( Eln \)) is significantly upregulated. This contributes to the stiffening of the tissues around which they are deposited. ‘Chondrocyte differentiation’ and stress fiber assembly leads to collagen production which leads to excessive deposition and or reduced turnover of ECM (key event 5) (Figure 19). This stress fiber assembly and excessive collagen deposition has been noted on human sections of patients’ submandibular salivary glands which had ductal obstruction (Figure 20). As opposed to what is shown in the network diagram of Figure 19, the adverse outcome (AO) in our mouse model would be salivary gland fibrosis.
Figure 19: Lung Fibrosis WikiPathway

Genes highlighted in red/orange are pathway components that are significantly increased in mouse salivary gland ductal ligation i.e. our cluster 0 dataset and genes highlighted in blue are significantly decreased in our dataset.
A. Hematoxylin and Eosin Staining

B. Picrosirius Red Stain

Figure 20: Human submandibular gland sections of a patient with chronic salivary gland obstruction
A. Human submandibular gland sections of a patient with chronic salivary gland obstruction showing excessive collagen deposition around a luminal structure with simple columnar cells (likely ducts). Hematoxylin and Eosin Staining showing the bright pink deposits of collagen. B. Picrosirius red staining showing intense collagen deposition. White boxes indicate field of magnification. Scale bars= 100 microns
Chapter 4: Discussion and Conclusion

The categorization of fibroblasts has been largely context specific. In recent times, however, studies have emerged to classify cross-tissue fibroblast subtypes (Buechler et al., 2021; Korsunsky et al., 2021). In the absence of a normal salivary gland fibroblast atlas, adequately identifying the fibroblast population and the changes therein was a challenge. We were, however, able to identify gross changes in the population like size and biological processes and pathways. These give us indications into what these population are doing. We have identified the active population by their coexpression of Pdgfra and b. A deeper understanding of fibroblast identities in salivary glands will help us to get one step closer to developing targeted cellular therapy addressing the issue of fibrosis. We show here the expansion of a fibroblast subtype that is driving fibrosis, likely through TGFβ signaling. Pdgfra and b-copositive fibroblasts are responsible for the significant expansion of the fibroblast population in the ligation model. They represent a population that arises in response to mouse salivary gland injury. This population exhibits characteristics of the myofibroblast phenotype. They increase their expression of ECM and rapidly proliferate (Figure 6 and Figure 8).

We did not detect a similar copositive population in the NOD/ShiLtJ mouse model but, that could be due to a disadvantage of the isolation technique used rather than a true reflection of the fibroblast subtypes in the disease model. The technique used yielded a small number of fibroblast cells. However, fibroblast subtypes which are not fibrotic in the CD-1 are fibrotic in the NOD/ShiLtJ model. The significantly higher expression of Fgfr2 and Pdgfrb in the NOD/ShiLtJ (Figure 10D) model suggests that only some fibroblasts might be targeted by Nintedanib in the diseased state. If TGFβ is driving the fibrosis process the drug would need to affect that pathway in some way to be effective.
The significant amount of collagen that arises in human obstructive injury is suggestive of similar stress fiber pathways being activated (Figure 20) as in our ligation model. In an early study, (van den Akker & Busemann-Sokole, 1983) seeking to validate sialolithectomies, it was shown that the removal of the stones from 21 patients saw recovery of salivary gland function in almost all the patients. Our data suggests that our mouse ligation model is a good representation of human salivary gland ductal obstruction. Unfortunately, in the clinic salivary glands containing stones are typically removed rather than the stones being removed.

Similar hypoxic pathways to those activated in ductal ligation will likely be activated in the human scenario, thus inducing oxidative stress and hence a similar molecular cascade of events driven by TGFβ signaling. Also, because the Wiki Pathway, WP 3932, is studied in homo sapiens, it suggests that there is concordance between the mouse and human species with respect to this specific pathway. It is likely that hypoxia was induced in the tissues from the ligation. The de-ligation of our mouse model, therefore, shows a similar recovery of salivary function in published studies. This recovery is likely due to recovery signals being able to overcome profibrotic signaling. Seeing how FGF2 stimulation favored regeneration and repair, perhaps stimulation with FGF2 might change the fibroblast phenotype from being myofibroblast like to becoming more homeostatic. The effectiveness of such an experiment might be contingent upon the removal or reduction of TGFβ signaling, as in our previous publication (Moskwa et al., 2022).

Numerous fibroblast subtypes have been identified in various biological contexts. With the advent of single cell RNA sequencing and spatial transcriptomic technology, the field is actively trying to come to a consensus on universal stromal sub-types (Cadinu et al., 2024; Korsunsky et al., 2021). Interestingly, these Pdgfra/b-co-positive cells do not express the known
myofibroblast markers Acta2, Myl9 and Myh11 (as seen in Figure 5) indicated by the yellow box indicating that they are a different cell-type and are most likely to be pericytes. This study brings us one step closer to subtyping fibroblasts in salivary glands in the diseased state. The study highlights that the co-expression of these two receptor groups (Pdgfra/b and Tgfbr2) likely leads to immune signaling, fibrosis, and fibroblast motility through its more metabolically active state (Figure 18). Indeed, we saw that inflammatory response pathway, prostaglandin synthesis and regulation, cytokines and inflammatory response and complement activation-the classical pathway were all activated in the fibroblasts of our dataset following salivary gland ductal ligation (Figure 14).

Sparc+Col3a1+ fibroblasts previously identified (Korsunsky et al., 2021) were seen in our dataset although not limited to Pdgfra/b-expressing fibroblasts (Figure 5 and Figure 13). The Pdgfra/b-copositive subtype did not coincide with the specific phenotype published in Korsunsky et al. (2021) that had SPARC and Col3a1 being co-expressed with Notch signaling. In our dataset, Pdgfra/b-copositive fibroblasts, along with other fibroblast subtypes, express Sparc and Col3a1 but Notch receptors were not expressed in Pdgfra/b fibroblasts (Figure 13). Therefore, Pdgfra/b fibroblasts after ductal ligation injury only partially satisfy this inflammatory fibroblast characteristic, as previously described. This might indicate a disease-specific fibroblast phenotype and that the disease process in SjD is different from solely fibrotic disease. Blood circulation and angiogenesis are showing up; however, as processes that are increased in the fibroblasts under fibrotic conditions (Figure 13). Further investigations would have to be done to determine the characteristics of fibroblasts regulating angiogenesis and blood circulation, for example, the genes involved. Moreover, the algorithm used in the cross-tissue
stromal study (Korsunsky et al., 2021) could be used to do a dataset overlay to determine the exact correlation between our dataset and theirs.

A new paper (Tsukui et al., 2024) added fresh perspective to the interpretation of our results. Their paper traced the lineage of fibroblasts in the lungs in response to fibrotic injury and showed that the cells that respond are inflammatory, interact with monocytes which release TGFβ1 and leads to the fibrotic phenotype. This was insightful because the most significant KE (Figure 19) is T-helper 2 or Macrophage 2 response, secretion/activation of interleukins and growth factors. This shows the macrophages to be having a central role in the initiation of the fibrotic process. Spp1 is likely making the TGFβ1 more bioavailable (Kramerova et al., 2019). TGFβ1 is related the KE3 and KE4 (Figure 19). This likely reflects the intermediary inflammatory stage of the fibroblasts which are responding to the injury and the result of recruiting monocytes to the site of injury (Tsukui et al., 2024). The implications of this then for an appropriate treatment regimen might mean addressing the fibrosis through abrogating the TGFβ while addressing the source of the TGFβ, the macrophages and monocytes.

Gene Ontology is a powerful tool that can help us to define the disease process in a system through analysis of high-throughput data. The Gene Ontology Biological Processes (GOBP) are different from biological pathways. A GOBP is a sequence of molecular events with an established starting and ending point that represents a specific objective an organism is genetically designed to do (Nguyen et al., 2015). On the other hand, a biological pathway is a system of molecules that interact through signals to create a molecular product or a change in the molecular state. The strongest upregulated pathway match in the fibroblast population was focal adhesion through the Pi3K, Akt, mTorc pathway followed by focal adhesion pathway (WP3932) (Figure 14). This possibly explains the ‘cell chemotaxis’ in Figure 12A Gene Ontology analysis:
the fibroblasts are likely moving in response to chemical stimuli (from the monocytes and macrophages) or due to pathogens in the environs (dysregulated microbiome). But the activation of this WikiPathway (WP3932) is also related to fibroblast proliferation, fibroblast differentiation, regulation of gene expression and cell survival through cell-matrix adhesion (Hanspers et al., 2024). This upregulated cell-matrix interaction through a complex series of pathways can lead to cell proliferation, angiogenesis, and DNA repair (ibid. 2024). Hypoxia activates the mTor complex, which phosphorylates various intermediary proteins to activate VEGF signaling/angiogenesis, cell growth, protein synthesis and autophagy.

This same hypoxia induced Ndufa4L2, in Pdgfraf/b copositive fibroblast cells specifically. NDUFAe4L2 has a promoter site for HIF1a, a molecule that is phosphorylated by the mTor complex. When induced, it reduces the activity of the electron transport chain to decrease mitochondrial oxygen consumption (Z. Liu et al., 2021). This is proposed to reduce reactive oxygen species production. In this study (Z. Liu et al., 2021), which involved the ligation of an artery to the muscle, it was proposed that this molecule drives the wasting that was noted in the muscle. In our reversible fibrosis model, salivary gland weights were found to be reduced after ligation along with salivary gland size. NDUFAe4L2 could be the chief molecule orchestrating this size reduction. This all suggests that hypoxia induces oxidative stress. There is a positive feedback loop between TGFβ signaling and oxidative stress where reactive oxygen species are able to activate latent TGFβ, and TGFβ in turn creates ROS species (Antar et al., 2023).

Having reviewed the molecular pathways that characterize fibrosis (Antar et al., 2023)(Figure 21), the differential expression analysis of the Pdgfraf/b copositive WikiPathways suggests that these are the cells driving fibrosis through TGFβ signaling (Figure 16, Figure 17, Figure 19). The Wnt signaling pathway activated by TGFβ has been found in fibrosis, cancer
invasion, and metastasis (Antar et al., 2023; S. Liu et al., 2018). But Wnt signaling is also required for TGFβ-mediated fibrosis and is known to regulate proliferation, migration and cell fate determination (Akhmetshina et al., 2012). The rapid and large expansion then of the Pdgfra/b copositive fibroblast population is likely related to a combination of the influence of Wnt, TGFβ, and PDGF signaling (Figure 6). Tissue inhibitor of matrix metalloproteinase (TIMP) is a possible connection between the ‘GPCR pathway’ (Figure 17) in Cluster 0 and the fibrosis pathway (Figure 21). Hem oxygenase 1 (Hmox1), also a part of the fibrosis pathway, is known to respond to TGFβ and to be related to stemness and cancer invasion (Ghosh et al., 2016). This link between fibrosis and cancer through TGFβ was thoroughly explored in a recent review (Massagué & Sheppard, 2023).

Ccn2/CTGF has been shown to be critical in the fibrosis process and is overexpressed whenever TGFβ1 is expressed under hypoxic conditions (Valle-Tenney et al., 2020). In turn, it upregulates TGFBR2 in vascular smooth muscle cells (Tejera-Muñoz et al., 2022). Whether the Tgfbr2 upregulation in response to ligation is directly due to Ccn2 overexpression remains to be determined but is a plausible cause (Figure 5). The role of Fgf2 is somewhat nuanced and is dependent on the tissue context (Sun et al., 2022) as in some organ systems it has been found to be antifibrotic (Dolivo et al., 2017; Pan et al., 2015), but in others, fibrogenic (Tsugeno et al., 2022). TGFβ and basic FGF2 are known to induce the expression of Pdgfr and aberrant PDGFR signaling drives pathological responses in fibroblasts, pericytes, and myofibroblasts in diverse fibrosis diseases (Paolini et al., 2022). Osteopontin is known to promote TGFβ1 activation or bioavailability and hence an activated fibroblast state (Dong & Ma, 2017; Kramerova et al., 2019). Therefore, the promotion of this TGFβ dysregulation arises from the ligand side and the receptor side.
This upregulation of TGFβ by this activated fibroblast cell type (Pdgfra/b-copositive) (Figure 21) is likely responsible for the activation of T-cells and hence the adaptive immune response (Figure 15). This fibroblast-immune system relationship is supported by this double enrichment of TGFβ ligands and receptors. As we discussed in the introduction, latent TGFβ is able to activate T cells and which in turn activate fibroblasts. These cells when activated produce TGFβ and activate other cells like myeloid cells and endothelial cells (Figure 12-adaptive immune response and Figure 15-T-cell activation inter alia). In a vicious cycle, the activation of TGFβ signaling in fibroblasts leads to an increased laying down of ECM products leading to fibrosis (Figure 19, Figure 20 and Figure 21). With so many routines of activating TGFβ, there are many possible pathways to compensation. The context specificity of fibroblasts does not help the case of fibrosis. It is no wonder that finding a key target for fibrosis in salivary gland disease has been so long coming and is yet to be realized. One possible solution is addressing the fibrosis alongside inflammation, that way both the upstream and downstream players are targeted.

**Limitations of Study**

We have not determined the specific lineage origin of this Pdgfra/b-copositive fibroblast sub-type, and it would be interesting to see the cell source of this fibroblast sub-type. We did not investigate whether this subtype aligns with CTHRC1+ fibroblasts, as described in multiple diseases, to be driving fibrotic disease (Tsukui et al., 2024). We think it is likely that the cells arose from the fibroblasts of the salivary glands and not from any other cell type, as in the case of lung fibrosis. It would be interesting to know if this fibroblast disease type also arises in human obstructive injury and if the pathways are consistent with those found in mice. This study also does not disaggregate the pathway analysis by 3-day, 7-day, and 14-day to determine which
pathways dominate at specific times. Although Gene Ontology is a powerful tool to uncover processes in biological systems, the information generated is not conclusive because the ontology annotations are predictions that are constantly being updated (Tomczak et al., 2018). Thus, we are cautious about drawing conclusions from these studies alone and encourage further in-vitro and in-vivo studies to confirm the findings. However, we have demonstrated that increased metabolic activity, Wnt signaling, and chondrocyte differentiation-like pathways all interact with TGFβ signaling to promote fibrosis in obstructed mouse salivary glands and require further investigation.

Figure 21: Summary of the molecules involved in the fibrosis pathway in Pdgfra/b copositive fibroblasts
Summary of the molecules in our dataset that is involved in the published Wikipathway. These are Spp1/Timp1/Ccn2/Tgfb1/Fgf2/Pdgfa/Hmox1. Created using Biorender.com
Appendix A: Supplemental Figures

A. Appendix Figure 1: Control, 3-, 7-, and 14-day ligated with homeostatic controls dataset
All cell type clusters are represented. Cluster 8 and 3 are fibroblast identified.
Appendix Figure 2: Feature Plot to determine which cluster is the fibroblast
Key fibroblast markers are *Col1a1, Pdgfra* and *Pdgfrb*
Appendix Figure 3: Heatmap of the gene expression level of fibroblast markers
Heatmap corroborates that clusters 2 and 9 are fibroblasts
Appendix Figure 4: Fibroblast subset using automated animation
(Aran et al., 2019)
Appendix Figure 5: Salivary Gland Fibroblast Expression Data Mapped on the Chondrocyte differentiation pathway.

Genes highlighted in red/orange are pathway components that are significantly increased in mouse salivary gland ductal ligation (i.e. our subcluster 0 fibroblasts dataset) and genes highlighted in blue are significantly decreased in our dataset.
Table 2: Top 3 upregulated WikiPathways in clusters 0 and 11

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Appendix B: Permission for figure use

Identifying fibrogenic cells following salivary gland obstructive injury © 2023 by Melinda Larsen is licensed under Creative Commons Attribution 4.0 International. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/
Appendix C: Approved Institutional Review Board form

Protocol Title: Albany Medical Center Tissue Consent
Version Number: 1.0            Version Date: 11/21/2021

Protocol title:  Albany Medical Center Normal Tissue Collection in the Head and Neck

Protocol number:

Date of original version: 11/21/2021

Date of revision: 11/21/2021

Revision number: 1

Effective date:

Clinical phase:

Sponsor: Albany Medical Center

Monitor: NA
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![Protocol Summary](image)

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<td>Study Centre(s):</td>
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<td>Clinical Phase:</td>
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<tr>
<td>Objectives:</td>
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<td>Methodology:</td>
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<td>Number of Subjects:</td>
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<td>Inclusion Criteria:</td>
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<td>Planned tissue removal (including normal tissues) from the head and neck.</td>
</tr>
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<td>Are scheduled for surgery or have already undergone surgery at Albany Medical Center.</td>
</tr>
<tr>
<td>Exclusion Criteria:</td>
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</tr>
<tr>
<td></td>
<td>Not scheduled or have not had surgery at above-mentioned hospitals.</td>
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1. INTRODUCTION

The purpose of this study is to develop patient centered regenerative medicine therapies for xerostomia (dry mouth). Extreme dry mouth can be a side effect of radiation therapy or autoimmune disease, as well as medication side effect with associated quality of life detriment (Almståhl 2016). The Larsen laboratory at University at Albany, SUNY has developed techniques to produce salivary gland organoids in a mouse model. The next step would be to see if this technology can be replicated in human tissues, and to characterize the cellular composition of the resultant salivary organoids. Multiple techniques, such as single cell sequencing and histologic analysis may be complementary to the approach. Specific tissue types of interest include skin, fat, and salivary gland tissue. Salivary gland tissue could include tissue from the major salivary glands (parotid, submandibular and sublingual) and minor salivary glands. These normal tissues would be removed in the course of treatment during head and neck surgery, so as not to have any interference with pathologic analysis of specimen or patient healing.

Study subjects are patients identified and referred for procurement and consent by their treating clinicians. Study subjects will give informed consent to donate excess tissues not utilized for routine diagnostic pathologic evaluations. Subjects names and medical record numbers will be stored on a secure database Key file on the Otolaryngology departmental Y-drive which will link to the Tissue Donation Identification Number (TDIN). Samples will be processed in laboratory under the TDIN, which does not contain any HIPPA protected identifiers. Patients can terminate participation in the study at any point, however, their names will be maintained as a record of tissue donation. Once the sample is handed off to the Larsen Laboratory it can still be recalled at any point by the patient if they request to cease participation in the study.
2. **STUDY OBJECTIVES**

**General Objective**

To provide a high-quality normal tissue for research purposes to help find cures for salivary gland dysfunction. There are two primary routes to achieve the goal.

1) Enable development of human salivary gland organoids derived from salivary tissue. Based on prior published work (Hosseini 2019) from the Larsen lab demonstrating that the salivary gland mesenchyme supports complex murine salivary epithelial organoid formation in vitro with robust ductal and proacinar cell differentiation, the Larsen lab plans to culture human salivary gland tissue with human mesenchymal stem cells (MSC) derived from adipose to from complex human salivary organoids exhibiting robust duct and acinar cell differentiation. Optimization of the numbers of MSCs, cell culture medium and other conditions will be required. Development of such complex human salivary organoids could enable in vitro testing of therapeutics that promote salivary gland survival and function, including novel treatments as well as diagnostic testing of xerostomia patient samples for response to therapeutics that would warrant use. This method requires salivary gland tissue.

2) Enable development of salivary gland organoids derived from induced pluripotent stem cells (iPSCs). In parallel, the Larsen lab plans to use patient-derived fibroblast cells to generate iPSCs which will be induced to form salivary gland organoids. This method requires skin tissue.

3. **STUDY POPULATION**

**Number of Subjects**

We expect to enroll up to 50 subjects each year depending on the volume of surgeries across the Albany Medical Center.

**Inclusion Criteria**

Age 18 and above.
Normal tissues may be collected from the head and neck as part of planned surgery. Are scheduled for surgery or have already undergone surgery at The Albany Medical Center participating sites.

Subjects meeting the above-mentioned criteria will be enrolled without regard to gender, race, or ethnicity.

Exclusion Criteria

- Unable to provide informed consent
- Prisoners
- Not scheduled or have not had surgery at Albany Medical Center

4. OBSERVATIONS

Safety

There are minimal safety concerns for subjects since they will not undergo any additional procedures specific to the study. All tissues will be collected as byproducts of routine care procedures. Specifically, fat or normal salivary gland tissue is often attached to a surgical specimen and can be trimmed ex-vivo. In terms of skin, the edges of the surgical incision can be trimmed very narrowly to provide a fresh surface to suture, the excess trimmed tissue can be donated. Thus, they will not experience additional risks over those entailed in the clinical care of their disease. Normal tissue submitted for research will not exceed 2 grams, as estimated during the time of surgery.

5. STUDY PLAN

Study Design and Plan
Patients that will be undergoing surgeries of the head and neck will be identified as potential candidates for benign tissue donation. Specifically, skin, adipose, and salivary gland tissue have been identified as candidate tissues of interest. Patients will provide informed consent to donate up to 2 grams as estimated intraoperatively of benign tissue.

These harvested tissues will be transferred fresh and de-identified to a representative of the Larsen lab, or courier.

There will be no specimens stored at Albany Medical Center, in other words there will be no associated biorepository. Tissue samples will be destroyed after 2 years.

The TDIN can be cross referenced with patient chart data to provide de-identified data from the medical record, such as any history of Sjögren’s disease or external beam radiation therapy.

Subject Preferences

While patients can end participation at any point. They could rescind consent prior to surgery in which tissue would not be donated. Consent could be terminated after the tissue reached the lab, in which case it would be cross referenced with the secure Key file, and the Larsen lab would be instructed to dispose of the sample and stop experiments. If experiments have already been completed on the tissue, and a patient withdraws consent, then no further donation of tissue would be obtained. The informed consent will explain that while cell development is being studied, there will be no long-term cell lines resulting from their tissue donation. Additionally, there will be no publication of identifying factors including genetic code.

Advertisements

There will be no public advertisement for enrollment in the study, patients will be individually offered participation by the treating clinician or delegate or collaborating physician.
6. **ADMINISTRATIVE MATTERS**

**Recruitment**

Approach for recruitment only takes place after discussion with the patient’s treating physician. Recruitment may take place if the patient demonstrates interest in the study.

**Informed Consent**

Informed consent of a subject is obtained using the appropriate IRB approved study informed consent document. Study staff will observe good clinical practices outlined in the Albany Medical Center IRB guidelines in all informed consent activities.

Copies of informed consent documents will be provided to the patient.

IRB- and HIPAA-trained and certified research personnel stipulated in the protocol will obtain informed consent from patients. Subjects identifying information will be removed from the specimen.

**Informed Consent and Vulnerable Subjects**

This study may enroll disadvantaged individuals. Economically or socially disadvantaged subjects will be treated no differently from other subjects. This study does not provide medical evaluation or therapy that these subjects could not obtain through the existing medical services already available without participation in the project. There are no financial incentives included in the research. Prisoners are not eligible for participation.

**Participation of Children**

The Albany Medical Center Normal Tissue Collection in the Head and Neck is a minimal risk study, however, for simplicity participants will be required to be age 18 and above.
**Adverse Events**

There are no procedures performed outside of routine clinical care, thus we do not anticipate any study-related adverse events. Adverse events unrelated to the study will be reported as per IRB guidelines.

**Events requiring Immediate Reporting**

Fatalities and Adverse Events classified as Serious are those that are:

- Serious Adverse Event (SAE)
- Immediately life threatening
- Permanently disabling
- Requires in-patient hospitalization
- Prolongs a current hospitalization
- Serious in the opinion of the investigator

If an event listed above occurs to an enrolled subject, and if the event is unanticipated and directly related to the subject’s participation in this research study, a written report to the IRB must be made within 24 hours after the site is made aware of the event. In all cases, the safety of the subject is the primary concern of the study staff. The principal investigator (PI) or co-investigators must be contacted by study coordinators, in or out of office hours by phone or beeper to discuss serious adverse event management if necessary.

Serious adverse event reporting consists of completion of a serious adverse event memo and IRB form 10. Both documents must be reviewed, signed and dated by the principal investigator or a co-investigator ONLY if the PI cannot be contacted in the 24-hour time reporting frame.

Determination of relationship to study should be made by the Principal Investigator or if she/he cannot be contacted, by a co-investigator.
The PI is alerted to all serious adverse events as soon as possible.

**Expected Adverse Events not related to Study**

Because of the observational nature of the study with minimal risk and the target study population, adverse events are expected to occur due to the study population’s underlying illness. Expected adverse events NOT meeting serious adverse event category and definitely NOT related to study participation are not reported to the IRB. For this study population these expected adverse events would include any new onset or worsening symptom, sign or diagnosis.

**Statement of Confidentiality**

Participants’ identity and research record will be kept confidential in any publication of the results of this study and to the extent permitted by law.

Specifically, each subject will be assigned a Tissue Donation Identification Number (TDIN) for each event of donation (some patients may be eligible for multiple donations if multiple surgeries are performed). The TDIN will take the form of “year-number”, for instance “2020-01”, the next patient may be “2020-02” and so on. The TDIN will be cross referenced in the secure Key file stored on the departmental Y-drive, linking to the patient name and medical record number. In this way, no patient identifiers will be released to the Larsen lab, decreasing risk of breach of security.

**References for Tissue Consent Protocol:**


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