UNIVERSITY OF CALIFORNIA,

IRVINE

Understanding centroacinar cells as a source for pancreatic cancer and as a beta-cell progenitor

DISSERTATION

Submitted in partial satisfaction of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Tori Raquel Tucker

Dissertation Committee:

Associate Professor Michael Parsons, Chair

Associate Professor Thomas Schilling

Associate Dean Peter Donovan

Associate Professor Ali Mortazavi

Associate Professor Ping Wang

2023

DEDICATION

Big thanks to my parents Petra and Douglas Tucker for always continuing to support my dreams and my sister Tami Tucker and grandma for their continued support. I especially want to thank my cats Sasha and Bentley for putting up with my countless complaints throughout this process.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Shoutout to all the members of the Parsons Lab throughout the years!

Chapter 1 - I would like to thank previous members of the Parsons Lab for their help in optimizing my *in-situ* hybridization experiments. The publication of this chapter would not have been possible without the efforts of Hannah Edelman and Sarah McClymont. I would also like to thank the members of the Schilling Lab for their ongoing support and help when I needed it while working with zebrafish.

Chapter 2 – I would like to specially thank Jeff Mumm from Johns Hopkins University for providing our lab with NTR2.0. This project would not have been possible without his efforts! I would also like to thank my undergrads Sheida Eshghi for helping with all the tedious tissue sectioning, Deena Khoury for putting up with my nonsense, carrying out numerous larval experiments with MTZ, and performing IHC experiments, Sophia Tran for helping with all the chronic hyperglycemia studies and coming in on countless weekends to complete the work needed for us to publish this paper. I would also like to thank Travis Wiles and his PhD student Courtney Knitter for helping with the details regarding the gut microbiome. Special thanks to Courtney for her help with the germ-free derivation experiment done in larval zebrafish.

Chapter 3 – I would like to thank so many people for making this project possible because without them I don't know what I would have done. Stephenson Chea from Anne Calof's Lab deserves a shout out for helping me understand how to analyze my single cell data. David Tatarakis (a former member of the Schilling Lab) helped walk me through Seurat during COVID. Praveer Sharma (a former member of the Schilling Lab) took the time to help me generate my zebrafish reference genome and taught me how to use CellRanger. Meritxell Rovira deserves the up most credit for

her expertise in helping me analyze and understand my single cell data. I want to thank her and Loris especially for providing their single cell analysis script that functioned as a scaffold for my project. I would like to also thank Dr. Qing Nie and the Mathfish group for their ongoing support in this project, especially Dr. Nie's postdoc Changhan who helped me so much with the downstream bioinformatics analysis. Lastly, I would like to thank Jenn Atwood for sorting my cells and those especially at the UCI genomics core facility (Melanie Oakes, Quy, and Valentina)!

Overall, I would like to thank members of my committee for their continued support throughout my time at UCI: Drs. Thomas Schilling, Ali Mortazavi, Ping Wang, and Peter Donovan. I would like to give a special thanks most of all to my mentor and thesis advisor Dr. Michael Parsons for his encouragements throughout my time in the lab and his ongoing help when I could not see the light at the end of the tunnel, and I thought all hope was lost. It was such a great pleasure working in the lab with you and getting to know your family! I truly enjoyed getting to know you as a person. We shared a lot of laughs and humor throughout my time in the lab. I look forward to watching the lab grow and hearing about all its amazing accomplishments as the years go on!

I thank Oxford Academic for permission to include copyrighted materials in **Chapter 1** of my dissertation, which was originally published in Human Molecular Genetics. I also thank The Company of Biologists for permission to include copyrighted materials in **Chapter 2** of my dissertation, which was originally published in Disease Models and Mechanisms. Financial support was provided by the University of California, Irvine, and the NIH NIDDK F31 Grant F31DK127688.

VITA

Tori Raquel Tucker

Institution	Degree	Years	Course of Study
University of California Irvine, Irvine CA	-	2018-2019	Teaching Assistant Professional Development Program
University of California Irvine, Irvine CA	PhD	2017-2023	Developmental and Cell Biology
University of California Irvine, Irvine CA	M.S	2017-2021	Developmental and Cell Biology **Cumulative GPA:** 3.979
University of California Irvine, Irvine CA	B.S	2013-2017	Developmental and Cell Biology **Cumulative GPA:** 3.71 **Science GPA:** 3.982
Los Angeles Trade Technical College (LATTC), Los Angeles CA		2010	Health Sciences
Great Oak High School, Temecula CA	HS Diploma	2009-2013	General Education **Cumulative GPA:** 4.3

Certifications:

June 2020: Three-part Nature Masterclasses: Scientific Writing and Publishing

Awarded Fellowships:

May 2021: NIH F31 Fellowship (impact score: 17)

March 2021: Graduate Dean's Excellence Fellowship

2018-2019: Graduate Assistance in Areas of National Need (GAANN) Fellowship

Honors & Awards

April 2021: Edward A. Steinhaus Teaching Award

June 2020: Developmental and Cell Biology Teaching Assistant (TA) of the year

April 2019:	National Science Foundation (NSF) Honorable Mention
June 2017:	Jayne Unzelman UC Irvine School of Biological Sciences Scholarship for Conducting Undergraduate Research
June 2017:	Undergraduate Research Award Bio199 Program UC Irvine
June 2017:	Developmental and Cell Biology Program Recognition UC Irvine
May 2017:	Student Research Spotlight UC Irvine School of Biological Sciences Newsletter
2015-2017:	Undergraduate Research Opportunities Program (UROP) Grant
2015-2017:	Summer Undergraduate Research Opportunities Program (SURP) Grant
2013-2017:	Dean's Honor List
2013-2014:	MiliporeSigma Scholarship Recipient
2013:	California Scholarship Federation Highest Honors Award
2012-2013:	International Baccalaureate (IB) Biology Certification

Conferences and Talks:

September 2021:	Medical Student Endocrinology Club Speaker, University of Touro Nevada
June 2021:	International Zebrafish Conference 2021—Abstract and poster presentation
June 2019:	Association for Biology Laboratory Education (ABLE) -- Abstract and presentation
May 2016:	10th World Biomaterials Congress, Montreal, Canada

Teaching and Mentorship Experiences:

2021:	Cell and Molecular Biology Mentorship Program
2021-Present:	SoCal BioEYES Outreach
2019-2023:	UCI Undergraduate BioSci Mentorship Program
2018-2019:	Participation in Association for Biology Laboratory Education active learning development through GAANN Fellowship
Fall 2018-2020:	3 years teaching undergraduate level Honors Bio97 Genetics UC Irvine TA

2014-2023:	UC Irvine Undergraduate Research Program Mentor (Bio199)
2017:	UC Irvine BioSci Alumni Speaker
2015-2016:	UC Irvine Peers Under BioSci (PUBS) Mentorship Program
2016:	Bio93 Discussion Tutor Undecided/Undeclared Office UC Irvine
2013-2016:	Biology, Organic Chemistry, and Calculus Tutor
2014:	UC Irvine Student and Parent Orientation Program (SPOP)

Other Experiences and Professional Memberships

2021-Present:	UCI MathFish bioinformatics and zebrafish collaboration group
2021- Present:	International Zebrafish Society (IZFS) member
Spring 2019:	Hosted Bio-EYES Outreach Program Talk
2017-Present:	Graduate Professional Success-Biomedical Sciences (GPS-BIOMED)
2017-Present:	Golden Key International Honour Society
2015-2017:	College Diabetes Network
2015-2017:	Summer Undergraduate Research Opportunities (SURP) Program
2015-2016:	Peers Under BioSci Mentorship Program, UC Irvine
2014-Present:	Undergraduate Research Program Mentor (Bio199), UC Irvine
2014-2016:	Chair at ONE Disease and Poverty Prevention Outreach Program
2014-2017:	Participation through the Pediatric-Adolescent Diabetes Research Education Foundation (PADRE)
2014-2017:	Undergraduate Research Opportunities Program (UROP), UC Irvine
2013-Present:	Participation through the Juvenile Diabetes Research Foundation (JDRF)
2022-Present:	Volunteer at Irvine Rotary and UCI Rotaract

FIELD OF STUDY

Since joining the Parsons lab in 2018, I have had three major accomplishments. My first major accomplishment was to systematically test different components of a zebrafish whole mount *in-situ* hybridization protocol and optimize each step for transcript detection in micro-dissected larval pancreata. This protocol is a very important tool for my research as I can now use it to study gene expression of the developing endocrine system. Using this protocol, I have a first-authored paper

published in Human Molecular Genetics. My paper describes how Sox9b-target genes in zebrafish pancreas are regulated in our b-cell progenitor. My second major accomplishment in the lab is developing a model that can be used to study chronic hyperglycemia and used as a more effective b-cell ablation transgenic line that requires less prodrug to perform b-cell ablations in the pancreas. At the levels of prodrug presently used, there are many undesirable side effects. With this new model, it has the potential to study long-term effects and complications associated with diabetes. I am in the process of publishing my second first authored paper in March 2023. My third major accomplishment was uncovering the cellular heterogeneity of a zebrafish pancreatic progenitor called centroacinar cells. To do this, I used fluorescent activated cell sorting (FACS) to sort CACs and performed single-cell RNA-seq (scRNA-seq) on those sorted cells. Once the sequencing data was returned to me, I learned how to bioinformatically analyze the CAC transcriptome using R and the Seurat package. I was the first person in our lab to establish scRNA-seq techniques and the scripts used to bioinformatically analyze the CAC population in zebrafish.

Research Presentations/Posters:

1. Tori R. Tucker, Jeff S. Mumm, Meritxell Rovira, Praveer P. Sharma, and Michael J. Parsons. "Uncovering the cellular and molecular mechanisms driving b-cell neogenesis during regeneration" International Zebrafish Society Conference 2021.

2. Deena M. Khoury, Zhanbo Ding, Juan Tao, Jeff S. Mumm, Tori R. Tucker, and Michael J. Parsons. "Generation of a New Model to Study the Effects of Chronic Diabetes." Undergraduate Research Opportunities conference, 2019 April, Irvine, CA.

3. **Observing the Effect Pro-Inflammatory Cytokines have on Porcine Islets when Cultured with Pro-Inflammatory Cytokines**
   University of California Irvine
   2017 Undergraduate Research Opportunities Program (UROP) Symposium

4. Pediatric-Adolescent Diabetes Research and Education (PADRE) Foundation speaking events 2017

5. **A Novel Strategy to Ameliorate Hypoxia in Encapsulated Islets using Perfluorodecalin**
   University of California Irvine
   2016 Undergraduate Research Opportunities Program (UROP) Symposium

6. **Verifying Non-invasive Optical Scanning of Vascularization with in situ Immunostaining**
   University of California Irvine
   2016 Undergraduate Research Opportunities Program (UROP) Symposium

7. **Strategies to Overcome Tissue Hypoxia in Islet Encapsulation**

Montreal Canada
2016 WBC: 10th World Biomaterials Congress Montreal Canada Talk and Poster Session for Combinatorial Approaches to Biomaterial Design

8. **Juvenile Porcine Islets Reverse Hyperglycemia in Diabetic Athymic Nude Mice after Xenotransplantation**
   University of California Irvine
   2015 Translational Research Symposium Presentation

9. **Studies of the Effect of Bioflavonoid Supplements on Zucker Diabetic Fatty Rat Blood Glucose Levels**
   University of California Irvine
   2015 Undergraduate Research Opportunities Program (UROP) Symposium

10. Juvenile Diabetes Research Foundation (JDRF) speaking events 2015

Publications:

1. Tori R. Tucker, Deena M. Khoury, Sheida Eshghi, Sophia Tran, Jeff S. Mumm, Michael J. Parsons. "A new model of chronic hyperglycemia." Pending submission to Disease Models and Mechanisms (2023)

2. Hannah E Edelman, Sarah A McClymont, Tori R Tucker, Santiago Pineda, Rebecca L Beer, Andrew S Mcallion, Michael J Parsons. "SOX9 modulates cancer biomarker and cilia genes in pancreatic cancer." *Oxford Human Molecular Genetics* (2021).

3. Christopher Kopan, Tori Tucker, Michael Alexander, M. Rezaa Mohammadi, Egest J. Pone, and Jonathan Robert Todd Lakey. "Approaches in Immunotherapy, Regenerative Medicine, and Bioengineering for Type 1 Diabetes." *Frontiers in Immunology* 9 (2018).

4. Tori Tucker, Kevin Labadie, Christopher Kopan, Michael Alexander, and Jonathan RT Lakey. "Strategies in Preventing Diabetes after Pancreatectomy Using Islet Auto- and Allo-Transplantation." *International Journal of Transplantation Research and Medicine* 3.1 (2017).

5. Rahul Krishnan, David Ko, Tori Tucker, Emmanuel Opara, Clarence E. Foster, III, David Imagawa, Michael Stamos, and Jonathan RT Lakey. "Strategies to Combat Hypoxia in Encapsulated Islet Transplantation." *Surgery: Current Research* 06.02 (2016).

Abstracts:

1. Tori R. Tucker, Jeff S. Mumm, Meritxell Rovira, Michael J. Parsons. “Uncovering the cellular and molecular mechanisms driving b-cell neogenesis during regeneration.” International Zebrafish Society (IZFS) Conference 2021.

2. Kristina Lackey, Barbara Waring, Tori Tucker and Debra Mauzy-Melitz. “Unzipping Your Genes: A Fahionable Tutorial in Gene Expression.” Association for Biology Laboratory Education (ABLE) 2019 conference.

3. Rahul, Krishnan, Huy Nguyen, Nick Neel, Nicole Corrales, Sally Padayao, Tori Tucker, Michael Alexander, John Mcquilling, Elliot Botvinick, Emmanuel Opara, and Jonathan Lakey. "Strategies to Overcome Tissue Hypoxia in Islet Encapsulation." *Frontiers in Bioengineering and Biotechnology* 4 (2016).

ABSTRACT OF THE DISSERTATION

Understanding centroacinar cells as a source for pancreatic cancer and as a beta-cell progenitor

by

Tori Raquel Tucker

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2023

Associate Professor Michael Parsons, Chair

Type one diabetes is an autoimmune condition characterized by the loss of insulin producing beta cells of the pancreas. A loss of beta cells leads to a dysregulation in glucose homeostasis and results in prolonged hyperglycemia. If left untreated, numerous secondary complications can result such as neuropathy, nephropathy, retinopathy, limb amputation, and eventually death. Current treatments for type one diabetes rely on the administration of exogenous insulin or cell transplantation methods which have a variety of problems such as donor scarcity, a high cost, and requires immunosuppressants to be taken. Our lab is interested in inducing the body to produce its own beta cells endogenously via neogenesis. Since the mammalian pancreas is limited in its capacity to regenerate beta cells, zebrafish function as an excellent model to study neogenesis because they have a beta-cell progenitor called a centroacinar cell. Zebrafish centroacinar cells are like mammalian Ngn3+ progenitor cells of the developing pancreas. However, zebrafish centroacinar cells retain their progenitor capacity in adult fish. From mammalian models, centroacinar cells have also been suggested as a potential origin of pancreatic cancer. This is because centroacinar cells express similar pathways utilized by pancreatic cancer. These pathways are also conserved in zebrafish centroacinar cells. **In this thesis**, I am interested in further understanding the biology of centroacinar cells in order to elucidate (**1**) how zebrafish

centroacinar cells can function as an *in vivo* model to better study the pathways involved in pancreatic cancer and (**2**) how the pathways utilized by zebrafish centroacinar cells allow them to behave as a beta-cell progenitor compared to mammalian centroacinar cells. In C**hapter 1** I focused on the importance of the SOX9 pathway in pancreatic cancer and related this pathway back to zebrafish centroacinar cells. Previous work in our lab used RNA-seq and ChIP-seq in PANC-1 cells to identify the targets of SOX9. One of these targets was *EPCAM*. Using zebrafish, I demonstrated that *epcam* is expressed in centroacinar cells and is regulated by levels of *sox9b*. In **Chapter 2** I focused on improving our lab's nitroreductase/metronidazole transgenic zebrafish line to facilitate beta-cell regeneration. Using this line, I have shown that a lower dose of prodrug is required to ablate beta cells. As a result of this lower dose, it reduces the toxic side effects experienced by both larvae and adult fish. Unlike our old nitroreductase transgenic line, adult fish expressing this improved nitroreductase can achieve complete beta-cell loss by simply immersing them in prodrug. Additionally, my improved nitroreductase model can also be used to study chronic hyperglycemia which could not be done using our old nitroreductase model. In **Chapter 3**, I took advantage of using single-cell RNA sequencing to uncover centroacinar cell heterogeneity. Uncovering heterogeneity is important because it has allowed us to further uncover the role of centroacinar cells as a beta-cell progenitor but also as a potential origin for pancreatic cancer. From my genomics study, I have identified various subpopulations of centroacinar cells including a potential transitioning subpopulation of acinar cells toward a centroacinar cell fate, an immune-like cell population of centroacinar cells, and a potential endocrine precursor cell population. Additionally, I used both an *in silico* and *in vivo* approach to uncover new marker genes of centroacinar cells that may have an important role in their progenitor and cancerous behavior.

Introduction

In my thesis, I use zebrafish as an animal model to explore pancreatic cancer (**Chapters 1 and 3**) and beta-cell regeneration (**Chapters 2 and 3**). I use zebrafish because they are an excellent model system to study pancreas biology (i.e. pancreatic cancer) and beta-cell neogenesis (Tiso, Moro, & Argenton, 2009). A major advantage of using zebrafish is their remarkable capacity to regenerate (Delaspre et al., 2015; Ghaye et al., 2015; Moss et al., 2009). In zebrafish, beta-cell neogenesis can be studied during embryogenesis and regeneration in the adult fish (S. Chen, Li, Yuan, & Xie, 2007; Delaspre et al., 2015; Parsons et al., 2009). Zebrafish have a beta-cell progenitor called a centroacinar cell (CAC) that allows them to regenerate so well. CACs have become the primary focus of my thesis and will be discussed in more detail later. Diseases like type one diabetes (T1D) result in a loss of beta-cell mass and one question that remains in the field is how to restore beta-cell mass to treat T1D. The ultimate goal would be to induce an endogenous stem cell or progenitor to restore beta-cell mass in those with T1D. Current research on beta-cell regeneration relies on rodent models. However, like humans, adult rodent models have a limited regeneration capacity, and therefore, they are not the most well-suited model to study beta-cell regeneration. The limited ability to study regeneration in rodent models has made it difficult for those in the field to uncover mechanisms that are needed to restore-beta cell mass. Thus, if we want to uncover regenerative mechanisms to treat human conditions like T1D, we need to use an animal model that can regenerate beta cells well. Using a highly regenerative model will enable the deconstruction of the mechanisms involved in regenerating beta cells. Since adult zebrafish retain the ability to regenerate beta-cells, they are a well-suited model to understand the cellular and molecular mechanisms driving beta-cell regeneration. The question that remains in the field is

how zebrafish can regenerate beta cells better than their mammalian counterparts. **In my thesis**, I have set out to understand the cellular and molecular pathways turned on in the fish that facilitate them to continue to regenerate beta cells in the adult fish. Although much work is still needed to understand these pathways utilized in the fish, my work presented here sets a solid foundation for future work in the field of pancreas regeneration. Here, I will provide an overview of the pancreas, diseases of the pancreas, current treatment attempts to restore beta-cell mass in humans, and how my work using zebrafish will further our understanding of both pancreatic cancer and beta-cell regeneration.

Overview of the pancreas

In vertebrates, the pancreas is an essential organ that is part of both the digestive (exocrine) and the endocrine system. The human pancreas is a gland that is located within the abdomen behind the stomach. Anatomically, the pancreas is divided into three main parts: the head, body, and tail. The head of the pancreas is located near the intestinal duodenum, the body of the pancreas stretches from behind the stomach, and the tail of the pancreas is located adjacent to the spleen. The exocrine compartment is responsible for the breakdown of food and the endocrine compartment regulates blood glucose homeostasis. 95% of the pancreas is made up of exocrine tissue while the other 5% of the pancreas is made up of endocrine tissue (Das et al., 2014).

The exocrine pancreas is made up of both acinar and ductal cells. Acinar cells are grouped into functional units called acini with a central lumen and come into contact with the intestine through an intricate ductal system (Ashizawa, Sakai, Yoneyama, Naora, & Kinoshita, 2005). The pancreas associated transcription factor 1a (PTF1A) specifically controls pancreatic organogenesis

in early development but primarily promotes acinar cell differentiation and maintains the specific gene expression of acinar cells in the adult pancreas (Beres et al., 2006; Dong, Provost, Leach, & Stainier, 2008; Duque, Amorim, & Bessa, 2022; Hoang et al., 2016). The primary function of acinar cells is to synthesize, store, and secrete digestive enzymes that breakdown carbohydrates, proteins, and fats. These enzymes include proteolytic enzymes, glycoside hydrolases, ribonucleases, lipases, and phospholipases (Rutter et al., 1968). Among these enzymes, there are many that have become acinar cell-specific markers including amylase, trypsin, carboxypeptidases, and elastase. Acinar cell morphology is characterized by their abundance of digestive zymogens which are seen to accumulate in the endoplasmic reticulum and are stored at the apical cytoplasm membrane as secretory granules (Han, Rall, & Rutter, 1986). Zymogens are an inactive precursor of an enzyme that become activated once they have reached the intestinal duodenum.

The ductal system of the exocrine pancreas is highly branched throughout the pancreas and contains terminal/intercalated ducts that meet with the intralobular ducts, and then the main pancreatic duct which opens into the duodenum of the intestine. Ductal cells are bound by connective tissue that produces columnar epithelium and communicates with the central lumen of each acinus. Ductal cells have two essential roles, first, they direct the passage of enzymes produced by acinar cells into the intestine and second, they secrete bicarbonate to neutralize acid entering the duodenum from the stomach which avoids the aggravation of digestive enzymes. Gene-specific markers of ductal cells include the expression of *cystic fibrosis transmembrane receptor (CFTR)*, various *cytokeratins*, *carbonic anhydrase II*, and *Sox9*. Specialized ductal cells called CACs are located within the center of acini at the duct terminuses. In the mammalian pancreas, CACs are located at the proximal tips of the pancreatic ductal tree. A major feature of

gene expression that distinguishes CACs from ductal cells is their continuous Notch activity, which is absent in mature ductal cells (Parsons et al., 2009; Rovira et al., 2010). In the literature, there are numerous controversies that persist regarding mammalian ductal cells or CACs behaving as an endogenous beta-cell progenitor (K. Hayashi, Takahashi, Kakita, & Yamashina, 1999; Inada et al., 2008; Kopinke et al., 2011; Kopinke & Murtaugh, 2010; Miyatsuka et al., 2014). This will be discussed in more detail later.

The endocrine compartment of the pancreas is made up of cell clusters called the islets of Langerhans, which are scattered throughout the exocrine pancreatic tissue. The islet is primarily composed of five different cell types including: insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, ghrelin-producing epsilon cells, and pancreatic polypeptide-secreting PP cells. The most abundant cell types in the islet include the beta cells which make up approximately 60-70% of the islet and alpha cells which make up approximately 20-30% (Yau & Kebede, 2021). In rodents and teleost fish (e.g., Zebrafish), beta cells are found within the islet core and are surrounded by a thick discontinuous mantle of alpha, delta, and epsilon cells (Bosco et al., 2010; Kinkel & Prince, 2009). This is somewhat less obvious in humans where controversies persist about the topographic arrangement of endocrine cells within the human islet (Bosco et al., 2010). It is no surprise that the islet is comprised mostly of beta cells and alpha cells since they are the primary cell types involved in regulating glucose homeostasis. Insulin is a hormone produced by beta cells to lower blood glucose levels and glucagon is a hormone produced by alpha cells to increase blood glucose levels. Blood glucose levels are always in oscillation, but the most obvious changes occur during periods of fasting and right after the consumption of a meal.

Diseases of the pancreas

When there is a disruption to either the exocrine or endocrine pancreatic compartment it can lead to numerous diseases of the pancreas including pancreatitis, pancreatic cancer, and diabetes. Although our lab is interested in all 3 conditions, the primary focus of my thesis is to study pancreatic ductal adenocarcinoma (PDAC) and type one diabetes (T1D). As mentioned, I use zebrafish as an animal model to study both diseases.

Pancreatitis is a disease associated with inflammation of the pancreas and can be categorized as either acute (temporary) or chronic (long term). Pancreatitis results when the digestive enzymes of the pancreas start to digest the pancreas itself. The most common cause of acute pancreatitis is excessive consumption of alcohol which accounts for approximately 30% of all cases and gallstones which accounts for up to 40% of all cases (Ashraf, Colombo, Marcucci, Rhoton, & Olowoyo, 2021). Chronic pancreatitis is characterized by persistent inflammation of the pancreas which results in permanent damage to the pancreas marked by fibrosis and ductal strictures (Brock, Nielsen, Lelic, & Drewes, 2013). Accumulating damage to the pancreas eventually leads to decreased function of both the exocrine and endocrine compartment. During pancreas inflammation or injury, acinar-to-ductal metaplasia (ADM) is a common process observed where the pancreas tries to facilitate the regeneration of damaged cells in the pancreas (Houbracken et al., 2011). The cellular and molecular process by which acinar cells transition into ductal cells is not fully understood. By studying CACs in the zebrafish pancreas, I have uncovered a potential population of cells that may be involved in ADM. These results are discussed in

Chapter 3. Although pancreatitis is not the primary focus of my thesis, my findings will expand upon future work in our lab to study ADM in the context of pancreatitis. Studying pancreatitis is important because about 40-75% of patients diagnosed with chronic pancreatitis will at some point require pancreatic surgery (pancreatectomy) to alleviate their pain symptoms (Parekh & Natarajan, 2015). If my work in studying zebrafish CACs can be applied to pancreatitis, we will have an *in vivo* way to study pancreatitis to discover better treatments for humans.

One of the pancreatic diseases that is the focus of my thesis is pancreatic cancer, which can affect both the exocrine and endocrine compartments of the pancreas. Cancer that affects the endocrine pancreatic compartment is referred to as neuroendocrine cancer and is characterized by neuroendocrine tumors (NETS). Endocrine pancreatic cancers are considered more rare than cancers that arise within the exocrine pancreatic compartment. The most common and lethal type of pancreatic cancer is PDAC which forms in the exocrine pancreatic compartment. One of the major questions that remains about PDAC is its cellular origin. It is thought that the cellular origin for PDAC could be ductal cells/CACs, acinar cells, or both (Kopp et al., 2012). From mouse models, PDAC is thought to involve the transdifferentiation of acinar cells to a more ductal like phenotype (Kopp et al., 2012). This finding suggests that acinar cells may be the cellular origin but must transition into ductal cells to give rise to PDAC. In **Chapter 1** of my thesis, I explore CACs as a potential origin for pancreatic cancer. Previous work in the Parsons Lab used a PDAC cell line called PANC-1 to uncover mechanisms involved in pancreatic cancer. In my thesis work, I show that these pathways in PANC-1 cells are similarly expressed in zebrafish CACs. Additionally, in **Chapter 3**, I study CACs at the single-cell level and uncover additional genes expressed by CACs that are also expressed by cells in PDAC. The significance of my work demonstrates that zebrafish CACs can function as an *in vivo* model to study pathways involved in

pancreatic cancer. Understanding these pathways utilized by CACs may explain how CACs go from non-cancerous to cancerous cells. The reason we should care about PDAC is because it is seldom detected in its early stages, which results in poor prognosis and a low survival rate. Symptoms of pancreatic cancer do not present until the cancer has metastasized to other organs. Most treatment options for pancreatic cancer include surgery, chemotherapy, radiation therapy, or a combination of therapies (Brunner et al., 2019). Understanding the origin of pancreatic cancer is important because it will allow for better prevention, diagnosis, and treatment of PDAC. Therefore, my research on CACs will have an impact in the field of pancreatic cancer research because it furthers our understanding of the cellular origin of PDAC. I will describe pancreatic cancer and CACs in the context of PDAC in greater detail later in **Chapter 1**.

Diabetes is the second disease I focus on in my thesis, specifically T1D. In general, diabetes is a chronic condition of the pancreas that is caused by a lack of insulin production or the inability of the body to respond to its own insulin. Diabetes is a global problem that is continuing to grow substantially. According to the Center for Disease Control and Prevention (CDC), 1 in 10 adults living in the U.S have diabetes and by 2025 it is predicted that 1 in 5 will have diabetes ("cdc.gov,"). In 2020, diabetes was the nation's 8th leading cause of death ("AmericasHealthRankings.org,").

There are two types of diabetes, type one (T1D) and type two (T2D). Both are diseases characterized by hyperglycemia caused by the dysregulation in blood glucose control. T1D is an autoimmune condition that results in the loss of insulin producing beta cells of the pancreas. It is well understood that the initial onset of T1D is characterized by a prediabetic asymptomatic period that results in the slow destruction of beta cells. During this period, there are various autoantibodies generated against several beta-cell antigens. One example includes autoantibodies against the beta-

cell specific enzyme glutamic acid decarboxylase (GAD_{65}) which can accurately predict T1D during development because anti-GAD antibodies are found in approximately 80% of newly diagnosed children (Belhiba et al., 2020; Towns & Pietropaolo, 2011). Autoantibodies are not only seen at the time of diagnosis but can persist even years after (Kikkas, Mallone, Larger, Volland, & Morel, 2014). Although T1D is primarily associated as a disease of the endocrine compartment of the pancreas, much of the effects on the exocrine pancreas have been understudied. Several studies have reported autoantibodies against exocrine cells of the pancreas which suggests that acinar and ductal cells may also be affected in T1D (Hardt et al., 2008; Panicot, Mas, Thivolet, & Lombardo, 1999; Taniguchi et al., 2003; Taniguchi, Tanaka, Seko, Okazaki, & Okamoto, 2001). The reason autoantibodies are generated against exocrine cells in T1D remains largely unknown. Beta cells are the only known cell type to be targeted and destroyed in T1D. By studying zebrafish CACs, I have found an undiscovered immune role of CACs/ductal cells that may increase in the autoimmune setting of T1D. These results are outlined in **Chapter 3** and are further described in the discussion section of **Chapter 3**. Although there are gaps in our knowledge regarding T1D in humans, my findings in zebrafish CACs could help us better understand how the exocrine pancreas is affected in a T1D setting.

The second form of diabetes is T2D, which is caused by either a defect in insulin secreted from beta cells or the inability of the body's tissue to respond appropriately to its own insulin that is secreted (e.g., insulin resistance) (Galicia-Garcia et al., 2020). Often patients diagnosed with T2D are obese, have a higher body fat index, and an increase in adipose tissue which promotes insulin resistance. Obesity has been viewed as the driving factor of T2D, however, the disease exists in a spectrum because genetic and environmental factors also play a significant role in disease development (Gaulton et al., 2008).

Although diabetes is mostly characterized as T1D or T2D, I want to emphasize that the disease is far more complex. Other forms of diabetes include maturity onset diabetes of the young (MODY) which is caused by the inheritance of monogenetic mutations (Fajans & Bell, 2011). MODY is not an autoimmune condition but like T1D, it results in abnormally high blood glucose levels. Cystic fibrosis-related diabetes (CFRD) is a type of diabetes that affects those with cystic fibrosis. CFRD can develop from excessive accumulation of mucous in the pancreas which can scar the pancreas and lead to endocrine islet dysfunction (i.e., a lack of insulin secretion and insulin resistance) (Ntimbane et al., 2009). Gestational diabetes is a temporary form of diabetes that only occurs during pregnancy when the body cannot make enough insulin (Quintanilla Rodriguez & Mahdy, 2023). Chronic pancreatitis, pancreatic cancer, and pancreatic surgeries can also lead to diabetes known as type 3c – a secondary complication resulting from a disease or injury to the pancreas (Hart et al., 2016). CFRD has also been considered a type 3c form of diabetes as it results from complications associated with cystic fibrosis.

Regardless of the etiology, diabetes often leads to a loss in beta-cell mass and results in elevated blood glucose levels (hyperglycemia). As mentioned, injuries to the pancreas can also lead to a loss in beta-cell mass. My thesis work is not only restricted to T1D but can be broadly applied to other forms of diabetes or pancreatic diseases that result in a loss in beta-cell mass. Treatments for diabetes are important because if left untreated, prolonged hyperglycemia can result in numerous secondary complications including limb amputation, heart disease, stroke, neuropathy, nephropathy, retinopathy, and eventually death (Deshpande, Harris-Hayes, & Schootman, 2008). It is also important to mention that many of these complications do not result from patient neglect. Simply having diabetes for a long period of time (many years) can result in secondary complications. Unfortunately, many of these secondary complications are not well

understood and are often difficult to prevent and treat. Major questions that remain are (**1**) how these secondary complications can be prevented and (**2**) how they can be treated once they manifest into a disease state. My thesis work presented in **Chapter 2** will help progress the understanding of secondary complications that can arise from chronic hyperglycemia. Within the zebrafish community, there lacks a well-suited zebrafish model to study chronic diabetes. In my thesis work, I have improved a transgenic zebrafish model to better study chronic diabetes. My zebrafish model will be accessible to the zebrafish community to use in order to better study secondary complications. The significance of this new model will help facilitate the understanding, prevention, and treatment of secondary complications associated with diabetes.

Treatments for diabetes

As of today, there is no cure for T1D. Given that diabetes is a global problem, we need better treatment methods to improve the quality of life of patients. The ultimate goal of the Parsons Lab is to understand how to induce an endogenous stem cell or progenitor of the pancreas to restore beta-cell mass and treat diabetes. Ideally, this could be achieved by simple drug treatment. Unfortunately, this treatment method has yet to be accomplished because one must understand the cellular and molecular components that must be targeted in humans to restore beta-cell mass. Here, I have outlined current T1D treatments and approaches that have been taken to try to restore beta-cell mass in T1D patients. I will then discuss how zebrafish can be used as an animal model to better understand the cellular and molecular mechanisms required for beta-cell regeneration.

Current T1D treatments primarily rely on the administration of exogenous insulin by either syringe injections or insulin pump therapy. T2D is often managed by medications (e.g.,

Metformin) or in extreme circumstances exogenous insulin therapy is required. Exogenous insulin injections require a single daily dose of long-acting insulin followed by multiple daily injections of short-acting insulin when a meal consisting of carbohydrates is consumed (Pathak, Pathak, O'Neill, Guduric-Fuchs, & Medina, 2019). Insulin pump therapy requires an insulin pump and continuous glucose monitor (CGM) to be worn daily to continuously monitor and regulate insulin requirements. These treatments can become exhausting for patients and especially difficult for parents who have very young and newly diagnosed children (especially if these children are too young to speak). Exogenous insulin administration is by no means a "cure" for T1D because there is no insulin-independence and there is no restoration of beta-cell mass. Instead, these treatments are an effective way for patients to better manage and control their diabetes to prevent secondary complications associated with poor diabetes management.

Finding a permanent cure for T1D has largely focused on restoring beta-cell mass by islet allotransplantations via the Edmonton Protocol (Shapiro et al., 2006), transplanting either human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) (Path, Perakakis, Mantzoros, & Seufert, 2019), or harnessing the pancreas's endogenous potential to regenerate beta cells (Zhong & Jiang, 2019). The most successful of these therapies to restore islet cell mass in humans is the Edmonton Protocol. This procedure requires transplanting beta cells from human cadavers into T1D patients (Marfil-Garza et al., 2022; Shapiro et al., 2006). Although this treatment is considered promising, there are several drawbacks to this approach that prevent this treatment from becoming a cure for T1D. First, most T1D patients selected to receive an islet transplant have severely uncontrolled diabetes, often called "brittle" diabetes (Gangemi et al., 2008). Thus, the treatment is not widely available to all T1D patients. Second, even if the treatment was made more readily available to all T1D individuals, not everyone would be able to afford the

procedure and insurance companies are not willing to pay the full cost. Third, cell transplants require a large cell load which means multiple donors (usually 2-3) are needed to treat a single patient with T1D (McCall & Shapiro, 2012). The requirement for numerous donors is problematic because human donors are scarce and as a result, this limits the number of transplants that can occur. Lastly, those receiving a transplant must be on immunosuppressants, namely sirolimus and tacrolimus, to enhance islet engraftment and survival. Taking immunosuppressants is not ideal because these treatments become toxic to the patient over time and can lead to other complications. It has been reported that tacrolimus can cause neuro and nephrotoxicity along with beta-cell damage (Chatenoud, 2008; Froud et al., 2006). The toxic side effects of sirolimus include hypertension, hepatotoxicity, nephrotoxicity, and neurotoxicity (Nizzi, Rees, Salzberg, & Ngwube, 2020). What diabetics really need is a long-lasting cure that is less invasive, less costly, and does not require immunosuppressants to be taken.

To overcome the problems associated with human donor-based transplants, focus has shifted to stem-cell based therapies that derive beta-cells *in vitro* using hESCs and iPSCs. Using these methods could eliminate the need for cadavers and pave the way for an unlimited supply of islets or beta cells required for transplantation. This means that more T1D patients could qualify for these treatments compared to the human donor-based approach. Although promising, one of the issues that remains in the field of deriving beta-cells from stem cells is having established protocols that can take a stem cell all the way to a functional beta cell (i.e., a cell that can produce insulin). hESCs can be differentiated successfully into pancreatic endoderm and endocrine precursor cells expressing NKX6.1, PTF1A, NGN3, and NKX2.2 when cultured for 12 days in specific media conditions (Kroon et al., 2008). Upon transplantation in SCID-beige, immunocompromised mice, these cells respond to glucose and produce insulin 30-60 days post-transplantation. hESCs can also

be differentiated into PDX1+ pancreatic progenitor cells *in vitro* and allowed to develop and mature further in mice into functional pancreatic endocrine cells (Rezania et al., 2012). One of the leading scientists in the derivation of functional human pancreatic beta cells *in vitro* is Doug Melton (Pagliuca et al., 2014). Melton's group developed a refined protocol that was able to extend the culture time of pancreatic progenitors *in vitro.* Their derived hESCs beta cells respond better to glucose compared to previous methods. They have also shown that these cells can ameliorate hyperglycemia in the NRG-Akita diabetic mouse model (Pagliuca et al., 2014). Biotech companies have also contributed to the treatment of T1D through stem cell-based approaches. ViaCyte is an example of a company focused on creating an implantable microencapsulation device that houses hESCs (Robert et al., 2018). This device allows for the flow of glucose in and insulin out while preventing the entrance of immune cells. Therefore, no immunosuppressants are required. As of 2022, Viacyte was acquired by the Biotech company called Vertex, which in early 2023 received FDA clearance to begin human trials using their new drug application for VX-264. Vertex utilizes allogeneic hESC derived islets encapsulated in a device to shield cells from the body's own immune system. We are now awaiting the results of this trial.

Although hESCs are a promising treatment for T1D, ethical issues surrounding the use of stem cells and the potential requirement for immunosuppressants is a challenge that remains for hESC based therapies. To overcome these issues, research has turned toward the generation of autologous pluripotent stem cells from a patient's somatic cells. These cells are called iPSCs and they were first reprogrammed from mouse fibroblasts in 2006 by the Yamanaka Lab at Kyoto University (Takahashi & Yamanaka, 2006). Four factors, Oct3/4, Sox2, c-Myce, and Klf4, were introduced to induce mouse fibroblasts to an ES like state. In one study, it was reported that hESC-like cells can also be derived from human skin cells when forced to express OCT4, SOX2, c-MYCE, and

KLF4. Using these hESC-like cells, insulin-producing islet-like clusters can be generated, which release insulin when stimulated with glucose (Tateishi et al., 2008). However, the main drawback of using iPSCs is their tumorigenic potential to spontaneously develop teratomas and teratocarcinomas (Volarevic et al., 2018). The only way to ensure that teratomas will not develop is to have protocols in place that can differentiate cells completely before their transplantation. Having protocols that can fully differentiate beta cells *in vitro* has posed a major challenge in the treatment of beta-cell loss using stem cell-based approaches.

Given that these treatments are headed in the right direction to treat beta-cell loss, they have a long way to go before they can replace exogenous insulin treatments for T1D patients. My thesis work is aimed at overcoming the challenges and risks associated with surgical transplants and *in vitro* derived stem cell-based therapies. Instead, I am focused on taking advantage of the body's endogenous potential to regenerate beta cells. One posing challenge in the pancreas field is how to induce endogenous cells of the human pancreas to restore beta-cell mass. It was originally thought that the pancreas was a non-regenerative organ and so it was thought that restoration of beta-cell mass could not be achieved by the pancreas itself. However, overtime we have learned that the pancreas possesses the ability to regenerate. Histological analysis of T1D cadaver autopsies revealed the presence of a small number of beta cells in the pancreas (Lohr & Kloppel, 1987; Maclean & Ogilvie, 1959; Meier, Bhushan, Butler, Rizza, & Butler, 2005). Consistent with these observations, other studies have shown there are low levels of circulating C-peptide in T1D patients (Eff, Faber, & Deckert, 1978). C-peptide is a short amino acid sequence that connects to insulin and is co-released with insulin into the body. Since C-peptide stays in the blood longer than insulin, it is useful in the indirect detection of insulin secretion. These observations suggest that there is ongoing beta-cell proliferation or regeneration in T1D patients. In T1D patients, there

should be no beta cells present because these cells are fully destroyed by the immune system in T1D. These results suggest that the pancreas has the capacity to regenerate but the ability to restore beta cells is hindered due to the autoimmune aspect outweighing the ability to restore beta cells.

The ability of the pancreas to regenerate is an example of cellular plasticity. Cellular plasticity is the ability of endogenous cells to change their cellular profile in order to respond to environmental cues (i.e., a loss in beta cell mass). Cell plasticity is not limited to the endocrine pancreas but can also be seen in the exocrine pancreas. One example of this is seen in patients who have been diagnosed with pancreatitis. As mentioned earlier, during pancreas inflammation or injury, ADM is a common process observed in these patients as the pancreas tries to facilitate the regeneration of exocrine cells damaged by inflammation (Houbracken et al., 2011). Cell plasticity also plays a role in the formation and progression of pancreatic cancer. For instance, the process of ADM is also seen throughout the progression of PDAC (Storz, 2017). Additionally, primary pancreatic tumors can be found to metastasize into secondary sites like the liver and have similar transcriptome profiles (Krieger et al., 2021) yet these cells can reside in the liver. This again, is another form of cell plasticity because tumors (i.e., pancreatic tumors) can integrate into other surrounding organs. Cellular plasticity is not a unique phenomenon of the pancreas. The brain is an example of another organ that was also believed to be non-regenerative because it was once thought that brain cells or neurons are finite and do not regenerate. However, the plasticity of the brain is more pronounced than once thought because findings have observed the generation of new neurons in the adult brain via neurogenesis (Ribeiro & Xapelli, 2021; Shetty & Hattiangady, 2016).

The discovery of cell plasticity will allow us to take advantage of harnessing a cell's ability to treat numerous diseases. An example of one treatment that relies on cell plasticity was discussed above regarding the use of iPSC-based therapies to treat diseases like T1D. From iPSCs, we have

learned that cells once thought to be terminally differentiated can be reprogrammed into a completely different cell type. A second treatment that relies on cell plasticity is the ability to harness the body's endogenous cells to give rise to various cell types to treat numerous diseases. Studies in animal models suggest that new beta cells can arise from three different endogenous cell sources within the pancreas including the proliferation of beta cells from pre-existing beta cells (Meier et al., 2008), the transdifferentiation of surrounding pancreatic cells into beta cells (Puri, Folias, & Hebrok, 2015; L. Ye, Robertson, Hesselson, Stainier, & Anderson, 2015; Ziv, Glaser, & Dor, 2013), or neogenesis, which is the process by which new cells arise from either an endogenous stem cell or progenitor (Delaspre et al., 2015; Y. Wang, Rovira, Yusuff, & Parsons, 2011; Xu et al., 2008).

Rather than relying on transplant methods or stem-cell based therapies to treat T1D, the best approach would be the *in vivo* expansion of beta-cell mass pharmacologically. This treatment approach would greatly reduce the side effects, invasiveness, and costs that are associated with islet transplantation and stem cell-based therapies. However, the challenge with this approach is understanding which pancreatic cells in humans should be targeted to restore beta-cell mass. In my thesis, pancreatic CACs could serve as a potential target to induce the regeneration of beta cells. If we want to learn how to target these cells to restore beta-cell mass, we need to understand the cellular and molecular mechanisms that facilitate CACs to do this. Much research relies on rodent models to try to understand CACs as a potential beta-cell progenitor. However, the use of rodent models poses a challenge because (1) the ability to study CACs in rodent models is difficult and (2) they may have a limited regeneration capacity. Since the goal is to study beta-cell regeneration, the best approach is to find an animal model that can regenerate beta cells well and try to understand how. In my thesis I use zebrafish as an animal model to address these challenges

regarding beta-cell regeneration because zebrafish CACs behave as a beta-cell progenitor in the adult fish. Understanding the cellular and molecular mechanisms in a highly regenerative animal model will enable us to understand how to induce mammalian CACs to regenerate in humans.

Developmental Biology of the Pancreas

One of the most common animal models used to study pancreas biology and pancreas regeneration are mouse models. In the mouse, there is a population of ductal cells that are Notch-responsive, express Nkx6.1, and behave as the major embryonic source of pancreatic progenitors during development (Kopinke et al., 2011; Kopinke & Murtaugh, 2010; Sander et al., 2000; Schaffer, Freude, Nelson, & Sander, 2010). These ductal cells eventually give rise to Ngn3+ endocrine precursors that then give rise to pancreatic islets (Kim & MacDonald, 2002). At E12.5 the secondary transition begins and at this stage the pancreas starts to form branched ductal epithelium that is divided into the tip and trunk domains (Afelik et al., 2012; Villasenor, Chong, Henkemeyer, & Cleaver, 2010; Q. Zhou et al., 2007). The tip domain of the duct contains Ptf1a expression that gives rise to acinar cells (Hald et al., 2008). The trunk domain contains both ductal and endocrine progenitors that eventually give rise to mature ductal cells (including CACs) and endocrine cells (Schaffer et al., 2013; Taylor, Liu, & Sander, 2013). Within the trunk domain, Notch signaling retains progenitors and prevents them from differentiating into endocrine cells. When Notch expression is high, it induces the expression of the transcription factor Hes1, which then promotes the expression of Nkx6.1 and Sox9. This consequentially inhibits the expression of Ngn3—the endocrine determinant factor (Afelik et al., 2012; Apelqvist et al., 1999; Hald et al., 2003; Kopinke et al., 2012; Shih et al., 2012). Over time, the expression of Hes1, Sox9, and Nkx6.1 become restricted to cells that are immediately adjacent to the tip domain of the developing pancreatic epithelium.

As the mouse develops into an adult, the progenitor cells of the developing mouse pancreas eventually lose their progenitor capacity. In the adult mouse, new beta cells primarily arise from mature beta cells that proliferate and in some cases other endocrine cells that transdifferentiate (Arnes, Hill, Gross, Magnuson, & Sussel, 2012; Chera et al., 2014; Courtney et al., 2013; Nir, Melton, & Dor, 2007; Thorel et al., 2010). After a partial ductal ligation in the adult mouse, it has been observed that Ngn3-expressing endocrine cell precursors appear in the regenerating mouse pancreas (Xu et al., 2008). Lineage tracing with several ductal markers showed that Ngn3-expressing cells give rise to new beta-cells in some studies but do not in other studies (Al-Hasani et al., 2013; Kopp et al., 2011; Van de Casteele et al., 2014; Xiao, Chen, et al., 2013; Xiao, Guo, et al., 2013). In mammals, there is debate as to whether ductal cells or CACs play a role in the regeneration of the adult pancreas. Several lineage tracing methods have used fluorescent "timers" (Miyatsuka et al., 2014) or numerous ductal and CAC promoters such as Hnf1b (Solar et al., 2009), Hes1 (Kopinke et al., 2011), Sox9 (Kopp et al., 2011), mucin (Kopinke & Murtaugh, 2010), and CAII (Inada et al., 2008) to observe whether adult ductal cells or CACs behave as endocrine progenitors. Most of these studies conclude that both endocrine and exocrine cells arise from ductal cells during development and do not come from adult ductal cells. Although many studies claim that ductal cells and CACs are not behaving as a beta-cell progenitor in adults, there is a caveat with these conclusions in mouse. Many of the lineage tracing experiments in the mouse only label a small portion of ductal cells/CACs (as low as 20% of the cell population) (Solar et al., 2009). It is possible that a subset of ductal cells and CACs contribute to regeneration but the inability to label these cells limits our ability to study them in this setting. Given these issues in mouse models, they may not function as the best animal model to study pancreas regeneration. Additionally, we

should also not rely on one animal model to draw upon conclusions regarding beta-cell regeneration.

Instead, my thesis uses zebrafish as an animal model to study both pancreatic cancer and beta-cell regeneration. The zebrafish pancreas is morphologically and functionally similar to the mammalian pancreas. Like the mammalian pancreas, the endocrine pancreas is made up of the islets of Langerhans comprised of beta, alpha, delta, and epsilon cells. As with the mammalian pancreas, the exocrine pancreas of the zebrafish contains acinar cells that synthesize and secrete digestive enzymes (i.e., zymogen) and ductal cells that transport these digestive enzymes into the lumen of the gut. Ductal cells in zebrafish are also made up of CACs, however, unlike the mammalian pancreas, these cells have been recognized for their progenitor capacity both during development and in the adult fish (Delaspre et al., 2015; Ghaye et al., 2015; Y. Wang et al., 2011). As mentioned earlier, CACs have also been suggested as a cellular origin for pancreatic cancer (Kopp et al., 2012; Miyamoto et al., 2003; Stanger et al., 2005). In my thesis I show that zebrafish CACs are an excellent model to study pancreatic cancer because they express similar pathways involved in the initiation and progression of pancreatic cancer. This will be described in greater detail in **Chapter 1** of my thesis.

At first glance, the mouse and zebrafish pancreas look vastly different during development (Shih, Wang, & Sander, 2013). These differences come from the difficulty of matching the developmental stages of the zebrafish pancreas with the developing mouse pancreas. This is because the speed at which the zebrafish pancreas develops is much quicker than the developing mouse pancreas. Regardless of these developmental differences, the cellular and molecular mechanisms that regulate endocrine cell differentiation during development are conserved in both the mouse and zebrafish.

At 5 days post fertilization (dpf) the zebrafish pancreas is a spoon shaped organ. The principal islet is found at the head of the pancreas and the intrapancreatic duct is made up of CACs that extend into the tail of the pancreas. During zebrafish development, the majority of endocrine cells come from CACs. In zebrafish, CACs behave as a beta-cell progenitor during development and can be seen differentiating along the pancreatic tail to give rise to new beta cells in a process (similar to the mouse) called the secondary transition (Parsons et al., 2009; Y. Wang et al., 2011). During this process, a wave of secondary islets can be seen appearing along the duct of the pancreas (Parsons et al., 2009). Lineage tracing of CACs during zebrafish development showed that larval CACs give rise to both ductal cells and endocrine cells (Y. Wang et al., 2011). The progenitor behavior of larval zebrafish CACs is equivalent to Ngn3+ cells found in the pancreatic trunk of the developing mouse pancreas. Like Ngn3+ cells, Notch activity also coordinates the differentiation capacity of larval zebrafish CACs into giving rise to endocrine cells of the pancreas. Decreasing Notch activity in CACs causes them to differentiate into NeuroD-expressing endocrine precursor cells (Parsons et al., 2009; Prince, Anderson, & Dalgin, 2017). CACs in the larval zebrafish express numerous transcription factors that regulate endocrine differentiation including Sox9b, Nkx6.1, and Nkx2.2a (Delaspre et al., 2015; Ghaye et al., 2015; Manfroid et al., 2012).

One major difference between mice and zebrafish is that zebrafish CACs do not lose their progenitor capacity as Ngn3+ progenitor cells do in the mouse pancreas. Instead, CACs retain their developmental progenitor properties in the adult fish. These properties that allow CACs to behave as a progenitor throughout the life of the fish remain unknown and is one of the primary interests of our lab and my thesis work. As mentioned earlier, one question that remains in the field is how to restore beta-cell mass in humans to treat T1D. In my thesis I address this question using a highly

regenerative animal model like zebrafish to deconstruct the cellular and molecular mechanisms used by zebrafish CACs.

In the adult fish, CACs are considered a facultative progenitor, which can be thought of as a cell that has a defined “day job” and an alternative “secondary job”. After development, a CAC fully differentiates into a functioning ductal cell. Thus, the day job of a CAC is to behave as a ductal cell by aiding in the passage of enzymes secreted by acinar cells. In both zebrafish and mammals, CACs express several aquaporins and calcium chloride ion channels including Cftr and Clcn1 which maintain the ion concentration of the ductal lumen and allows fluid to flow (Burghardt et al., 2003; Marino, Matovcik, Gorelick, & Cohn, 1991). The second behavior of adult zebrafish CACs is to act as a beta-cell progenitor by regenerating beta-cell mass when beta cells are either damaged or destroyed.

The Parsons Lab was the first lab to show that CACs behave as a beta-cell progenitor in adult fish. This was done by developing a nitroreductase (NTR)/metronidazole (MTZ) method in the zebrafish to specifically ablate >99% of beta cells (I will describe this method in greater detail in **Chapter 2**). As beta cells regenerate, single beta cells and beta-cell clusters can be observed along the pancreatic duct and in close proximity with CACs. The Notch-responsive *tp1* promoter or the *nkx6.1* promoter was used to drive GFP expression in CACs during regeneration. It was observed that GFP+ CACs begin to express insulin during regeneration this suggested that CACs contribute to beta-cell regeneration (Delaspre et al., 2015; Ghaye et al., 2015). Delaspre et al. further confirmed that CACs directly contribute to beta-cell regeneration by using an inducible genetic lineage tracing zebrafish line that utilized the Notch-responsive *tp1* promoter to label CACs before beta cells were ablated. During regeneration, lineage traced CACs were seen to directly contribute to the process of regeneration by co-expressing insulin. One of the contributions of my thesis is to

facilitate our understanding of zebrafish CACs during beta-cell regeneration. Although our NTR/MTZ transgenic zebrafish model can be used to study beta-cell regeneration, there are several drawbacks that limit our ability to study CACs in the context of regeneration. In my thesis, I have improved our NTR/MTZ beta-cell regeneration model to overcome these limitations (Tucker et al., 2023). As mentioned earlier, we can also use my new and improved model to study complications that arise from chronic diabetes. This improved NTR/MTZ transgenic model will be described in more detail in **Chapter 2**.

In the adult mammalian pancreas CACs are abundant but there is little evidence of them behaving as a beta-cell progenitor (Al-Hasani et al., 2013; K. Hayashi et al., 1999; K. Y. Hayashi et al., 2003; Sancho, Gruber, Gu, & Behrens, 2014). Instead, CACs in mammalian model systems seem to primarily behave as ductal cells. These observations suggest that there is a property in zebrafish CACs that facilitates their ability to regenerate beta-cells which appear to be lacking in mammalian CACs. These properties are largely unknown; however, it is thought that zebrafish CACs may retain the expression of genes utilized during development.

To initially understand the progenitor behavior of adult zebrafish CACs, our lab performed bulk RNA-sequencing to uncover the transcriptome of these cells. The CAC transcriptome was found to be enriched for progenitor markers including: *HES1* homologs (*her6, her9, her15.1, her15.2*), *nkx2.2a*, *nkx6.1*, *cftr*, *clcn1b*, and *sox9b* (Delaspre et al., 2015). Interestingly, adult zebrafish CACs retain the developmental expression of *nkx2.2a* and *nkx6.1*. The expression of these genes is normally restricted to only the beta cells in the adult mammalian pancreas (Binot et al., 2010; Papizan et al., 2011; Sander et al., 2000; Schaffer et al., 2013). This finding was an indication that the continual expression of both *nkx2.2a* and *nkx6.1* in zebrafish CACs could be one possible explanation as to why adult zebrafish CACs readily behave as a beta-cell progenitor.

However, much research is needed to determine whether this is true. In zebrafish, there are two orthologues of SOX9 expressed called *sox9a* and *sox9b*, but only *sox9b* is expressed in the pancreas (Chiang et al., 2001). Previous work done by our lab discovered two phenotypes that are different between *sox9b* heterozygotes and wildtype homozygotes. One phenotype found was that the expression of *sox9b* in larval CACs is regulated by retinoic acid signaling. The second phenotype found was that *sox9b* heterozygous adult zebrafish have an accelerated beta-cell regeneration phenotype (W. Huang et al., 2016).

In my thesis work, I set out to characterize adult zebrafish CACs in greater detail to better understand them as both a beta-cell progenitor and as the potential origin of pancreatic cancer. Although bulk RNA-seq was previously performed on adult zebrafish CACs, this approach conceals CAC heterogeneity that may exist in the zebrafish pancreas. Cellular heterogeneity is important because it could reveal subpopulations of CACs that are more primed at regenerating beta cells. Identifying a subpopulation of CACs that is more primed at regenerating beta-cells will allow for further understanding of this population so that we can understand how to target it to induce beta-cell regeneration. In my thesis work, I uncovered CAC heterogeneity and variation across the CAC population by performing single-cell RNA sequencing (scRNA-seq) and taking a bioinformatics approach to analyze the CAC transcriptome. From my work, I have found several subpopulations of CACs that may explain their behavior as both the cellular origin of pancreatic cancer and as a beta-cell progenitor. These findings will be discussed in greater detail in **Chapter 3**.

The overall goal of the Parsons lab is to understand the cellular and molecular mechanisms that promote CACs to regenerate beta cells. Ideally, understanding these mechanisms would provide insights into inducing beta-cell regeneration in humans. My thesis work in **Chapter 3** sets

the foundation for understanding how adult zebrafish CACs behave before beta-cell regeneration is induced. It is important to understand zebrafish CACs under non-regenerative conditions first so that when regeneration is induced, we can understand which subpopulations readily contribute to regeneration and what cellular and molecular mechanisms must change in order for CACs to become beta cells. Additionally, the work I have generated in **Chapter 3** greatly expands upon our knowledge of adult zebrafish CACs. The genomics work I have accomplished using zebrafish CACs can be utilized by others in the field to understand what properties in non-regenerating zebrafish CACs are active compared to mammalian CACs. Understanding these transcriptome differences between zebrafish and mammalian CACs could explain why adult zebrafish CACs behave more readily as a beta-cell progenitor. Comparing and contrasting zebrafish and mammalian CACs is beyond the scope of my thesis, however, the data that I have generated in **Chapter 3** can be used by others to tease out these cellular and molecular differences between zebrafish and mammalian models. Provided our goal is to study CACs during regeneration, I created an improved NTR zebrafish transgenic line (**Chapter 2**) that can be used to facilitate our understanding of CACs during beta-cell regeneration using scRNA-seq. In the future, it is with high hopes that the Parsons Lab and others in the field will use the work I have generated in **Chapter 2** and **Chapter 3** to uncover the cellular and molecular mechanisms utilized by zebrafish CACs to regenerate beta cells. The most exciting aspect of my work is that it raises numerous hypotheses that will need to be further addressed beyond my thesis, and as such, it will lead to future research projects. The work that I am presenting here will progress the field of both pancreas biology and our understanding of beta-cell regeneration.

Summary

In summary, **Chapter 1** describes how I have utilized zebrafish CACs as a model to uncover a potential mechanism involved in the initiation and progression of pancreatic cancer via the SOX9 pathway. **Chapter 2** focuses on the creation of an improved NTR/MTZ model that can now be used to better study beta-cell regeneration and for the first time, to study secondary complications associated with chronic hyperglycemia. **Chapter 3** describes scRNA-seq studies to reveal CAC heterogeneity and its potential implications. The efforts presented here in my thesis have led to an improved transgenic line that will contribute to the field of zebrafish pancreas regeneration and the ability to study the secondary complications associated with chronic hyperglycemia. Additionally, my genomics work has revealed novel findings that could explain why CACs are the potential cellular origin of source for pancreatic cancer and could further our understandings of beta-cell regeneration prior to the induction of regeneration.

Chapter 1: SOX9 modulates cancer biomarker and cilia genes in pancreatic cancer

The work presented here has been published in Human Molecular Genetics.

a) Introduction

Pancreatic cancer can form in both the endocrine and exocrine compartment of the pancreas. According to the American Cancer Society, the vast majority of pancreatic cancers (95%) affect the exocrine compartment while the other 5% of pancreatic cancers arise in the endocrine compartment.

Neuroendocrine pancreatic cancer affects the endocrine pancreatic compartment and as mentioned earlier, it is often associated with NETs. NETs are categorized as functioning and non-functioning tumors. Functioning NETS include insulinoma, gastrinoma, VIPoma, and glucagonoma that produce specific hormones with hypersecretion activity (Ro, Chai, Yu, & Yu, 2013). Insulinoma is the most common neuroendocrine tumor of the pancreas and patients diagnosed with insulinoma oversecrete insulin. An overproduction of insulin causes patients to experience hypoglycemia and neuroglycopenic symptoms (Hirshberg et al., 2000). Non-functioning NETS are the largest subtype of NETs but they do not produce excess hormones. Instead, these tumors have a greater chance at invading and metastasizing within normal pancreatic tissue and surrounding tissue. The exact cause of NETS is unknown although there is growing research evidence in the genetic causes of familial and sporadic NETS.

The remaining 95% of pancreatic cancers arise from the exocrine pancreatic compartment. Exocrine pancreatic cancer is a bit more complex than endocrine pancreatic cancer. There are four types of exocrine pancreatic cancers including: **1)** squamous cell carcinoma, **2)** adenosquamous carcinoma, **3)** colloid carcinoma, and **4)** adenocarcinoma. Squamous cell carcinoma is considered the rarest type of pancreatic cancer that originates from the pancreatic ducts (Al-Shehri, Silverman,

& King, 2008). This type of pancreatic cancer is atypical because it is purely made up of squamous cells which are not typically seen in the pancreas. Adenosquamous carcinoma is another rare type of pancreatic cancer with a poor prognosis. It makes up approximately 0.38% to 10% of all exocrine pancreatic cancers (Borazanci et al., 2015). These tumors have characteristics of both ductal adenocarcinoma and squamous cell carcinoma. Colloid carcinoma is a third rare type of pancreatic cancer that accounts for approximately 1 to 3% of all exocrine pancreatic cancer. These tumors develop from benign cysts called an intraductal papillary mucinous neoplasm (IPMN) (Gao, Zhu, & Yuan, 2015). This type of pancreatic cancer has a much better prognosis rate because the tumors float in a gelatinous substance called mucin which makes it much less likely for the tumors to metastasize compared to other pancreatic cancers (Plerhoples et al., 2011).

The most common and aggressive type of exocrine pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). PDAC accounts for more than 90% of all pancreatic cancers and has an overall 5-year survival rate of less than 8% (Siegel, Miller, & Jemal, 2018). According to the American Cancer Society, PDAC is the 9th most commonly diagnosed cancer in women and 10th in men. Overall, PDAC has the 4th highest mortality rate. The low survival rate seen in PDAC patients is due to its late detection (Niederhuber, Brennan, & Menck, 1995), a predicted early spread of cancer cells during tumor progression (Rhim et al., 2012), and its strong resistance to chemotherapy treatment (Adamska et al., 2018).

PDAC was given its name based on the morphology of its cancer cells resembling the ductal cells of the pancreas, however, the exact cellular origin of PDAC is still largely unknown. It is thought that PDAC may arise primarily from acinar cells since they are the most abundant cell type making up the pancreas. However, CACs and ductal cells of the pancreas have also been considered as two other possible cellular origins of PDAC although these cells are vastly

outnumbered by acinar cells. During the early stages of PDAC formation, acinar cells lose their cellular characteristics and take on a more ductal cell profile including ductal cell morphology and the expression of ductal marker genes (Kopp et al., 2012). This transition of acinar cells to a more ductal cell like state is called acinar-to-ductal metaplasia (ADM) and is thought to be a unique property of pancreatic cancer. These observations suggest that acinar cells could be the origin of PDAC but may need to first transition through a ductal-like state during the early stages of PDAC in order to give rise to pancreatic cancer. Regardless of the cellular origin of PDAC, it is thought to arise from preneoplasmic lesions that form in the exocrine pancreas. These lesions are called pancreatic intraepithelial neoplasias (PanINs). Following oncogenic activation, PanINs are thought to accumulate numerous mutations over time that allow them to initiate and eventually progress into full on PDAC (Hruban, Goggins, Parsons, & Kern, 2000). Many of these mutations occur in numerous signaling pathways including the hedgehog signaling pathway, KRAS, FGF, NOTCH, TGF-b, retinoic acid, EGF, and WNT (Rhim & Stanger, 2010). The most common mutation for initiating PDAC is found in KRAS. This is because more than 90% of all PDAC cases have a mutation in KRAS (Giri et al., 2017) and these mutations are found throughout the progression of PanINs (Kanda et al., 2012).

The majority of PDAC is caused by sporadic somatic mutations. Pancreatitis is one of the most well known risk factor for accumulating these sporadic mutations (Lowenfels et al., 1993). About 5-10% of those diagnosed with PDAC have a hereditary component (Permuth-Wey & Egan, 2009) and a significant amount of these cases are caused by a genetic susceptibility to developing pancreatitis. 92% of sequenced PDAC samples had tumors with mutations that activated KRAS (Almoguera et al., 1988; P. Bailey et al., 2016; Jones et al., 2008). Other molecular pathways that were found to be affected in patient PDAC samples include mutations in the G1/S checkpoint

machinery (78% of cases), TGF-b signaling (47% of cases), and in histone modifications (24% of cases) (P. Bailey et al., 2016).

As stated previously, mutations in KRAS are the number one cause of PDAC seen in patients. These mutations cause KRAS to be constitutively active, which consequentially leads to an increase in cell proliferation and differentiation (di Magliano & Logsdon, 2013). These mutations also lead to an increase in the activation of the KRAS downstream target gene SOX9 (H. Zhou et al., 2018). *SOX9* is a gene that encodes a transcription factor, which is part of the SRY-related box (SOX) gene family. All Sox genes contain a high mobility group (HMG) DNA-binding domain (Stevanovic, Lovell-Badge, Collignon, & Goodfellow, 1993), homodimerization domain, and a C-terminal activation domain (Lefebvre & Dvir-Ginzberg, 2017). Sox9 is considered an activator of transcription, but it can also function as a repressor (Garside et al., 2015; Leung et al., 2011). The repressive behavior of SOX9 involves the recruitment of cofactors that enable SOX9 to behave as a repressor (Kamachi & Kondoh, 2013). In humans, SOX9 is expressed in almost every single organ and haploinsufficiency of SOX9 causes campomelic dysplasia – a disease that affects multiple structures and leads to heart complications, skeletal abnormalities, sex reversal (Foster et al., 1994; Wagner et al., 1994), and of interest to our lab, abnormal pancreatic islets (Piper et al., 2002).

During pancreas development, Sox9 has been shown to have an important role in maintaining pancreatic progenitors and regulating endocrine differentiation (di Magliano & Logsdon, 2013; H. Zhou et al., 2018). In mouse models, it has been observed that the dysregulation of Sox9 accelerates the formation and progression of PDAC (Kopp et al., 2012). Another pathway important in PDAC is the Notch-signaling pathway (Miyamoto et al., 2003). This pathway also activates the expression of SOX9 (Shih et al., 2012). It has been thought that SOX9 plays multiple

and critical roles in both the initiation and progression of PDAC. A study conducted by Kopp et al. showed that the overexpression of Sox9 in transgenic mouse models causes acinar cells to decrease the expression of their acinar gene markers and upregulate ductal cell markers (Kopp et al., 2012). The ability of a cell to transform from an acinar cell into a ductal cell is consistent with characteristics associated with ADM. This same group also showed that PDAC formation increases even more when both KRAS and SOX9 are manipulated in the pancreas. It was also shown that Sox9 depletion blocked the formation of PanINs and PDAC even when KRAS was overexpressed within the mouse pancreas (Kopp et al., 2012).

Although CACs and ductal cells express high levels of Sox9, there is little evidence of them being the origin of PDAC. For instance, mouse models that only overexpress oncogenic KRAS suggested that ductal cells may be more resistant to becoming PDAC (Kopp et al., 2012). On the other hand, the overexpression of oncogenic KRAS activity with a loss of function of tumor suppressor genes suggested that ductal cells (including CACs) may be more readily 'primed' at giving rise to PDAC (J. M. Bailey et al., 2016; A. Y. L. Lee et al., 2019). Regardless of the debate over the cellular origin of PDAC, CACs seem to express genes and utilize the same molecular pathways that are required for the initiation and progression of PDAC. Therefore, CACs could function as an excellent model to study pancreatic cancer.

To further elucidate the role of SOX9 in the pancreas and in PDAC biology, the Parsons Lab took advantage of using two different model systems: (**1**) a human cell line called PANC-1 and (**2**) the developing zebrafish pancreas. PANC-1 cells are derived from a resected PDAC patient tumor and because PANC-1s express high levels of SOX9 they can function as a useful source for conducting both RNA and chromatin facilitated genomic studies. Here, both RNA-seq and ChIP-seq studies were performed to identify direct transcriptional targets of SOX9. One of the SOX9

targets identified from our work was an epithelial cell adhesion molecule (EPCAM). *EPCAM* encodes a transmembrane protein that is expressed in both stem cells and progenitor cells and in various epithelial tissues. Epcam is an interesting target to study not only in terms of pancreatic cancer but also pancreatic progenitor function. This is because EpCAM has been found to be expressed in stem/progenitor cells of the liver (Dolle et al., 2015). Provided this finding, EpCAM may also have a role in the progenitor behavior of the pancreas.

Here, in the introduction of **Chapter 1** will report on findings by previous members of the Parsons Lab (Hannah E Edelman and Rebecca L Beer) and the McCallion Lab (Sarah A McClymont). These results are included in the introduction as the premise for the experiments I performed and describe in the results section of **Chapter 1**. My contribution to this study and paper was to use the developing zebrafish pancreas to determine whether zebrafish CACs could function as a potential model to study pancreatic cancer via the sox9 pathway. I was responsible for optimizing a detailed *in-situ* hybridization protocol that can be used on the developing zebrafish pancreas to detect gene expression of SOX9 target genes in CACs. I then used this protocol to determine whether these target genes are regulated in a *sox9* dependent manner. The results that I generated are reported in the results section. As mentioned, the work presented here in **Chapter 1** is published in Human Molecular Genetics.

Previous members of the Parsons Lab performed RNA-seq on PANC-1s. Knocking down *SOX9* in PANC-1s was done using siRNA against SOX9. RNA-seq was then carried out to identify transcripts that were dependent on the expression of SOX9. To confirm the successful knockdown of SOX9, two methos were utilized. One method used western blotting performed on PANC-1 lysates to verify the knockdown of SOX9 (**Figure 1.1A**). A second method used immunofluorescence on fixed PANC-1s to detect the expression of SOX9 (**Figure 1.1B**). After

confirming the knockdown of SOX9, total RNA was then extracted and sequenced from PANC-1s transfected with either control or *SOX9* siRNA. Comparing gene expression results from control cells to *SOX9* siRNA knockdown cells, 93 differentially expressed (DE) genes were identified upon the knockdown of SOX9. Of these 93 genes, 60 of them were found to be upregulated and 33 of them were found to be downregulated **(Figure 1.1C, Table 1.1**). To validate whether these changes in gene expression were true, the top 5 upregulated DE genes and the top 5 downregulated DE genes were confirmed by performing qRT PCR (**Figure 1.1D**).

Gene ontology (GO) term analysis was performed to assess the biological role of both the downregulated and upregulated DE genes identified. Downregulated genes had terms that were associated with cancer motility (*ESRP1* (Ishii et al., 2014)), cell to cell adhesion (*TINAGL1*, (D. Li et al., 2007; Tajiri et al., 2010)), obesity and insulin resistance (*RGCC*, (Cui, Luan, Ye, & Chen, 2015)), and cancer stem cell maintenance (*EPCAM*, (Schnell, Cirulli, & Giepmans, 2013)). GO term analysis also suggested that these genes are involved in the Notch-signaling pathway and have a role in negatively regulating cell proliferation (**Figure 1.1E**). Upregulated genes had terms associated with cilia development, assembly, and movement (**Figure 1.1F**). The most highly upregulated gene was *LRRC6* and it was found to be involved in the formation of the axoneme, which is part of the cilia core (Horani et al., 2013; Kott et al., 2012).

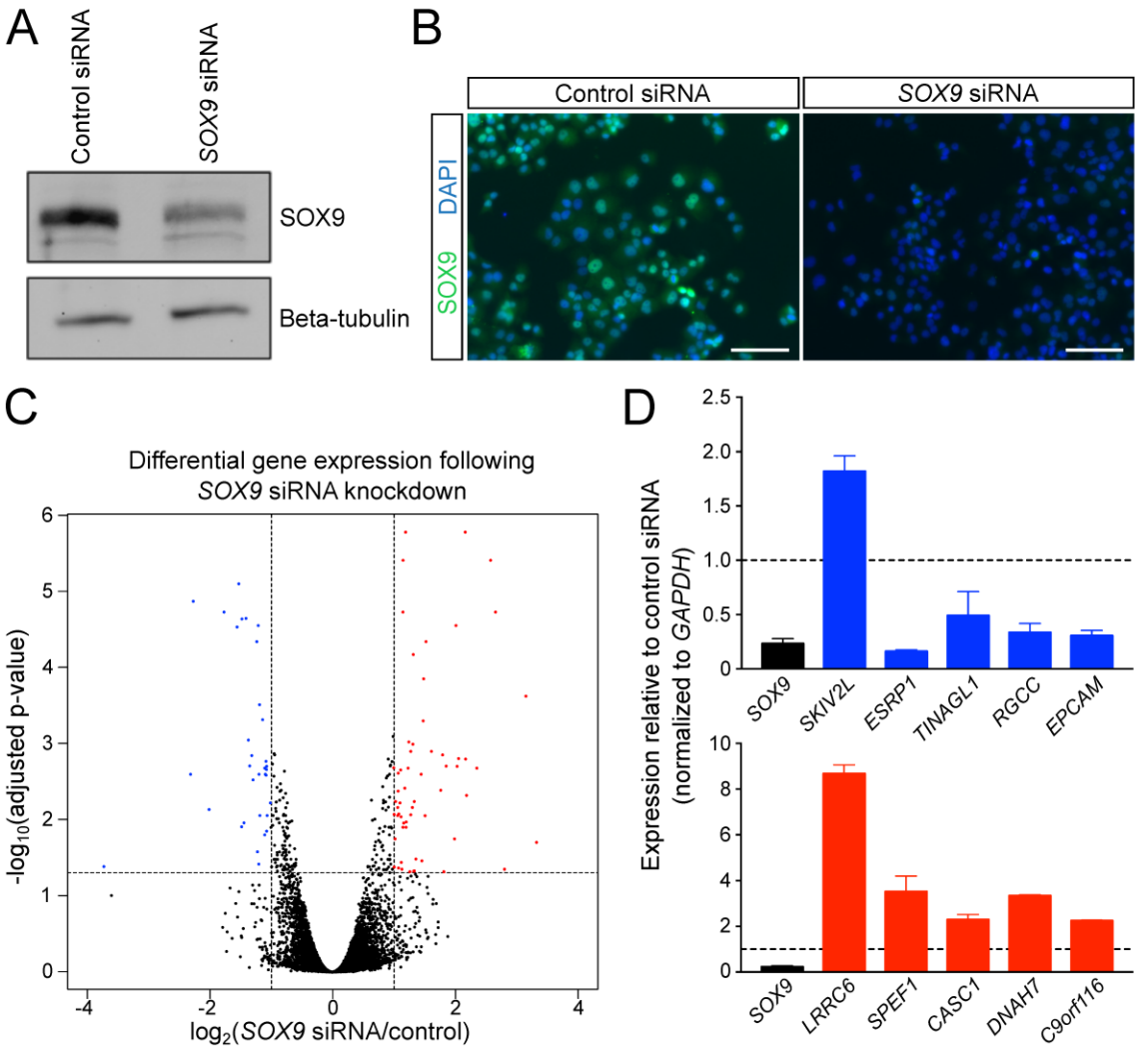

A
Control siRNA
SOX9 siRNA
SOX9
Beta-tubulin
B
Control siRNA
SOX9 siRNA
SOX9
DAPI
C
Differential gene expression following
SOX9 siRNA knockdown
-log10(adjusted p-value)
log2(SOX9 siRNA/control)
D
Expression relative to control siRNA
(normalized to GAPDH)
SOX9
SKIV2L
ESRP1
TINAGL1
RGCC
EPCAM
SOX9
LRRC6
SPEF1
CASC1
DNAH7
C9orf116
E
Downregulated
GO Biological Process - Combined Score
Pos. reg. of transcription of Notch receptor target
Neg. reg. of cell proliferation involved in kidney development
Neg. reg. of cell proliferation in bone marrow
Neg. reg. of fibroblast proliferation
Neg. reg. of cell proliferation involved in compound eye morphogenesis
Neg. reg. of hemocyte proliferation
Neg. reg. of cell proliferation involved in heart morphogenesis
Neg. reg. of fat cell proliferation
Neg. reg. of myoblast proliferation
Neg. reg. of germ cell proliferation
F
Upregulated
GO Biological Process - Combined Score
Motile cilium assembly
Inner dynein arm assembly
Outer dynein arm assembly
Non-motile cilium assembly
Axoneme assembly
Ciliary basal body-plasma membrane docking
Epithelial cilium movement
Cilium movement involved in cell motility
Ciliary transition zone assembly
Cytosolic ciliogenesis

Figure 1.1. Knockdown of SOX9 results in an increase of ciliary gene expression and a decrease in expression of genes negatively regulating proliferation.

(**A**) Western blot and (**B**) immunofluorescence confirms SOX9 knockdown in PANC-1s following transfection with siRNA against SOX9. (**C**) A volcano plot of adjusted *P*-value versus fold change upon SOX9 knockdown shows a total of 93 genes have significantly different expression. 60 of these genes increase in expression and 33 of these genes decrease in their expression. (**D**) Quantitative PCR was performed to confirm the top five upregulated and top five down regulated genes seen by RNA-seq. Error bars indicate the standard deviation from three biological replicates. (**E**) GO term analysis of downregulated genes reveals a role for SOX9 in Notch signaling and regulation of proliferation. (**F**) GO term analysis of upregulated genes shows terms for cilia development and function. Neg, Negative; Pos, Positive; reg, regulation.The scale bar size is 100 uM (**B**).

Table 1.1

	Gene	Pearson correlation with PAAD *SOX9*	Direct target of SOX9?	Enriched ductal expression?	CAC marker?
Upregulated	*LRRC6*	0.21	Yes	No	No
	SPEF1	-0.13	Yes	No	No
	CASC1	-0.14	No	No	No
	DNAH7	-0.02	No	No	No
	C9orf116	0.07	No	No	No
	CFAP69	-0.22	Yes	No	No
	CCDC13	-0.50	No	No	No
	AXIN2	-0.17	No	No	No
	C6orf165	-0.31	No	No	No
	ROPN1L	-0.35	No	No	No
Downregulated	*TUB*	-0.56	Yes	No	No
	LIN7C	0.04	Yes	No	No
	SLC45A3	0.48	Yes	No	No
	DDAH1	0.35	Yes	No	No
	CENPM	0.24	Yes	No	No
	EPCAM	0.58	Yes	Yes	Yes
	RGCC	-0.30	No	Yes	No
	TINAGL1	0.57	Yes	Yes	No
	ESRP1	0.67	Yes	Yes	No
	SKIV2L	0.03	Yes	No	No

To further validate our results, 178 publicly available RNA-seq data were looked at from pancreatic adenocarcinoma samples found in The Cancer Genome Atlas (TCGA). In all 178 samples, SOX9 was found to be highly expressed. It was predicted that there must be a direct effect that is opposite to what was observed when *SOX9* was knockdown in our PANC-1 experiments. DE genes were clustered together based on their expression patterns in each of the tumor samples looked at. Upregulated genes were found to cluster together and they were lowly expressed in TCGA tumor samples. Downregulated genes also clustered together with SOX9 and were highly expressed in TCGA tumor samples (**Figure 2.1A**). SOX9 expression was correlated with each of the top 10 DE genes to examine how close the genes are co-regulated in the tumor samples. A high degree of correlation was seen between SOX9 expression and the DE genes (**Table 1.1**). An example of this is seen with *CCDC13,* which is a gene that encodes a centriolar satellite protein required for ciliogenesis (Staples et al., 2014). Upon the knockdown of *SOX9*, *CCDC13* is found to be upregulated and had the strongest degree of negative correlation with SOX9 expression (r = -0.50). This finding is consistent with SOX9 negatively regulating the expression of *CCDC13* in both PANC-1 cells and in PDAC samples (**Figure 2.1B**). A second example is seen when looking at the *Epithelial splicing regulatory protein 1* (*ESRP1*). *ESRP1* has a strong positive correlation with SOX9 expression (r = 0.67) and suggests that SOX9 positively regulates the expression of this gene (**Figure 2.1C**). Overall, comparing our data to data found in TCGA validated our RNA-seq analysis and provided additional support for the observations seen regarding the transcriptional targets of SOX9. Additionally, these results support the biological relevance of our *in vitro* model and suggest that target genes of SOX9 have an important role in human disease.

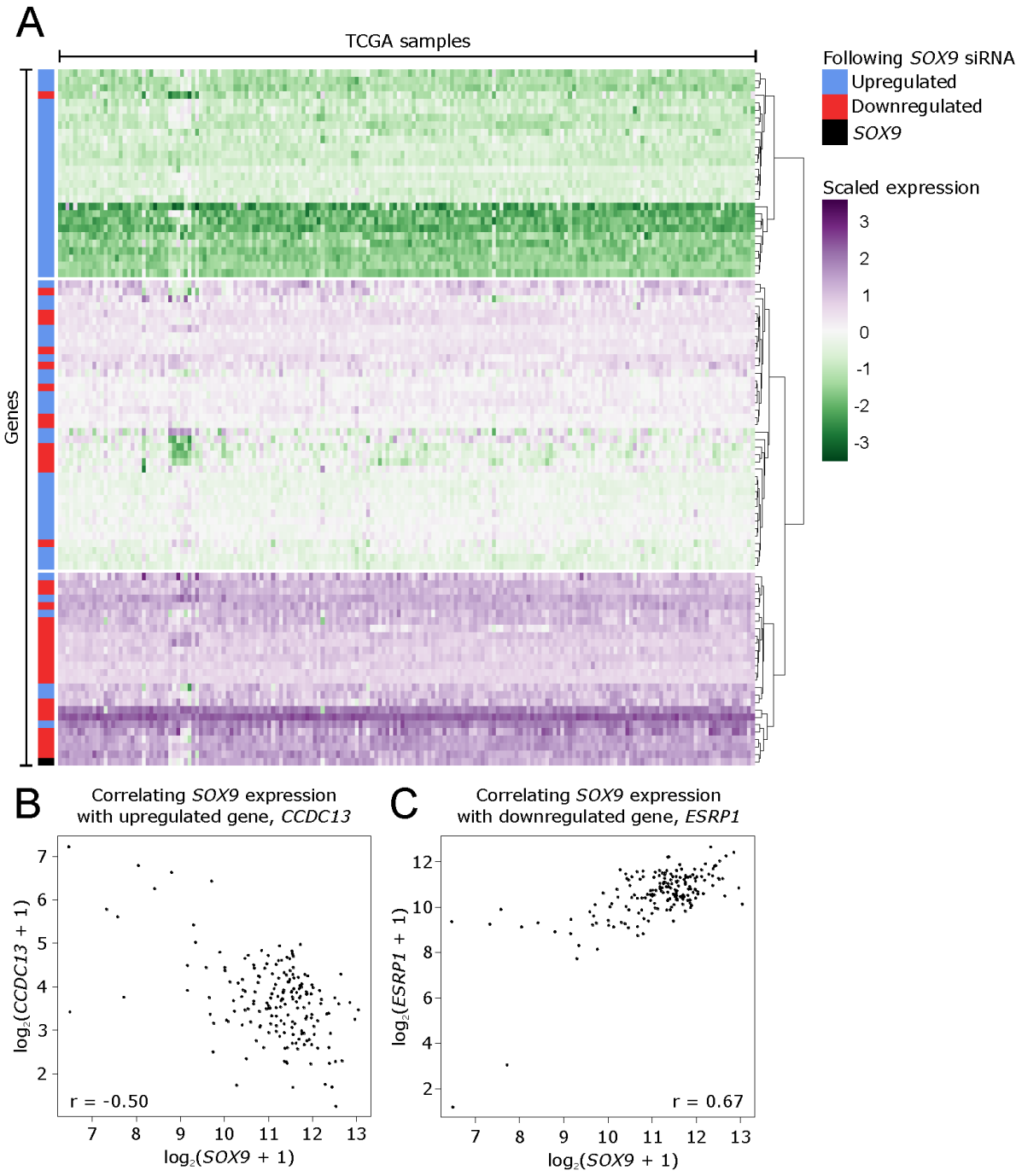

A
TCGA samples
Genes
Following SOX9 siRNA
Upregulated
Downregulated
SOX9
Scaled expression
3
2
1
0
-1
-2
-3
B
Correlating SOX9 expression with upregulated gene, CCDC13
log2(CCDC13 + 1)
r = -0.50
log2(SOX9 + 1)
C
Correlating SOX9 expression with downregulated gene, ESRP1
log2(ESRP1 + 1)
r = 0.67
log2(SOX9 + 1)

Figure 2.1. Expression data from PDAC samples corroborate the expression patterns seen in PANC-1s.

(**A**) Expression analysis of the TCGA pancreatic adenocarcinoma sample data set shows genes that are upregulated after the siRNA knockdown of SOX9 in PANC-1s cluster together and are lowly expressed. Genes that are downregulated upon the knockdown of SOX9 tend to cluster together and with *SOX9,* they are highly expressed. (**B**) *CCDC13* is an example of a gene that is upregulated following the knockdown of *SOX9.* This gene is negatively correlated with the expression of *SOX9* and this suggests that SOX9 negatively regulates *CCDC13*. (**C**) *ESRP1* is an example of a gene that is downregulated following the knockdown of SOX9. This gene is positively correlated with SOX9 expression and this suggests that SOX9 positively regulates *ESRP1*.

ChIP-seq was performed using PANC-1 chromatin and an antibody against SOX9 to identify SOX9 regulatory regions. Following pull-down, sequencing, and alignment, 47,858 SOX9 binding sites were identified in PANC-1s. The sequence that underly the top 1,000 most significant SOX9 binding events were analyzed to identify the presence of transcription factor motifs present. The 'head-to-head' palindrome motif was the most enriched motif identified. This sequence had the highest similarity to the SOX9 consensus motif (**Figure 3.1A**). This finding is supported by findings that observed that SOX9 can function as a homodimer in chondrocytes (Bernard et al., 2003). The FOS::JUN binding sequence was the second most highly enriched motif found (**Figure 3.1B**). This binding sequence has been found to bind in conjugation with SOX9 in chondrocytes (He, Ohba, Hojo, & McMahon, 2016). Additionally, SOX9 binding was found consistently at the promoters of genes (8.8% of SOX9 peaks, n = 4219 versus 1.1% of peaks expected **Figure 3.1C**). As the distance from the transcription start site increased, the number of SOX9 binding events lessened (**Figure 3.1D**). These findings are similar to what has been previously reported regarding SOX9 binding events (Kadaja et al., 2014; Z. Shi et al., 2015; Shih et al., 2015).

The potential outcome of SOX9 binding in PANC-1s was interrogated by performing a functional annotation on the genes that were proximal to the binding sites of SOX9. Many of these GO terms matched already known roles of SOX9 (**Figure 3.1E**). The terms identified include (**1**) 'endocrine pancreas development', a consistent finding of SOX9's role during pancreas embryogenesis (Kopp et al., 2011; Seymour et al., 2008; Shih et al., 2015), (**2**) 'stem-cell maintenance, which is consistent with the fact that SOX9 maintains pancreatic progenitor cell identity (W. Huang et al., 2016; Scott et al., 2010; Seymour et al., 2007), and (**3**) 'ossification and osteoblast differentiation which is a known function of SOX9 that is restricted to bone

development (Bi, Deng, Zhang, Behringer, & de Crombrugghe, 1999; Dy et al., 2012) but may also become dysregulated in cancer (Kawai et al., 2016).

Next, we sought to identify direct target genes of SOX9. This was done by looking at our RNA-seq data set of genes affected by the knockdown of SOX9 and comparing these results to our ChIP-seq data that found genes associated with SOX9 binding sites. 43% of upregulated genes and 64% of downregulated genes were found to be bound by SOX9 directly. 3 out of the top 10 upregulated genes showed evidence of being directly bound by SOX9. Of the top 10 down regulated genes, 9 out of 10 were found to be direct targets of SOX9 (**Table 2.1**).

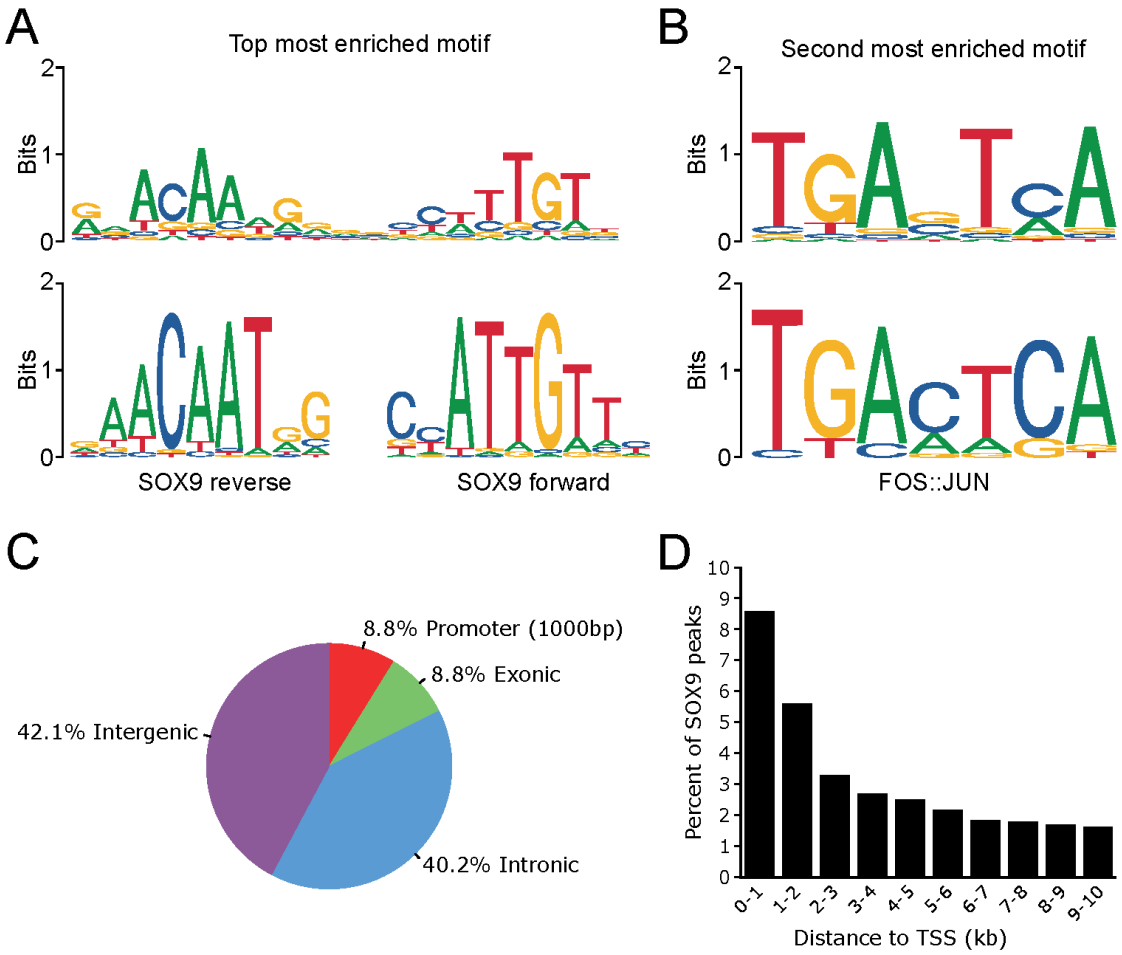

A
Top most enriched motif
Bits
SOX9 reverse
SOX9 forward
B
Second most enriched motif
FOS::JUN
C
8.8% Promoter (1000bp)
8.8% Exonic
42.1% Intergenic
40.2% Intronic
D
Percent of SOX9 peaks
Distance to TSS (kb)
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
E
Binomial Fold Enrichment
Endocrine pancreas development
Pancreas development
Somatic stem cell maintenance
Stem cell maintenance
Osteoblast differentiation
Ossification
Prostate gland morphogenetic growth
Prostate gland growth
Male sex determination
Embryonic placenta morphogenesis
Labyrinthine layer development
Embryonic placenta development
Placenta development
Endocardial cushion morphogenesis
Heart valve morphogenesis
Lactation
Mammary gland development
Reactive oxygen species metabolic process
Secretion by tissue
Body fluid secretion
2.82
2.23
3.19
2.19
2.44
2.20
6.95
4.23
4.06
4.76
3.94
2.80
2.77
3.91
3.72
3.74
2.07
3.28
3.18
3.01

Figure 3.1. Characteristics of SOX9 gene regulation include promoter proximal binding and regulation of genes important in pancreatic biology.

(**A**) The head-to-head SOX9 binding sequence was the most common motif found. (**B**) The FOS::JUN motif was the second most common motif found at SOX9 binding sites. (**C**) Promoter regions (≤1000 base-pairs upstream of a transcription start site) were enriched for SOX9 binding. (**D**) The number of SOX9 binding events were found to decrease as the distance from the transcriptional start site increased. (**E**) Genes that were found to be the closest to the SOX9 binding sites were enriched for GO terms related to known SOX9 biology. This includes terms associated with endocrine pancreas functions.

Table 2.1

Gene name	log2FoldChange	p value adjusted
SKIV2L	-3.720556577	0.041148556
ESRP1	-2.307411013	0.00251694
TINAGL1	-2.26393585	1.33E-05
RGCC	-2.005664194	0.007300057
EPCAM	-1.762243949	1.85E-05
CENPM	-1.549786505	2.91E-05
DDAH1	-1.521941045	7.86E-06
SLC45A3	-1.47538869	0.012325132
LIN7C	-1.470115402	2.28E-05
TUB	-1.436777273	0.010946958
NOTCH2	-1.404349386	2.24E-05
RTN4R	-1.364906312	0.000891839
ADAMTS1	-1.344226063	0.001952963
CYR61	-1.310365854	0.001426397
RAB11FIP4	-1.289578433	0.00297676
ARHGAP19	-1.228655751	4.52E-05
SMIM11	-1.217459433	0.026287665
OCRL	-1.204651296	2.78E-05
AIF1L	-1.195506932	0.038165605
CCDC117	-1.192380903	0.00251694
CAPN15	-1.183313247	0.000305734
TMEM156	-1.177820546	0.008768087
MBP	-1.133983528	0.000481696
PTMA	-1.099490435	0.015763551
RNF38	-1.09937516	0.002088331
SEPT11	-1.088646559	0.00251694
SLC7A1	-1.077960768	0.001697217
GTF2A2	-1.076524994	0.0025877
LRRC8B	-1.071493112	0.002181255
DUSP6	-1.0677417	0.014080344
ISOC1	-1.067349594	0.001976801
WDYHV1	-1.059553563	0.008768087
CTSV	-1.007205014	0.005980316
IQCG	1.003234741	0.002065062
PTRF	1.010195728	0.008475993
F2RL1	1.011130902	0.04172519
ZC3H6	1.025825335	0.017782031
CCDC113	1.032707909	0.005739877
MEGF6	1.066934518	0.008768087
ZNF214	1.067971619	0.006876023
IQCK	1.075083503	0.004190549
TTC30A	1.076580447	0.002434421
TNFRSF1B	1.08080392	0.0429372
UBXN11	1.086783937	0.008367014
CEP19	1.104998369	0.005980316
NPHP1	1.116314331	0.00220692

Since PANC-1s are derived from PDAC (Lieber, Mazzetta, Nelson-Rees, Kaplan, & Todaro, 1975), our lab was interested in determining whether PANC-1s share similarities with ductal cells since they are a possible origin for PDAC (A. Y. L. Lee et al., 2019; C. Shi et al., 2019). Our PANC-1 dataset was compared to two previously published bulk RNA-seq datasets that looked at two ductal cell populations using the adult zebrafish pancreas. One study was done by Ghaye et al., which used a *nkx6.1* fluorescent reporter to isolate all ductal cells (including CACs) from adult transgenic zebrafish via FACS (Ghaye et al., 2015; Tarifeno-Saldivia et al., 2017). The second study was done by Delaspre et al., which instead used a Notch-responsive fluorescent reporter (*tp1:eGFP*) to specifically isolate CACs from the adult zebrafish pancreas (Delaspre et al., 2015). In both transcriptome studies, the expression of Sox9b was highly enriched in ductal cells and CACs of the zebrafish pancreas. This finding confirmed the expression of Sox9b in the adult zebrafish pancreas. **Table 1.1** compares our PANC-1 sequencing data to the transcriptome of zebrafish ductal cells and CACs. From the knockdown of SOX9 in PANC-1s, none of the top 10 upregulated genes are expressed in adult zebrafish ductal cells or CACs. However, it was found that 4 out of the 10 downregulated genes were expressed in the ductal cells of the zebrafish pancreas (**Table 2.1**). These results demonstrated that *sox9b*-expressing ductal cells are enriched for homologues of direct targets of SOX9 in PANC-1s. One gene we found to be highly enriched in the CACs of the zebrafish pancreas and in our PANC-1 study was *epcam* (**Table 2.1**).

Based on these findings and knowing that SOX9 and EpCAM are found in healthy pancreatic tissue (Cirulli et al., 1998), we were interested in examining the relationship between these two genes further. To do this, we sought to determine how SOX9 might be regulating the expression of EPCAM. ChIP-seq (red) and RNA seq (blue) were used to examine reads from our PANC-1 experiments at the *EPCAM* locus (black). From our ChIP-seq data there was a SOX9 binding event

that overlapped with the EPCAM transcriptional start site and its promoter sequence (**Figure 4.1A**). The expression of *EPCAM* was found to decrease when *SOX9* was knocked down in PANC-1s (**Figure 4.1A**). To validate this observation, immunofluorescence was performed against EpCAM and SOX9 to detect their expression in PANC-1s transfected with either control or *SOX9* siRNA. In PANC-1s transfected with control siRNA, the expression of SOX9 was detected in the nuclei and the expression of EpCAM was detected at the plasmid membrane in all cells (**Figure 4.1B**). In PANC-1s transfected with *SOX9* siRNA, there was a reduction in the expression of SOX9 as expected and there was either a reduction or absence in the expression of EpCAM (**Figure 4.1C**). These findings support the idea that SOX9 directly bindings to the EPCAM locus and increases its expression in PANC-1s. When looking at TCGA PDAC samples, there was a co-regulation of *SOX9* and *EPCAM* found. A strong positive correlation was seen between SOX9 and *EPCAM* expression ($r = 0.58$, **Figure 4.1D**). This finding further confirmed that SOX9 positively regulates EPCAM in both PANC-1 and PDAC cells.

In the results section of this Chapter, I will highlight the role I had in determining whether zebrafish CACs (**1**) express *epcam* normally and (**2**) whether *epcam* expression in CACs is regulated in a *sox9b* dependent manner. The importance of this research is based on the notion that ductal cells (CACs included) could be the potential origin of pancreatic cancer. The Parsons Lab is interested in using zebrafish as an *in vivo* model to study pancreatic cancer because zebrafish CACs utilize many of the pathways involved in pancreatic cancer, particularly the sox9 pathway.

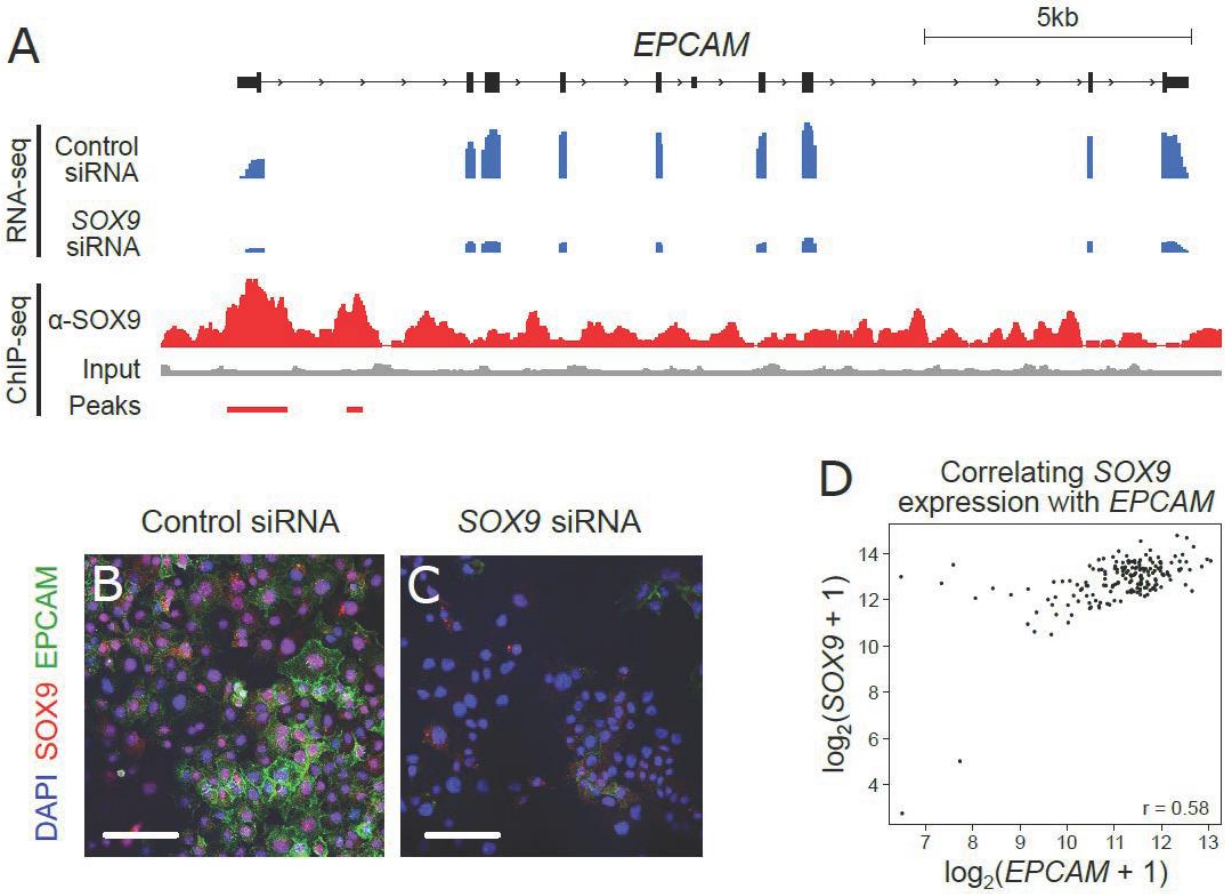

A
EPCAM
5kb
RNA-seq
Control siRNA
SOX9 siRNA
ChIP-seq
α-SOX9
Input
Peaks
Control siRNA
SOX9 siRNA
DAPI SOX9 EPCAM
B
C
D
Correlating SOX9 expression with EPCAM
log2(SOX9 + 1)
log2(EPCAM + 1)
r = 0.58

Figure 4.1. EPCAM *is a direct target of SOX9.*

(**A**) Shows RNA-seq (blue) and ChIP-seq (red) reads over the *EPCAM* locus. This confirms the siRNA knockdown of *EPCAM* and the binding of SOX9 at the transcription start site. (**B**, **C**) Immunofluorescence was performed to detect the expression of SOX9 and EPCAM in PANC-1s transfected with either control siRNA (**B**) or *SOX9* siRNA (**C**). (**D**) Looking at TCGA pancreatic adenocarcinoma samples, a positive correlation of *EPCAM* and *SOX9* expression was found.

b) Materials and Methods

Whole mount in situ hybridization

Adult *sox9b*^{fh313} heterozygous fish were incrossed, and the resulting offspring were maintained in E3 media up to 5dpf. Initially, I found that the *epcam* locus was highly polymorphic because my riboprobes were not binding to the *epcam* transcript. To ensure that the *in situ* riboprobe I made would bind specifically to the *epcam* locus in my *sox9b*^{fh313} fish used, I sequenced the full length of the *epcam* gene in larvae from a single pair of fish homozygous across the *epcam* locus. This pair of fish I identified as being homozygous for *epcam* was used to generate larvae for all my *in-situ* experiments. From 0-5dpf larvae were raised under normal conditions and then euthanized on ice. Larval cadavers were cut into two at the level of the cloaca. The tail of each larva was placed into an individual well of a 96-well plate. The tail was digested to obtain genomic DNA for genotyping analysis (Meeker, Hutchinson, Ho, & Trede, 2007). The body of the fish was placed into 4% PFA in a separate 96-well plate (in the same well position as tail) overnight at 4°C. The tail DNA was genotyped for *sox9b* (Chin, Toy, Vandeven, & Cairo, 1989). Pancreata of known *sox9b* genotypes were dissected the next day for each genotype. Each pancreas was then placed into 3 separate tubes corresponding to their correct genotype. To form a visible pellet, no less than 20 dissected pancreata were added to each tube. Pancreata were kept in 100% methanol overnight at -20°C and then whole mount *in situ* hybridization (WMISH) was performed the following day (Parsons et al., 2009). The original WMISH protocol was modified for dissected larval zebrafish pancreata as described below.

Whole mount *in situ* hybridization protocol modifications

Samples were removed from 4% paraformaldehyde (PFA) and were dissected in phosphate buffered saline containing 20% tween-20 (PBT). Each dissected pancreas was then placed into a tube containing PBT. Once dissections were completed, PBT solution was removed and pancreata underwent a gradient of methanol changes until they were in 100% methanol. Dissected pancreata were kept in 100% methanol for no more than 24 hours at -20°C. The entire WMISH protocol for dissected pancreata was carried out in tubes and then 24-well plates for color development on day 3. I made WMISH modifications that included the following: (1) Proteinase K (1:1000) digestion was performed for 8 minutes at room temperature without agitation (no shaking of the tubes), (2) fixation was carried out in 4% PFA after proteinase K and did not exceed 20 minutes of fix, (3) both pre-hybridization and hybridization buffers contained 65% formamide/0.5mg/mL tRNA and were performed at 65°C (and not allowed to cool at any point during the washes), (4) the *epcam* riboprobe stock solution was diluted at a concentration of 1:100 in hybridization buffer following a standard transcription reaction when probe is eluted in 20uL of RNAse free water, (5) 100uL of diluted *epcam* riboprobe was added to each sample tube, (6) Excess probe was removed by performing high stringency washes at 65°C which include two 20 minute washes in a 1:1 dilution of pre-hybridization buffer and 2x SSC, two 20 minute washes in 2x SSC, two 30 minute washes in 0.05x SSC followed by three gradient washes of 75%, 50%, and 25% in 0.05x SSC/ PBT for 10 minutes each at room temperature, (7) antibody blocking was carried out for 2 hours at room temperature without agitation in PBT containing 2% goat serum, (8) antibody dilution for anti-DIG was performed at 1:3000 and was incubated overnight at 4°C without agitation, (9) the NBT/BCIP color reaction was done by adding 2.25uL NBT (50mg/mL) and 1.75uL BCIP (50mg/mL) to 1mL of staining buffer and pancreata were transferred carefully with a disposable

pipette to a 24 well plate to perform and watch color development, (10) NBT/BCIP color reaction for pancreata was performed at room temperature for 2-4 hours (at least for the *epcam* riboprobe this is how long it took to develop), (11) probe background was cleared by performing ethanol washes using an ethanol gradient (30%, 50%, 75%) in PBT for 5 minutes, and then one final wash in 100% ethanol for 10 minutes, this was then followed by rehydration into PBT and clearance of background using the traditional glycerol gradient and storage in 70% glycerol at 4°C until imaging.

Designing of the *epcam* riboprobe

The *epcam* riboprobe (1.2 kb) was made from larval cDNA which was derived from combining approximately 5 sibling larvae into a single tube and extracting RNA using a zymo directzol kit. The *epcam* riboprobe used the following primers: forward— 5'-GGCCAGAGAGGGGATATCTT -3' and reverse— 5'- GTTAATCCAATTGAAGAGAAGC -3'. These primers were designed using primer3 and then blasted to the zebrafish reference genome to ensure that there were no off-targeting effects. To make a stock riboprobe of *epcam*, the transcription reaction was carried out for 2 hours using 1ug of DNA in a total volume of 20uL. The probe was transcribed using dig rNTP labeling mix (Roche). The transcription reaction was then cleaned up using the RNAeasy elution kit following the manufacturers guidelines (Qiagen). The probe was eluted in 20uL of RNAse free water. To establish a working *epcam* probe for my WMISH experiments, I diluted 1uL of the eluted riboprobe in 99uL of hybridization buffer (1:100 dilution). All WMISH images were collected using a Zeiss Axioplan2 microscope.

c) Results

In the developing mammalian pancreas, SOX9 has the ability to maintain both the endocrine and ductal cell progenitor population (Seymour, 2014). In order to test whether SOX9 regulates the expression of EPCAM during zebrafish pancreas development, I performed numerous *in situ* hybridizations on dissected pancreata from larval zebrafish at 5 days post fertilization (dpf).

I first established an optimized WMISH protocol that works robustly on dissected larval zebrafish pancreata (specifically CACs). Here, I tested several elements to establish this protocol including the length of the riboprobe used, the concentration of the riboprobe used for hybridization, time spent in proteinase k to digest larval pancreatic tissue for probe penetration, pre-hybridization and hybridization wash buffer concentrations for both formamide and tRNA, time spent in washes involving the removal of excessive probe, the concentration of the color reaction (NBT/BCIP) solution, staining buffer contents, and the inclusion of the ethanol gradient washes after staining was completed. Details on my established WMISH protocol can be found in the materials and methods section of this chapter.

Not only did I have to establish a robust WMISH protocol, but I also encountered issues with the *epcam* locus being highly polymorphic in our fish. I found that my initial *epcam* probe only worked in larvae from some fish pairs used. This was evident by the *in-situ* hybridization working only 50% of the time or less. This suggested to me that the probe was either not a robust probe or that the probe is not always binding to the *epcam* transcript in my fish. To overcome this issue, I (**1**) screened for adult *sox9b*^{fh313} fish that were homozygous at the *epcam* locus and (**2**) made multiple *epcam* riboprobes to target various regions of the *epcam* locus that I then tested by WMISH. For the first point, I had to design multiple PCR primers against various regions of the *epcam* locus (exons 1-10). Multiple PCR primers to the same exon were used because the reference

sequence online did not exactly match the *epcam* sequence in my fish. Many of these PCR primers I designed did not work because the locus was highly polymorphic in my fish. PCR primers that did work were seen to amplify a PCR product by gel electrophoresis. The PCR product was extracted from the gel, purified, and then sent off for sequencing. Once I analyzed the sequence, I designed additional PCR primers to this sequenced region captured in order to obtain the remaining *epcam* sequence missing. I did this until the entire *epcam* sequence was captured (exons 1-10). Approximately 20 adult fish were sequenced at the *epcam* locus and out of 20 fish I found 3 pairs of fish where the *epcam* locus was homozygous. These 3 pairs of fish were kept for generating larval fish I then used for WMISH. For the second point, I tested approximately 10 *epcam* riboprobes that I designed specifically for these 3 pairs of fish. The *epcam* probe that worked the best was used to generate my WMISH results for *epcam*. Since fish were maintained in pairs and sequenced at the *epcam* locus, I knew that my riboprobe would work in these fish because the *epcam* riboprobes were designed to the *epcam* locus of these fish specifically. Provided that I had an optimized WMISH protocol, pairs of fish ready to use for my experiments, and an *epcam* probe at hand, I was ready to carry out the results for this project.

Once fish reach the 5dpf larval stage, acinar cells of the pancreas can be found surrounding a large principal islet located at the head of the pancreas and extending posteriorly down the tail of the pancreas. Ductal cells are organized equatorial to the islet and are found to run down the center of the pancreatic tail (Beer, Parsons, & Rovira, 2016; Parsons et al., 2009; Yee, Lorent, & Pack, 2005). During early development, all ductal cells are Notch responsive and express *sox9b* (Beer et al., 2016). As stated previously, this Notch activity eventually becomes restricted to CACs in the adult fish.

To determine whether Sox9b activity affects the expression levels of *epcam* in zebrafish CACs, I took advantage of using our zebrafish line containing a mutant null allele named *sox9b*^{fh313} (Delous et al., 2012; Manfroid et al., 2012). I performed an incross between heterozygous *sox9b*^{fh313} fish (*sox9b*$^{+/-}$) pairs and then screened for *epcam* homozygosity as described early. I next used my established WMISH protocol to detect the expression of *epcam* in the CACs of the resulting larval progeny at 5dpf. I observed the expression of *epcam* in the ductal region of the pancreas for all three corresponding *sox9b* genotypes: wildtype, heterozygotes, and homozygous mutants (**Figure 5.1A-C**). Interestingly, the expression of *epcam* was harder to detect in the pancreata of wildtype larvae (n = 33, representative image seen in **Figure 5.1A**). In contrast, the expression of *epcam* was seen to be noticeably stronger in the pancreata of heterozygous larvae (**Figure 5.1B**, n = 55). I found the expression of *epcam* to appear to be even stronger in the CACs of homozygous mutant pancreata (**Figure 5.1C**, n = 23). In previous studies, it was found that Sox9b homozygous mutants have fewer CACs than wildtype (Delous et al., 2012; Manfroid et al., 2012) or heterozygotes (W. Huang et al., 2016). Since I observed the strongest staining of *epcam* in pancreata with the least number of CACs, this suggests that the loss of *sox9b* resulted in an increase in the expression of *epcam*. As was shown previously in the introduction of this chapter, there is a clear correlation between the expression of a SOX9 homolog and an *EPCAM* homolog in PANC-1s. Here, I have shown that in zebrafish CACs there is also a clear correlation between the expression of *sox9b* and *epcam*. However, the expression of *epcam* appears to be upregulated when the levels of *sox9b* are lowered in zebrafish CACs. This is the opposite of our finding in PANC-1s. Together, these findings suggest that the regulation of *EPCAM* by SOX9 appears to function as an EPCAM activator in PANC-1s and as a repressor in the CACs of the developing zebrafish pancreas.

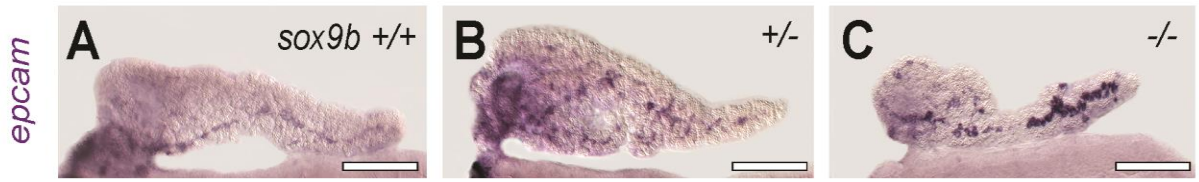

epcam
A
sox9b +/+
B
+/-
C
-/-

Figure 5.1. *epcam* is regulated in a *sox9b* dependent manner in the zebrafish pancreas

(**A-C**) Whole mount *in situ* hybridization was performed to detect the expression of *epcam* in the pancreas of 5 dpf zebrafish larvae with the following *sox9b* genotypes: (**A**) +/+, (**B**) +/− and (**C**) −/−. The expression of *epcam* is detected the highest in *sox9b* homozygous mutant pancreata. Scale bar 100 um.

EpCAM was initially discovered as a gene associated with congenital tufting enteropathy (CTE)— a diarrheal disorder that presents during the neonatal period. CTE is caused by a mutation in EpCAM at the donor splice site of exon 4 and results in a decrease in EpCAM expression (Sivagnanam et al., 2008). Not only is EpCAM linked to CTE, but it is also considered a biomarker for cancer (Went et al., 2006), including pancreatic cancer (van der Gun et al., 2010). Given these findings and that EpCAM is also regulated by SOX9, we wanted to learn more about the function of Epcam. Thus, CRISPR/Cas9 technology was used to generate an *epcam* mutant zebrafish line called *epcam*^{jh79}. This mutation should have resulted in a 12base pair (bp) deletion at the donor splice site of exon 4 (**Figure 6.1A**). The goal was to create an *epcam* mutant line that mimicked the mutation seen in human CTE patients. This was done so that the biological relevance of *epcam* could be understood in relation to the human mutation.

My role was to characterize the *epcam*^{jh79} mutation. To do this, I first confirmed that the 12bp deletion occurred on the genomic level. I did this by sequencing genomic DNA extracted from the tail of both wildtype *epcam*$^{+/+}$ fish and *epcam*$^{jh79/jh79}$ fish. **Figure 6.1B** shows the chromograph for both *epcam*$^{+/+}$ and *epcam*$^{jh79/jh79}$ fish which confirmed the 12bp deletion. Next, this 12bp deletion should have created a premature stop codon resulting in nonsense-mediated mRNA decay (NMD). NMD is a process by which mRNAs with a premature stop codon are degraded by the cell. This process avoids the synthesis of truncated proteins and therefore should result in lower levels of the mRNA detected. To determine whether the *epcam*^{jh79} allele expressed lower levels of *epcam,* I performed an incross between *epcam*$^{+/jh79}$ fish and *epcam*$^{jh79/jh79}$ fish to generate 50% heterozygous and 50% homozygous mutant offspring. Since other groups have shown that there is a clear developmental abnormality in the developing ear of other mutant alleles of *epcam,* (Slanchev et al., 2009) I verified whether this phenotype holds true in our new *epcam*

mutant line of fish. I found that our *epcam*$^{jh79/jh79}$ mutant fish have a clear ear abnormality up to 2dpf. By 1dpf, wildtype embryos have otic vesicles that contain two large otoliths (**Figure 7.1A**) and *epcam* homozygous mutants have either an absence of these otoliths or very small otoliths (**Figure 7.1D**). I then verified that *epcam* homozygous mutants express lower levels of *epcam*. I did this by incrossing *epcam*$^{jh79/+}$ and *epcam*$^{jh79/jh79}$ mutant fish and phenotyped the resulting offspring at 2dpf for otolith formation. These fish were separated into two groups, large and small otoliths. Larger otoliths correspond to an *epcam*$^{jh79/+}$ genotype whereas smaller otoliths correspond to an *epcam*$^{jh79/jh79}$ genotype. Once both groups of fish reached 5dpf, I used WMISH to confirm that having two copies of the *epcam*^{jh79} allele (smaller otoliths) resulted in lower levels of *epcam* being expressed. This can be seen by the higher expression of *epcam* detected in *epcam*$^{jh79/+}$ larvae (**Figure 6.1C**) compared to the much lower *epcam* expression detected in *epcam*$^{jh79/jh79}$ homozygous mutant larvae (**Figure 6.1D**). Having confirmed that we have a mutant line that expresses lower levels of *epcam,* we next wanted to explore the biological relevance of *epcam* in zebrafish.

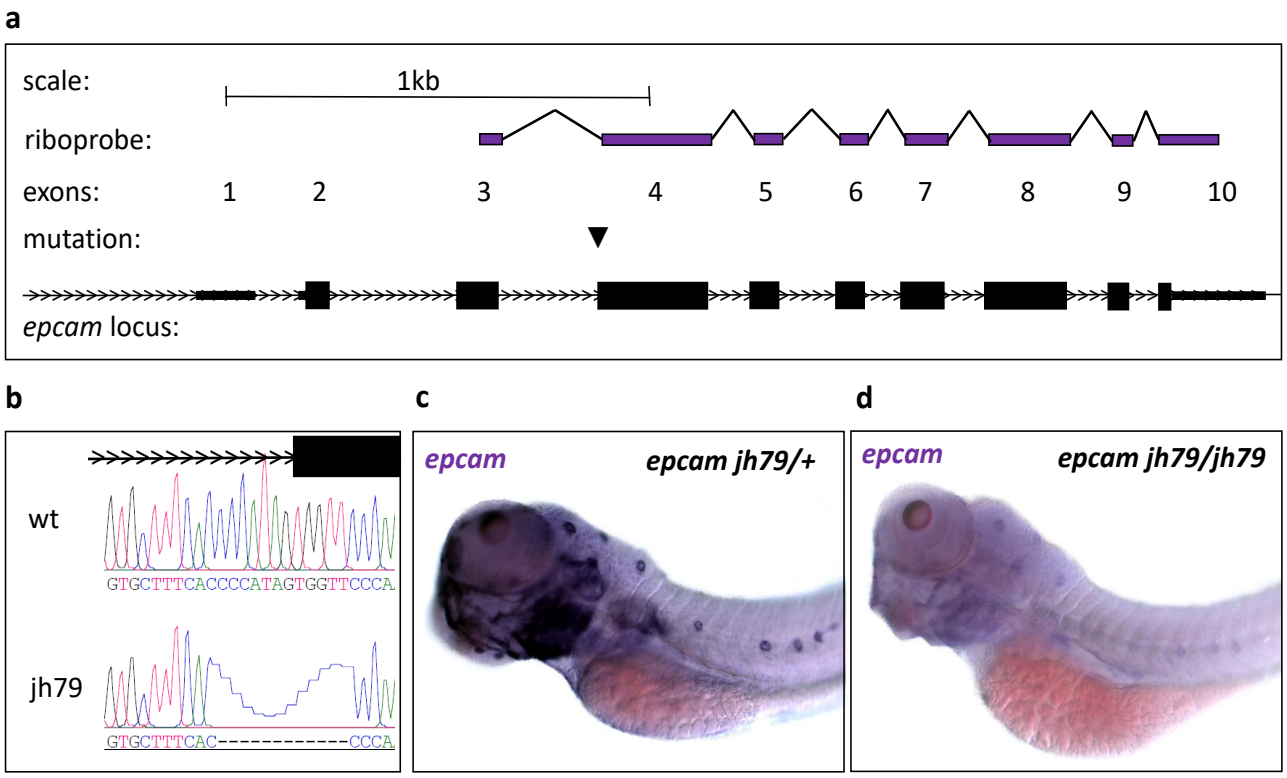

a
scale:
1kb
riboprobe:
exons:
1
2
3
4
5
6
7
8
9
10
mutation:
epcam locus:
b
wt
GTGCTTTCACCCCATAGTGGTTCCCA
jh79
GTGCTTTCAC----------CCCA
c
epcam
epcam jh79/+
d
epcam
epcam jh79/jh79

Figure 6.1. *epcam*^{jh79} mutants are nulls

(**A**) Schematic of the *epcam* locus showing where the ribprobe was designed to target the *epcam* transcript (exons 3 to 10). (**B**) Chromograph of wildtype (wt) and epcam (jh79) exon 4 genomic DNA sequence. Whole mount *in situ* shows the expression of *epcam* in *epcam* hets (**C**) and *epcam* homozygous mutants (**D**).

From **Figure 1.1**, it was discovered that SOX9 may have a role in regulating cilia. When SOX9 was knocked down in PANC-1s it led to the upregulation of GO terms associated with cilia development and function. We also found that SOX9 directly regulates EpCAM. As mentioned, one of the phenotypes associated with *epcam* mutants (including our new *epcam*^{jh79} mutants) is delayed otolith development. Interestingly, otolith formation is linked to cilia function in the otic epithelium (Stooke-Vaughan, Huang, Hammond, Schier, & Whitfield, 2012). Defects in cilia have also been known as a hallmark of cancer (H. Liu, Kiseleva, & Golemis, 2018) and PanIN cells that overexpress KRAS in transgenic mice were found to have abnormal primary cilia (Seeley, Carriere, Goetze, Longnecker, & Korc, 2009). Provided that SOX9 and EpCAM may be linked to cilia development, *epcam*^{jh79} mutants were used to understand whether *epcam* regulates cilia development. Having established an otolith phenotype for the *epcam*^{jh79} mutants, Santiago Pineda (a former member of the Parsons Lab) used the developing zebrafish ear as a model to detect cilia in 1dpf wildtype and maternal zygotic (MZ) epcam mutant embryos (**Figure 7.1**). Cilia was detected by performing immunofluorescence and staining for acetylated tubulin in 1dpf wildtype (**Figure 7.1B, C**) and MZ epcam mutants (**Figure 7.1E, F**). Using confocal microscopy, Santiago quantified the number of cilia in 20 larval MZ epcam mutants. At 1dpf he found that wildtype otic vesicles (n = 18, **Figure 8.1A**) had on average 6.17 ± 0.44 cilia, whereas MZ *epcam* mutants had significantly fewer cilia at 2.55 ± 0.3 with a P value < 0.01 (two-tailed t-test) (**Figure 8.1A**). It was also found that cilia in the MZ epcam mutants were significantly shorter (P < 0.01, two-tailed t test) with an average length of 4.01 ± 0.2 microns (**Figure 8.1B**) compared to wildtype cilia being at 5.44 ± 0.09 microns (**Figure 8.1B**). Santiago also looked at two other structures that are highly ciliated to see if the aberrant cilia phenotype he found was conserved. These two structures included the developing olfactory pits (Reiten et al., 2017) (**Figure 8.1C**) and the pronephric duct

(Kramer-Zucker, Wiessner, Jensen, & Drummond, 2005) (**Figure 8.1D**). It was found that there was a lack of cilia in both of these structures in MZ epcam mutants (**Figure 8.1C, D**).

Together, these results show that Sox9b activity negatively regulates the expression of *epcam* in the developing zebrafish pancreas. The overall significance of the e*pcam*^{jh79} mutants shows that EpCAM is required for normal ciliogenesis to occur in multiple epithelia observed. These results suggest that there may be a link between Sox9b, EpCAM, and cilia in the pancreas however, further investigation is needed.

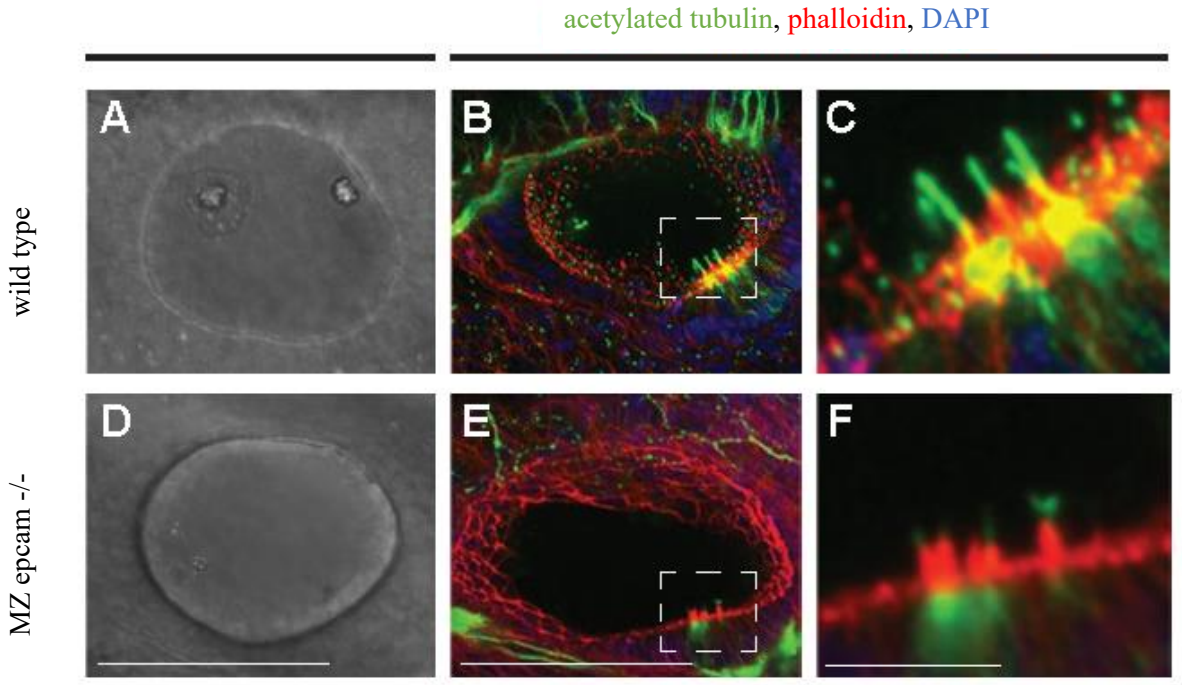

acetylated tubulin, phalloidin, DAPI
wild type
MZ epcam -/-
A
B
C
D
E
F

Figure 7.1. MZ epcam mutants have a cilia defect in the otic vesicle

(**A-F**) Cilia was phenotyped for both wildtype and MZ *epcam*$^{jh79/jh79}$ mutants. (**A**) Bright-field images are shown of the otic vesicle in wildtype larvae. (**D**) Bright-field image of otic vesicle in MZ epcam mutants are also shown and can be seen as missing. Immunofluorescence was performed to detect acetylated tubulin (green), phalloidin (red), and DAPI (blue) using confocal microscopy in wildtype (**B, C**) and MZ epcam mutants (**E, F**). The dashed square represents the areas enlarged in (**C**) and (**F**) to visualize cilia easier. Scale bar 100uM (**A, B, D, E**) and 5um (**C, F**).

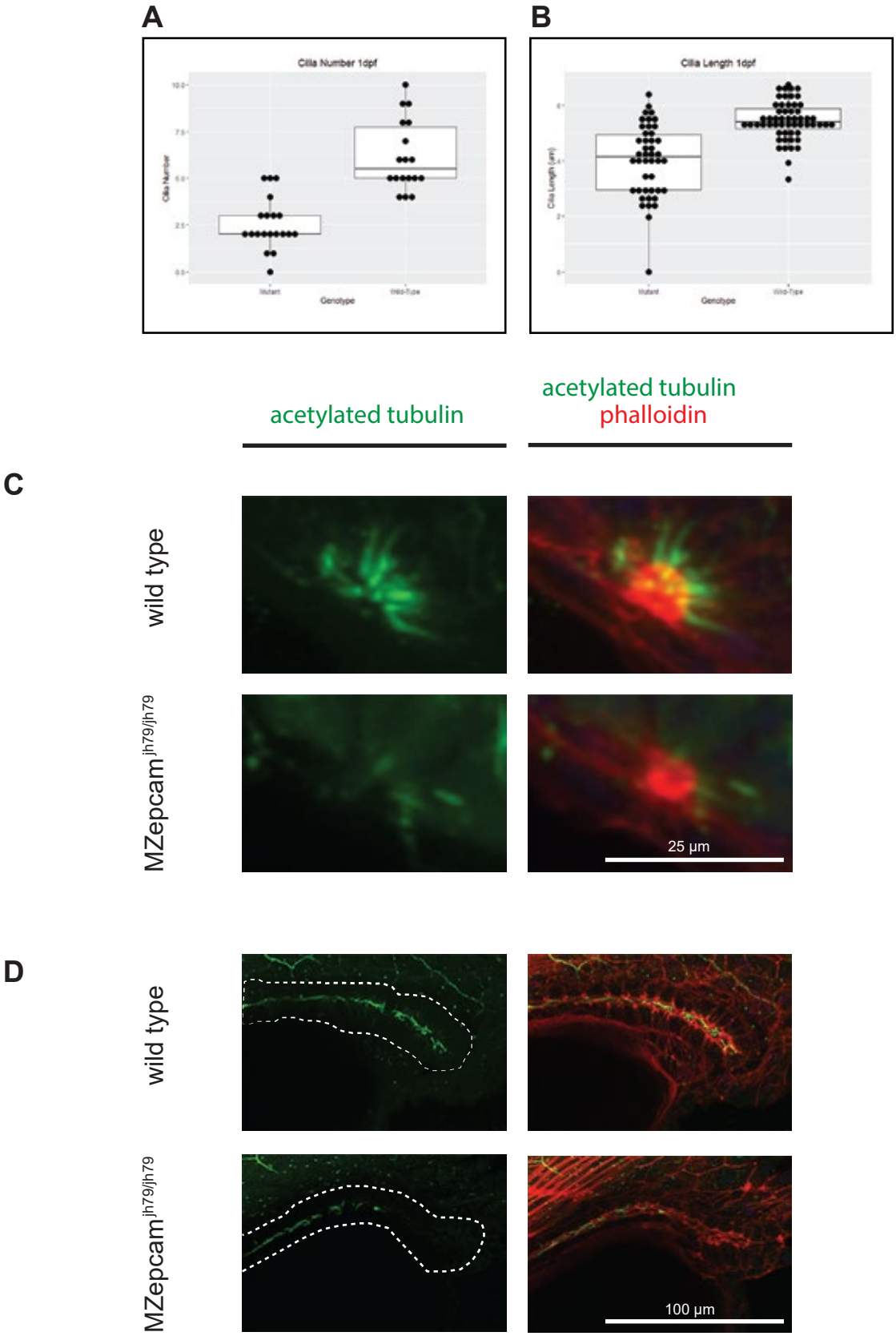

A
B
Cilia Number 1dpf
Cilia Length 1dpf
Genotype
acetylated tubulin
acetylated tubulin
phalloidin
C
wild type
MZepcam jh79/jh79
25 µm
D
wild type
MZepcam jh79/jh79
100 µm

Figure 8.1. Cilia quantification in wildtype and MZ epcam mutants

Quantification of cilia number is shown (**A**) and cilia length (**B**) for both wildtype and MZ epcam mutant larvae observed. Immunofluorescent staining was performed to detect the expression of acetylated tubulin (green) and phalloidin (red) in the olfactory pits (**C**) and the pronephric duct (**D**) of wildtype and MZ epcam mutant larvae.

d) Conclusion

To better understand the role of SOX9 in PDAC, our lab used PANC-1s and two genomics methods to identify genes that are regulated by SOX9. As outlined in the introduction, the following two methods were performed: 1) a knockdown of SOX9 in PANC-1 cells to obtain a list of DE genes that depend on the expression levels of SOX9 and 2) ChIP-seq using an antibody against SOX9 to determine direct targets of SOX9. After doing these experiments, 93 DE genes were identified and grouped into downregulated and upregulated genes upon the knockdown of SOX9.

When SOX9 was knocked down, 9 out of the 10 downregulated genes were found to be directly bound by SOX9 at their promoter sequence. This was seen from the ChIP-seq results. These genes were predicted to be positively regulated by SOX9 but were also found to be expressed in *sox9b*-expressing pancreatic ductal cells in the zebrafish. One gene identified was *EpCAM*, which is also expressed in CACs. As mentioned, the relationship between SOX9 and EpCAM was opposite than what was found in our PANC-1 experiments compared to the relationship found between *sox9b* and *epcam* in the CACs of the zebrafish pancreas. It is known that SOX9 and corresponding orthologues regulate the transcription of downstream targets by binding to cis-regulatory elements within the genome. However, it is the presence of cell-specific cofactors that determine whether Sox9 will act as a transcription activator or repressor (Geraldo, Valente, Nakajima, & Martins, 2016; Kamachi & Kondoh, 2013; Leung et al., 2011). I suspect that there are numerous reasons why there could be a difference in the relationship between SOX9 and *EPCAM* between these two model systems used. From both studies done in PANC-1 and zebrafish CACs, our results are consistent with the fact that there may be different species-specific cofactors that are used or different cofactors that are used in healthy versus cancerous cells. Regardless of our observations,

these differences seen in the ability of SOX9 to behave as either an activator or repressor will need to be further investigated to draw upon these conclusions.

EpCAM is best known as an epithelial cell adhesion molecule, an approximately 40kDA transmembrane glycoprotein. Like SOX9, EpCAM is also often found as a biomarker for cancer, including pancreatic cancer (Went et al., 2006). Having a high expression of EpCAM in cancer is often associated with a poor prognosis and survival rate (Patriarca, Macchi, Marschner, & Mellstedt, 2012; van der Gun et al., 2010). Although EpCAM has been implicated in cancer, its exact role still remains largely unknown (Schnell et al., 2013). It was originally thought that EpCAM had the sole role of being a cell adhesion molecule, however, its role may be more complex than what was originally thought. For instance, it has been shown that EpCAM may function as an activator of transcription. It was found that EpCAM can become cleaved at both the extra-cellular and intra-cellular domain (Maghzal, Vogt, Reintsch, Fraser, & Fagotto, 2010). When the intracellular domain becomes cleaved, EpCAM can enter the nucleus and interact with the transcription factor called LEF1. This interaction controls the transcription of other downstream target genes related to this pathway (Maetzel et al., 2009). These findings suggest that EpCAM may have a greater role in the cell than functioning only as a cell adhesion molecule, however. many of these roles have yet to be fully understood.

Given our results, we hypothesized that there is a direct link between KRAS activity, SOX9 activation, EpCAM, and cilia. In our study, we provide the first evidence showing that the loss of EpCAM leads to a defect in cilia. This is seen in zebrafish MZ epcam mutants where every otic vesicle looked at displayed a significant delay in otolith production. This observation is consistent with there being a defect in cilia – both in cilia number and in cilia length at 1dpf. Future work is still needed to determine whether regulating cilia is a general role of EpCAM or if this role is

specific to the otic vesicle. Since our primary interest is to understand the EpCAM pathway in the pancreas, further understanding is still needed to determine whether EpCAM regulates cilia in the pancreas. Loss of cilia has been implicated as a hallmark of carcinogenesis (H. Liu et al., 2018). It has been shown that PanIN cells that overexpressed of KRAS in transgenic mice had a lack of primary cilia (Seeley et al., 2009). Although the connection between EpCAM and cilia in the pancreas was not found here, our findings still provide a potential cancer-driven pathway that requires further investigation. For the first time, our lab was able to make a direct link between the SOX9 and EpCAM pathway which has never been done before. We also suggest that zebrafish CACs can serve as a useful *in vivo* model to further study the SOX9 pathway and other potential pathways implicated in pancreatic cancer.

Chapter 2: A new model of inducible chronic hyperglycemia

This chapter is published in Disease Models and Mechanisms

a) Introduction

Both T1D and T2D are characterized by chronic hyperglycemia. Prolonged hyperglycemia in diabetic patients leads to damage to various tissues including the eyes, kidneys, blood vessels and nerves (Pourghasem, Shafi, & Babazadeh, 2015; Shukla & Tripathy, 2022; R. Singh, Kishore, & Kaur, 2014). Various animal models have been used to model hyperglycemia which can reflect the pathophysiological changes associated (Acharjee, Ghosh, Al-Dhubiab, & Nair, 2013; Bakker & de la Garza, 2022; Kottaisamy, Raj, Prasanth Kumar, & Sankaran, 2021; Wolf, Braun-Reichhart, Streckel, & Renner, 2014; Zettler et al., 2020). The most used animal models to study diabetes includes rats, mice, and pigs. Each of these models have their own advantages and disadvantages, but no single animal model can fully represent the clinical symptoms that reflect human diabetes. In this chapter, I will describe an improved transgenic zebrafish animal model I developed that can be used to better study CACs as a progenitor during beta-cell regeneration and the downstream complications associated with diabetes due to chronic hyperglycemia.

One of the most widely used rodent models to study diabetes is the non-obese diabetic (NOD) mouse which has a genetic predisposition for autoimmune diabetes that mimics features of human T1D (D. Chen, Thayer, Wen, & Wong, 2020). Injection of streptozotocin (STZ) in rodents is an often-used method to ablate beta cells and model diabetes (Furman, 2015). STZ is an alkylating agent that enters cells through the Glut2 receptor and causes DNA damage that leads to cell death. Since beta cells highly express Glut2, this makes them more susceptible to STZ treatment. Models of T2D include the Zucker diabetic fatty rat which develops both obesity and insulin resistance at a young age (Shiota & Printz, 2012). As these animals age they progressively become more and

more hyperglycemic as a result of impaired beta-cell function, loss of beta-cell mass, and decreased responsiveness of tissue to insulin and glucose. Another animal model of T2D is the $Lep^{ob/ob}$ mouse model which has a deficiency in leptin (Fang, Lin, Huang, & Chuang, 2019). Leptin leads to satiety which causes these animals to become hyperphagic and thus leads to obesity from overeating.

Other larger animal models used to study diabetes include dogs, pigs, and monkeys (Grussner et al., 1993; Ionut et al., 2010). T1D can be induced in canine models by either a partial pancreatectomy or the injection of STZ (Seita et al., 2013). Both canine models have been successful in studying insulin resistance and defects within beta-cell mass. The pig is another well suited animal model to study diabetes because the development and morphology of the pancreas is similar to the human pancreas. Pigs have been genetically engineered to study diabetes including the use of transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor (GIPR) and the expression of a ubiquitous dominant-negative human hepatocyte nuclear factor 1a (HNF1A) (Renner et al., 2010; Umeyama et al., 2009). To study T2D in the pig, high fat diets can be fed to these animals (Xi et al., 2004). Non-human primates (monkeys) have also been used as models for T1D and T2D (Harwood, Listrani, & Wagner, 2012). T1D induced models usually involve treatment with STZ, however, the progression of T1D is extensive and the percentage of animals displaying diabetes is small. T2D induced models in non-human primates rely on diet-induced obesity which leads to the progression of insulin resistance and prolonged hyperglycemia (Harwood et al., 2012).

Non-mammalian animal models for diabetes also exist including *c. elegans* (round worms), *drosophila* (fruit flies), and zebrafish. *c. elegans* and *drosophila* have been primarily used to study T2D because both can be easily fed a high fat diet. *c. elegans* function as a useful model to study glucose toxicity, which is the affect high glucose concentrations have on the animal. When fed a

high glucose diet, the life expectancy of *c. elegans* decreases due to an increase in reactive oxygen species (ROS) and advanced glycation end products (AGEs) (Schulz et al., 2007). ROS and AGEs are a result of hyperglycemic induced stress. Insulin resistant *drosophila* can be generated by feeding them a high-sugar diet which causes them to become hyperglycemic (Musselman et al., 2011).

More recently, zebrafish have emerged as a model to study diabetes and have become the animal model used in my thesis to study beta-cell regeneration. Unlike the other animal models mentioned above, zebrafish are highly regenerative. They can regenerate their heart, retina, spinal cord, brain, liver, and pancreas (Gemberling, Bailey, Hyde, & Poss, 2013). Since zebrafish can regenerate the pancreas so well, this makes them an excellent model to also study beta-cell neogenesis (Kimmel & Meyer, 2016; Kinkel & Prince, 2009; Prince et al., 2017; Tarifeno-Saldivia et al., 2017). There are various zebrafish models that are used to study the pathologies associated with diabetes, namely hyperglycemia, which I have outlined below:

i. Glucose submersion method

The easiest way to induce hyperglycemia in zebrafish is submersing fish in glucose. This method requires fish to be immersed in a solution of glucose treated with an appropriate glucose concentration to induce hyperglycemia. For fish, 74 ± 8.5mg/dL is considered the normal fasting blood glucose range and anything three times the normal value (>200mg/dL) is considered hyperglycemic. Adult zebrafish can be submerged in 0 to 2% glucose solutions every other day for 28-30 days or can be chronically exposed to 2% glucose solutions for 14 days to induce diabetic phenotypes. The caveat to this method is that adult fish must be immersed for a long period (1-2 months) to achieve a hyperglycemic state and the glucose concentrations required vary based on

the age of the fish (Connaughton, Baker, Fonde, Gerardi, & Slack, 2016). Additionally, there is a 20-30% mortality rate since high glucose concentrations are toxic to the fish (Capiotti et al., 2014).

ii. Diet method

To study T2D in zebrafish, adult fish are fed a high fat diet which leads to hyperglycemia, hyperinsulinemia, and insulin resistance. The first diet-induced zebrafish model of T2D was established by overfeeding adult fish freshly hatched *Artemia* which are high in fat content (Oka et al., 2010). In a second study conducted by the same research group, fish were overfed commercially available fish food and fasting blood glucose levels were monitored (Zang, Shimada, & Nishimura, 2017). Fasting blood glucose levels were significantly increased in fish with diet-induced obesity compared to fish fed a normal amount of food. An intraperitoneal and oral glucose tolerance test further revealed impaired glucose tolerance caused by overfeeding. The production of insulin was determined by measuring green fluorescent protein (*GFP)* signal strength in overfed fish whose beta cells transgenically expressed *GFP*. Insulin expression increased in overfed fish compared to controls.

iii. Surgical method

In 1689, Johann Conrad Brunner removed the pancreas from a dog and was the first person to describe symptoms of polyphagia, polyuria, and polydipsia. In 1889, the same pancreatectomy was performed which linked the pancreas for the first time to diabetes (Keck & Duntas, 2007). A partial pancreatectomy can be performed in an adult zebrafish by removing a single lobe of the pancreas, which causes the fish to become hyperglycemic and diabetic (Delaspre et al., 2015; Moss et al., 2009). One of the main issues with this model of diabetes is that it is highly invasive and

damages other surrounding tissue outside the beta-cell compartment. Thus, this procedure is traumatic to the fish and can result in low survival rates. Additionally, this procedure does not truly mimic a chronic hyperglycemic state because a partial pancreatectomy does not remove all beta cells. Most beta cells also regenerate from pre-existing beta cells rather than other surrounding cells of the pancreas. Therefore, a partial pancreatectomy model may not be the best model to study beta-cell neogenesis.

iv. Drug induced method

Like in mammalian models, STZ is also used in zebrafish to chemically induce diabetes. The intraperitoneal injection of STZ into adult fish induces beta-cell apoptosis which leads to an increase in blood glucose levels and a reduction in insulin levels (Olsen, Sarras, & Intine, 2010; Olsen, Sarras, Leontovich, & Intine, 2012; Sarras, Leontovich, & Intine, 2015). These zebrafish develop diabetic complications including nephropathy and caudal fin aplasia. Although the induction of hyperglycemia was successful, the toxic side-effects of STZ (Goyal et al., 2016; Moss et al., 2009) complicate interpretation of acute ablation and preclude its use in chronic studies. For instance, to maintain zebrafish in a chronic hyperglycemic state over long periods of time, multiple intraperitoneal injections of STZ are required which puts increased stress on the fish.

v. Genetic methods

There are zebrafish genetic mutants that provide models for MODY including a *pdx1* and *hnf1ba* mutation. MODY4 in humans is caused by a haploinsufficiency in PDX1. Zebrafish that lack pdx1 (*pdx1*$^{-/-}$ mutants) have reduced numbers of beta cells as larvae and the few fish that survive to adulthood have a smaller body size, elevated blood glucose levels (Kimmel et al., 2015),

and retinopathy (Wiggenhauser et al., 2020). The caveats to this model include the high mortality rates and the persistence of some beta cells (and insulin) through development, which complicates parsing of phenotypes caused by chronic diabetes from developmental defects. MODY 5 is another form of MODY found in humans but is caused by a defect in HNF1b. Zebrafish that *are hnf1ba*$^{-/-}$ mutants have a decrease in embryonic beta-cell number (Lancman et al., 2013). It was also shown for the first time that there may be a direct link between *hnf1b* and the WNT signaling pathway. Unlike *pdx1*$^{-/-}$ mutants, *hnf1ba*$^{-/-}$ mutants survive to adulthood and do not appear to have an abnormal developmental phenotype.

vi. Transgene methods

Transgenesis can provide temporal control – allowing the induction of both acute and chronic hyperglycemia after development. Several different transgenic approaches in the zebrafish have been used to ablate beta cells, namely, a cre-enabled tetracycline-inducible transgenic system for the specific expression of a cytokine in the beta cells, diphtheria toxin gene A chain (DTA)-mediated targeted ablation in beta cells, and the ganciclovir-HSVTK system to selectively ablate proliferating beta cells (Ibrahim et al., 2020; Z. Li, Korzh, & Gong, 2009; Moro, Gnugge, Braghetta, Bortolussi, & Argenton, 2009). The most widely used transgenic method is the nitroreductase/metronidazole (NTR/MTZ) method (Carril Pardo et al., 2022; Curado et al., 2007; Ghaye et al., 2015; Pisharath, Rhee, Swanson, Leach, & Parsons, 2007; S. P. Singh et al., 2022; L. Ye et al., 2015; J. Yu et al., 2023). The NTR/MTZ method in zebrafish was first developed by the Parsons Lab to specifically ablate beta cells.

Specific tissue and cell expression of NTR enzymes converts MTZ into a cell-restricted cytotoxin, enabling inducible, targeted cell ablation. The original NTR was cloned from the *E. coli*

nfsB gene and is referred to here as NTR1.0. The insulin promoter was used to express both the NTR1.0 enzyme and a fluorescent marker in beta cells to create a line capable of facilitating the destruction of beta cells and a loss of insulin production. Hyperglycemia was induced in adult transgenic fish by the injection of single dose (30 mmol/L) of MTZ dissolved in PBS at a dose of 0.25g/kg body weight. Beta cells regenerate to normal blood glucose levels after two weeks (Delaspre et al., 2015).

Although the NTR1.0/MTZ system has worked well in studying beta-cell regeneration in adult fish, it is not the most suitable method to induce chronic hyperglycemia (modeling diabetes). This is because keeping fish in a high dose of MTZ is detrimental to the health of the fish. In this chapter, I show that treatments with acute high concentrations of MTZ (10 mM) lead to lethality and inflammation-associated aberrant gut morphology in surviving adults and larval zebrafish.

Over the last 15 years, many colleagues have informed our lab of their interest in studying the pathologies associated with chronic beta-cell loss – both during development and in the adult setting. Within the zebrafish community there is a lack of zebrafish models that can be used to study diabetes pathologies due to the limitations of current zebrafish models I outlined above. Unfortunately, the NTR1.0 enzyme has low activity and requires a high concentration of MTZ to ablate beta cells. Consequentially, this limits our ability and others to use NTR1.0/MTZ to maintain a chronic beta-cell loss and associated chronic hyperglycemia. Thus, I saw this as an opportunity to improve the NTR/MTZ system by developing an improved NTR/MTZ model to study chronic hyperglycemia.

Recently, Sharrock et. al. (2022) engineered an alternative NTR called NTR2.0, which was derived from the *Vibrio vulnificus nfsB* gene (*nfsB_Vv F70A/F108Y*). NTR2.0 has a higher MTZ conversion activity and therefore enables effective cell ablation using ~100-fold lower doses of

MTZ. Like NTR1.0, we also used the insulin promoter (Pisharath et al., 2007) to drive the expression of NTR2.0 in transgenic zebrafish. I have demonstrated that complete beta-cell ablation can be achieved when using dramatically lower doses of MTZ. These lower doses of MTZ are also better tolerated by fish of all ages. The higher ablation efficacy of NTR2.0 allows us to adopt immersion as the method of MTZ delivery in both larvae and adult fish. We can also use this method to carry out chronic hyperglycemia trials that were not possible with NTR1.0. In this chapter, I report on protocols that can be used to keep larvae hyperglycemic for 10 days and adults for 16 days. Furthermore, I have shown that chronic hyperglycemia in adults leads to severe weight loss, which is consistent with the pathology of diabetes. As stated previously, the work shown here in **Chapter 2** is published to Disease Models & Mechanisms (Tucker et al., 2023).

b) Materials and Methods

Zebrafish lines

Several zebrafish lines were maintained and created for **Chapter 2**. *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} transgenic fish constructs were made and injected into one cell staged fish embryos by Dr. Mike Parsons. Dr. Parsons made the NTR2.0 constructs using the transgenes: *ins:mCherry-P2A-nfsB_Vv-F70A/F108Y* and *ins:YFP-P2A-nfsB_Vv-F70A/F108Y* . A *YFP-P2A-nfsB_Vv-F70A/F108Y* cassette from the construct *5xUAS:GAP-tagYFP-P2A-nfsB_Vv-F70A/F108Y* (Sharrock et al., 2022), was cloned downstream of the insulin promoter in a tol2 transposable element (Pisharath et al., 2007). The YFP gene was removed and swapped for mCherry to create *ins:mCherry-P2A-nfsB_Vv-F70A/F108Y* . For the *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} transgenic fish, I raised multiple F0s that I then outcrossed to generate F1 founders. The F1 adult fish I generated were then screened by outcrossing. I observed the resulting progeny of these fish to identify F1 fish transmitting the transgene at 50%. This was done to find a fish with a single copy of the transgene.

The *8xNFκB:GFP*^{ir2019} transgenic fish used in **Chapter 2** were also made by Dr. Parsons. The eight NFκB binding elements and the CMV minimal promoter (from the plasmid pSGNluc NFκB reporter (Kuri, Ellwanger, Kufer, Leptin, & Bajoghli, 2017)), were cloned upstream of GFP in a tol2 transposable element to create *8xNFκB:GFP*. Dr. Parsons then injected the construct into one cell staged embryos to generate *8xNFκB:GFP*^{ir2019} fish. Dr. Parsons screened and raised these fish to F1s. When Dr. Parsons made the DNA constructs, all of the constructs mentioned above were mixed with Tol2 transposase mRNA and phenol red (as per (Kawakami, 2004)). In **Chapter 2**, I also used casper fish which lacked pigment in the body (White et al., 2008). These fish have

homozygous mutations for, *mitf1a* (Lister, Robertson, Lepage, Johnson, & Raible, 1999) and *mpv17* (D'Agati et al., 2017). Using casper fish allowed me to visualize adult internal organs easier.

Drug dependent cell ablation

While carrying out acute and chronic ablation experiments in both larvae and adult fish, water/MTZ solutions were changed completely each day. All MTZ (AC210341000 Thermo Scientific) solutions were made fresh. Making fresh solutions was critical for optimal function of the MTZ drug treatment. I noticed that when I used different commercial sources of MTZ, there was considerable batch variability in terms of ablation efficacy and toxicity. Larvae (3dpf – 5dpf, n = 25- 50) were maintained in MTZ dissolved in E3 (Westerfield, 1995) in petri dishes at 28 °C. Adult fish were kept individually in 200mL beakers containing system water and they were fed once per day (Gemma 500, Skrettering). For chronic beta-cell ablation studies in larvae, 3-5dpf larvae were treated the same as above. Once larvae reached 6dpf (n = 20), they were transferred to small beakers and like adult fish, they were fed once a day (Gemma micro 75, Skrettering). After fish were fed, a 100% water/MTZ treated water exchange was performed. For adult chronic beta-cell ablation studies, fish were kept individually in 1L tanks containing 250mL of water (control) or MTZ dissolved in system water. Again, adults fish were fed once per day with 0.77g of Gemma 500 food. Food was weighed out precisely each day to ensure that over or underfeeding did not affect fish weight at the end of the experiment. Fish were allowed to eat for 3 minutes before a complete (100%) MTZ/water exchange was done.

Hematoxylin and eosin staining of paraffin sections.

Each individual fish was given an ID#. I kept the corresponding treatment information associated with each fish in a separate excel document. When it was time to collect tissue samples, adult fish were euthanized on ice, a small incision was made along the midline of the fish before each fish was placed individually into a 15mL tube containing 10mL of 10% non-buffered formaldehyde. Fish were left overnight in formaldehyde at room temperature. I noticed that adult guts can be fixed indefinitely. For pancreatic tissue, I noticed that the pancreas can only be kept in fixation overnight. Exceeding overnight fixation for the pancreas caused pancreatic tissue to not stain as well. After fixation, I transferred the guts/pancreas into individual 1.5mL tubes containing 500uL of 70% ethanol for at least 1 hr. I then removed the tissue and placed the individual tissue samples into histology cassettes (62500 series Tissue Tek) and then proceeded to process the tissue in a tissue processor (Leica TP1020) overnight. The next day, the samples were removed from the tissue processor and left to cool for a couple hours before embedding each sample individually into a paraffin block (Sabaliauskas et al., 2006). Once embedded, 5 uM sagittal sections were cut using a microtome and then stained with H & E. I looked at the gut histology of 5 fish from each group (control and treatment groups). This added up to 25 histological slides per one fish. Each histological slide contained 4-5 sections of sectioned gut/pancreas tissue. When I looked at the H&E staining of the gut, I scored each fish blindly for gut abnormalities. The corresponding ID# for each fish was then referenced back to my excel document containing information about the treatment each fish received. I used this method to draw upon conclusions for each group regarding gut health.

For larval fish, I cut off the head of the fish after fixing them overnight in 10% non-buffered formaldehyde at 4°C. The rest of the body was then transferred into a tube containing 500uL of

70% ethanol. Larval samples were sent to UCI's histology core facility where they were sectioned and stained by H&E. When I got the slides back from the core facility, I then examined the gut histology of 5 fish for each group. Around 3-4 slides were obtained for a single fish. Each slide contained 3-4 sections of gut tissue. All of these slides were stained by H&E and were scored by me to determine the effect MTZ has on the gut of larval zebrafish.

Immunofluorescence

Immunofluorescence staining was performed on adult sections of guts containing pancreas tissue (Parsons et al., 2009). When performing deparaffinization and rehydration steps, all microscope slides were placed in a slide rack that was fully submerged in each solution. I used histoclear (national diagnostics) as a substitute for xylene since xylene is extremely toxic. I performed antigen retrieval on paraffin sections using a citrate standard procedure outlined by Cell Signaling Technology (see supplier's protocol online). While in a slide rack, I submerged the slides in a histology container containing room temperature 1x antigen retrieval buffer (100x Citrate Buffer ab93678) for 10 minutes. Still in the slide rack, I then transferred the slides to a second histology container containing pre-warmed 1x antigen retrieval buffer. I incubated these slides in this buffer for 10 minutes. Without removing the samples, I then placed the histology container containing the pre-warmed antigen retrieval buffer on ice for 30 minutes. Once cooled, I removed the slides from their slide rack and dried them carefully with a Kimwipe^{TM}. I then placed the slides into an incubation chamber that contained DI water. I used a pap pen to carefully draw a circle around each individual tissue on each of the slides. Next, the slides were incubated in 15-20uL of permeabilization buffer containing 0.5% Triton and 1X PBS for 20 minutes. This was done to increase the penetration efficiency of the primary antibody. I next made fresh block buffer solution

which contained 10% goat serum, 0.2% Triton, and 1x PBS. 15-20uL of block buffer was added to each tissue section. I allowed the slides to incubate in this buffer for 1 hr at room temperature.

For all primary antibodies used, I diluted them in freshly made block buffer as follows: 1:200 rabbit anti-insulin (Genetex), 1:200 mouse anti-glucagon (Sigma), and 1:200 rat anti-mCherry (Invitrogen). Primary antibody solutions were incubated overnight at 4 °C. The next day, I removed and kept the primary antibody and then proceeded to wash the slides completely with a wash buffer which contained 0.2% triton and 1X PBS. These washes were performed 3 times for 5 minutes each. I used a Kimwipe™ to carefully dry each slide. I then used the PAP pen again to draw a circle around each tissue sample on the slide. 10-15 µL of freshly made block /wash buffer containing 10% goat serum, 0.2% Triton and 1X PBS was added to each tissue section for a 5-minute wash and then pipetted off. Each tissue sample was incubated for 2 hrs in block/wash buffer which contained 1:2000 DAPI, 1:250 anti-rabbit Alexa 488 (Abcam), 1:250 anti-mouse Alexa 594 (Abcam), and 1:250 anti-rat Alexa 594 (Abcam) secondary antibodies. After, slides were washed completely with wash buffer, and then a quick 2-minute wash was performed using 1x PBS. Once staining was completed, I mounted each slide with a cover slip for confocal imaging.

Fasting Blood Glucose Assays

All adult fish were fasted for 24 hrs to normalize all blood glucose readings before the fish were euthanized on ice. The next day, all fish were decapitated, and a test strip was used to draw up blood using a Freestyle lite (Abbott) glucose meter. The maximum BG reading of the glucose meter I used is 500mg/dL. Since blood glucose readings cap out at 500mg/dL using this meter, it is important to note that hyperglycemia in adult zebrafish can be much higher than this blood glucose reading (Delaspre et al., 2015).

Image acquisition

All fluorescent images were acquired using a Leica SP8 confocal. Before imaging 5dpf embryos, they were anesthetized with tricaine and then mounted in 1% low melt agarose spread out onto a microscope slide. A cover slip was added on top of the fish and sealed with clear nail polish. I used an EVOS XL Core microscope to obtain all H&E images.

PCR analysis

To ensure that the NTR2.0 transgene entered the fish genome via Tol2 mediated transgenesis, Dr. Parsons performed a PCR analysis. This analysis was initially carried out to answer a comment made by the reviewers in order to address whether the transgene was at a single insertion in the genome. Dr. Parsons performed a standard touchdown PCR using PCRBIO Taq Mix Red (PCR Biosystems). The reaction was carried out at 95^{o}C for 4mins followed by 10 cycles of 95^{o}C for 30 secs, 69^{o}C for 30 secs, and 72^{o}C for 30 secs. Each progressive cycle's annealing temperature dropped by 1^{o}C. The cycles then followed 27 cycles of 95^{o}C for 30 secs, 59^{o}C for 30 secs, 72^{o}C for 30 secs, and then 72^{o}C for 4 minutes. The sequence of primers that were used in Figure 4 are the following:

F1 TGTTCCACACAGGTCAGAGG; R1 TTGAGTAGCGTGTACTGGCATT;

F2 CACCATCGTGGAACAGTACG; R2 CAACGTGAGAAGCATCCAAA;

F3 GGTTCTTGACCCCCTACCTT; R3 ATTAATGCAGCTGGCACGAC;

F4 CCGACCACTACCAGACCAAC; and R4 CACTGCTCGCGACAATAAAA.

Germ-free derivation

In a collaboration with the Travis Wiles Lab at UCI, Courtney Knitter (Dr. Wiles' Graduate student), derived "germ-free" (GF) *8xNFκB:eGFP* embryos as was previously described (Melancon et al., 2017) but with slight modifications. I helped collect fertilized eggs from adult fish pairs set up in our fish facility. After collecting, I provided Courtney with the embryos so that she could carry out the GF derivation procedure in the Wiles Lab. Courtney incubated the embryos in sterilized embryo media (EM) in the Wiles Lab. EM contained ampicillin (100 ug/ml), gentamicin (10 ug/ml), amphotericin B (250 ng/ml), tetracycline (1 ug/ml), and chloramphenicol (1 ug/ml) for approximately 5 hours. After a 5-hour incubation period, Courtney removed the embryos from this media and washed the embryos in EM containing 0.1% polyvinylpyrrolidone-iodine followed by EM containing 0.003% sodium hypochlorite. Courtney distributed these surface-sterilized embryos into sterile T25 and T75 tissue culture flasks at a density of one embryo/ml. Courtney then proceeded to making "Conventionalized" (CVZ) embryos by inoculating the flasks containing GF embryos with undefined microbial communities derived from parental zebrafish feces. This was done immediately following GF embryo derivation. While the embryos were kept in the Wiles Lab, they were incubated at 28.5°C with a 14 hours/10 hours light/dark cycle. Embryos were not fed during this experiment.

I provided Courtney each day with freshly made 10mM MTZ dissolved in EM. Courtney then proceeded to sterilize the media containing MTZ using a vacuum-driven filter (Genclone Cat #: 25-233). Sterile technique was used to replace the original EM in flasks with EM containing MTZ. MTZ-containing EM exchanges were performed on 3 and 4 dpf larvae followed by Courtney assessing the GF status of the larvae using an inverted microscope in the Wiles Lab. Courtney checked GF status of larvae by inspecting the flask water for microbial contaminants. Once the

larval fish reached 5dpf, I picked up the larvae from the Wiles Lab. I then carried out the confocal imaging on all fish groups to inspect the activation of the corresponding transgene.

Statistical analysis

To perform statistical analysis of blood glucose and body weight results, I used a student's t-test in GraphPad (Prism). I defined significance as having a p-value <0.05. I calculated standard deviation (SD) for both blood glucose and body weight changes in order to determine the variation of the data in regard to the mean.

c) Results

NTR2.0 requires less MTZ to ablate beta cells.

To improve beta-cell ablation and facilitate our ability to study beta-cell regeneration in zebrafish, Dr. Mike Parsons cloned the insulin promoter upstream of a cassette (*GAP-tagYFP-P2A-NTR2.0*). This was done to express both membrane tagged YFP (GAP-tagYFP) and NTR2.0 (Hall, Astin, Mumm, & Ackerley, 2022; Sharrock et al., 2022) (**Figure 1.2**). Using confocal microscopy, I observed the expression of the initial membrane tagged transgene. This is when I noticed that there were fewer beta cells labeled than expected. These cells also displayed evidence of cell stress, which was seen by blebbing that occurred in these cells (**Figure 1.2B-C**). From these observations, I suspected that this cell stress might be a result of the membrane tagged reporter we initially used to make our new NTR2.0 transgenic lines (W. Y. Huang, Aramburu, Douglas, & Izumo, 2000; H. S. Liu, Jan, Chou, Chen, & Ke, 1999). With Dr. Parsons' additional help, he created two new transgenic lines that did not have the membrane tag in the construct. These two new lines were named: Tg(*ins:mCherry-P2A-nfsB_Vv F70A/F108Y)*^{ir2018} (abbreviated to *ins:mCherry NTR2.0*^{ir2018}) (**Figure 2.2A**) and Tg(*ins:YFP-P2A-nfsB_Vv F70A/F108Y)*^{ir2050} (abbreviated to *ins:YFP NTR2.0*^{ir2050}) (**Figure 3.2A**). Dr. Parsons microinjected these constructs into wildtype fish embryos at the 1 cell stage. I then proceeded to screen these injected F0s for expressing NTR2.0 (both the mCherry and YFP versions of the transgene). Numerous F0s that expressed the transgene were then maintained as independent lines which became different allele numbers. Once these F0 lines became adult fish, I further outcrossed these fish to wildtype fish and then screened the resulting F1 offspring to ensure that the transgene went germline for each line of fish I maintained. I carefully screened each line of fish to ensure that I had the correct expression of the transgene being maintained. Many of the lines I maintained had multiple copies

of NTR2.0 (again, both for the mCherry and YFP versions). The initial expression being transmitted to the offspring was 90%. To get the transgene down to a single copy, I outcrossed adult fish for numerous generations. After I outcrossed the lines for multiple generations, transgenic fish from both lines were found to transmit their transgenes to 50% of their progeny. This indicated to me that both transgenes inserted at a single locus in the fish genome. To ensure that the transgene entered the genome via Tol2-mediated transgenesis, Dr. Parsons performed a PCR analysis (**Figure 4.2, results generated by Dr. Parsons**). The significance of using transgenic lines that only have a single site of insertion is that all related hemizygous transgenic fish will contain the same number of insertions. Ensuring identical number of insertions reduces experimental variation and facilitates maintenance of a functional line of transgenic fish. Additionally, having a single copy of the transgene is important because it ensures that we have a more active NTR, and it is not the multiple copies of NTR2.0 that make it more active than our original NTR1.0 line. I then sought to functionally analyze my two new NTR2.0 lines for their efficiency in ablating beta cells and then later, beta-cell labeling.

To test that my new NTR2.0 enzyme can ablate beta cells using a lower dose of MTZ, I tested a range of MTZ concentrations (0, 10 mM, 100 uM, and 10 uM) on larval fish expressing *ins:mCherry NTR2.0*^{ir2018} (3 to 5 days post fertilized (dpf)). I compared ablation results to controls that were treated in a similar manner. These controls include larvae lacking NTR (*ins:dsRed*^{m1018}) (**Figure 5.2A**) and larvae from our lab's old NTR1.0 models, namely: *ins:NTR1.0-mCherry*^{jh4} (Pisharath et al., 2007) and *ins:PhiYFP-nfsB;sst2:TagRFP*^{lmc01} (abbreviated to *ins:YFP NTR1.0*^{lmc01}) *(Delaspre et al., 2015; Walker et al., 2012)* (**Figure 2.2**). As a control, I also incubated *ins:dsRed*^{m1018} larvae (n =30) in 10 mM MTZ for 48 hrs. This was done to show that without NTR expressed, there are no effects on beta-cell numbers. This verifies that beta-cell

ablation is dependent on both MTZ treatment and NTR expression (**Figure 5.2B**). As we have previously reported, fish treated with 10 mM MTZ could achieve a complete loss of beta cells in *ins:YFP NTR1.0*^{lmc01} (50/50) and *ins:NTR1.0-mCherry*^{jh4} (50/50) larvae (**Figure. 2.2H, 2.2I**). In this study, I found that lower doses of MTZ are required to ablate beta cells in *ins:mCherry NTR2.0*^{ir2018} larvae. I found that *ins:mCherry NTR2.0*^{ir2018} larvae treated with 100 uM MTZ achieve the complete ablation of all beta cells (50/50 larvae) (**Figure. 2.2J**). Lowering the MTZ dose to 10uM only led to 70% (35/50) of *ins:mCherry NTR2.0*^{ir2018} larvae having a complete loss of beta cells (**Figure. 2.2M**). *ins:YFP NTR2.0* ^{ir2050} larvae (n=30) were also treated with same MTZ concentrations (0, 10 mM, 100 uM, and 10 uM) and the results were identical (**Figure. 3.2C, 3.2D**). Based on these findings in both transgenic lines, I adopted 100 uM MTZ as the lowest working dose of MTZ required to ablate beta cells in all my NTR2.0 larval studies.

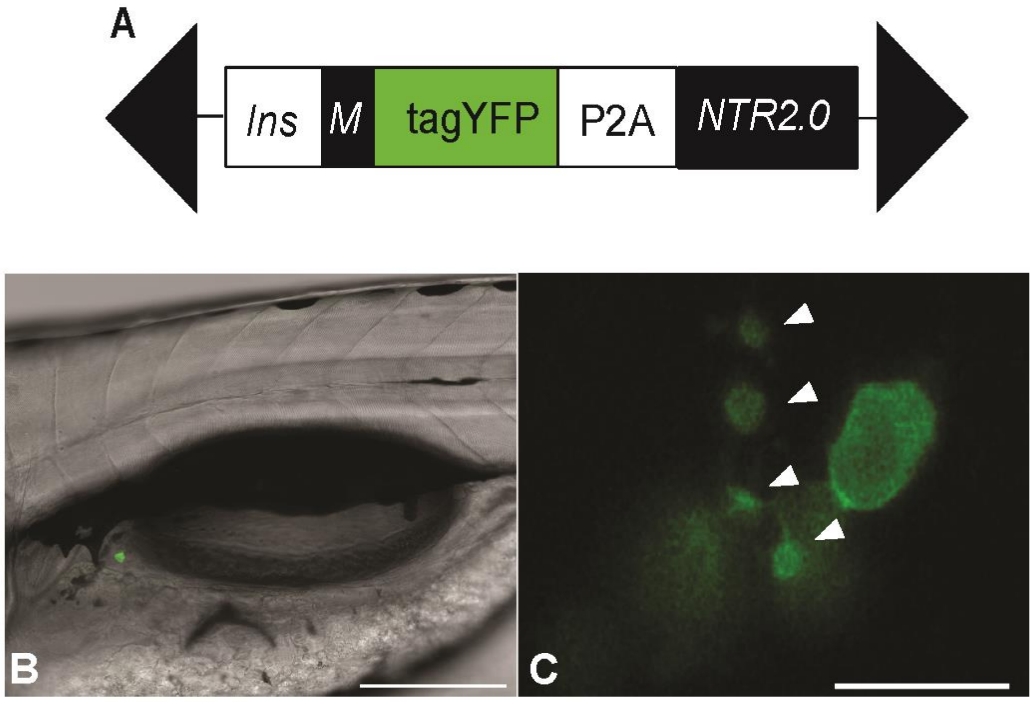

A
Ins
M
tagYFP
P2A
NTR2.0
B
C

Figure 1.2. Membrane expressing NTR2.0 YFP transgenic fish (**A**) The construct used to generate transmembrane NTR2.0 YFP transgenic fish was *ins:tagYFP-P2A-NTR2.0*. Tol2 arms are shown by black triangles, the insulin promoter (box- ins), the palmitoylation sequence from GAP43 (box- M) that confers membrane localization to the fluorescent protein (tag YFP) and the gene that encodes NTR2.0 (box-NTR2.0) (M) (**B**) A lower magnification image is shown of a 5dpf larvae via confocal microscopy. Only a single cell with fluorescence where the pancreas is located is shown (**C**). A higher magnification image of larvae via confocal microscopy shows the cells expressing transmembrane YFP and are blebbing (white arrows). This observation is consistent with cell stress. Here, I have concluded that high expression of the membrane tagged YFP is toxic to beta cells.

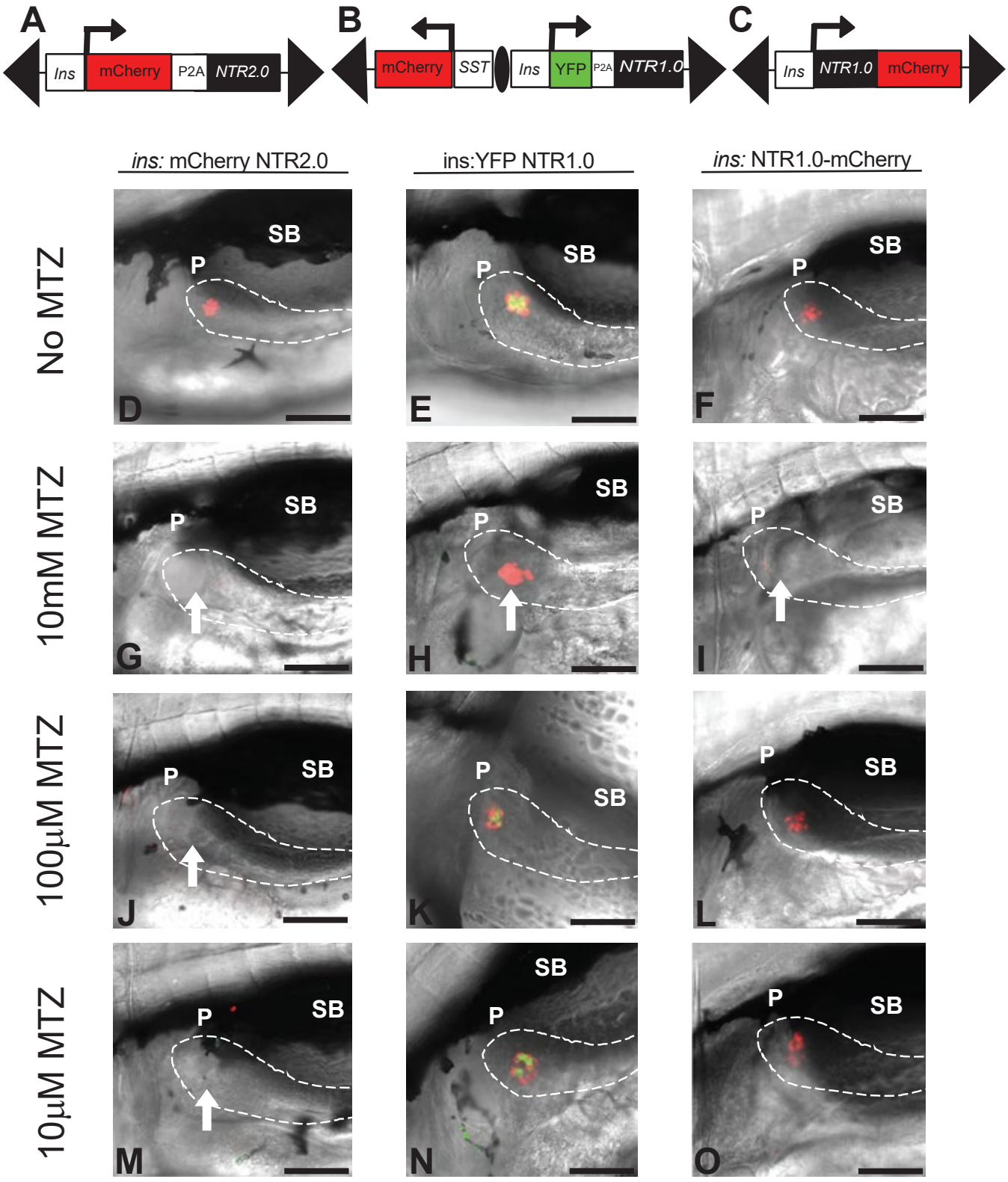

A
Ins
mCherry
P2A
NTR2.0
B
mCherry
SST
Ins
YFP
P2A
NTR1.0
C
Ins
NTR1.0
mCherry
ins: mCherry NTR2.0
ins:YFP NTR1.0
ins: NTR1.0-mCherry
No MTZ
10mM MTZ
100μM MTZ
10μM MTZ
SB
P
D
E
F
G
H
I
J
K
L
M
N
O

Figure 2.2 Use of NTR2.0 improves efficacy in ablating beta cells. A schematic representation of NTR2.0 is shown (**A**). The NTR1.0 based transgenes scematic is also shown (**B, C**). Positions of Tol2 arms (black triangles), HS4 insulator (black oval) and promoters (arrows) are indicated in the schematic. Larvae were incubated from 3 to 5dpf (48 hrs) in various concentrations of MTZ and were imaged after treatment (**D-O**). A white arrow points to the position of ablated beta cells. Results from *ins:mCherry NTR2.0* larvae (**D, G, J, M**) were compared to two older NTR1.0 lines: i*ns: YFP NTR1.0*^{lmc01} (**E, H, K, N**) and *Ins:NTR1.0-mCherry*^{jh4} (**F, I, L, O**) larvae. Larvae expressing NTR2.0 were able to achieve a complete loss of beta cells when they were treated with 100uM MTZ (**J**) compared to older NTR1.0 lines of fish. **SB:** Swim bladder, **P:** Pancreas – outlined in dashed white line. All images were taken at 40× (scale bar = 100uM) with anterior to the left.

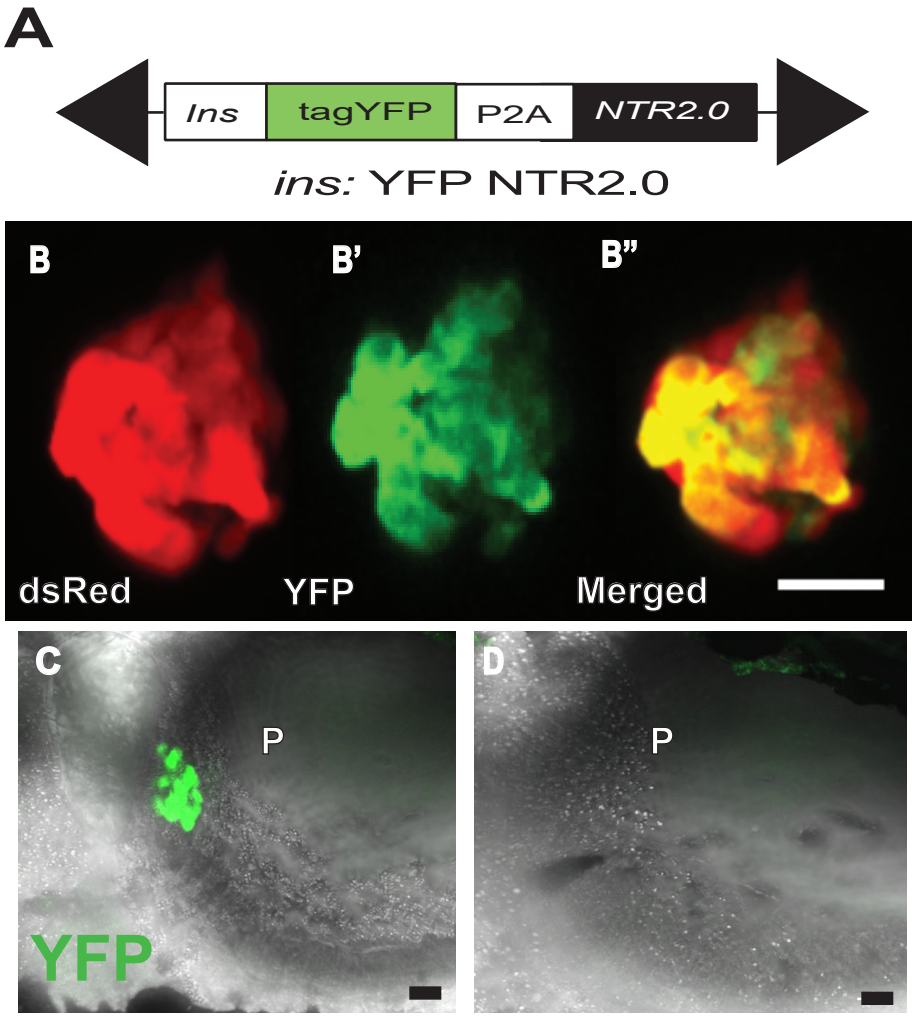

A
Ins
tagYFP
P2A
NTR2.0
ins: YFP NTR2.0
B
B'
B"
dsRed
YFP
Merged
C
P
YFP
D
P

Figure 3.2. *ins:YFP NTR2.0* transgenic line

(**A**) We created a new non membrane expressing YFP version of NTR2.0 to both label and ablate beta cells. Tol2 arms (black triangles), the insulin promoter (box - ins), and sequences encoding the yellow fluorescent protein (tagYFP) and the gene that encodes NTR2.0 (box-NTR2.0). Incorporation of the P2A sequence ensures that the expression of YFP and NTR2.0 produce separate proteins. Live imaging using confocal microscopy was performed on 5 dpf larvae (n=20) expressing a transgene known to mark the cytoplasm of all beta cells (*ins:dsRed*) and tagYFP (YFP) (**B-D**). (**B-B''**) Shows that tagYFP labels all beta cell in 5 dpf larvae pancreas. (**C**) Represents a control tagYFP in larvae not treated with MTZ. (**D**) larvae (n=20) treated with 100mM MTZ from 3dpf to 5dpf have a complete loss of beta cells. Images were taken via the confocal at 40× (scale bar = 20 uM). P marks the position of the head of the pancreas and where the islet should be located.

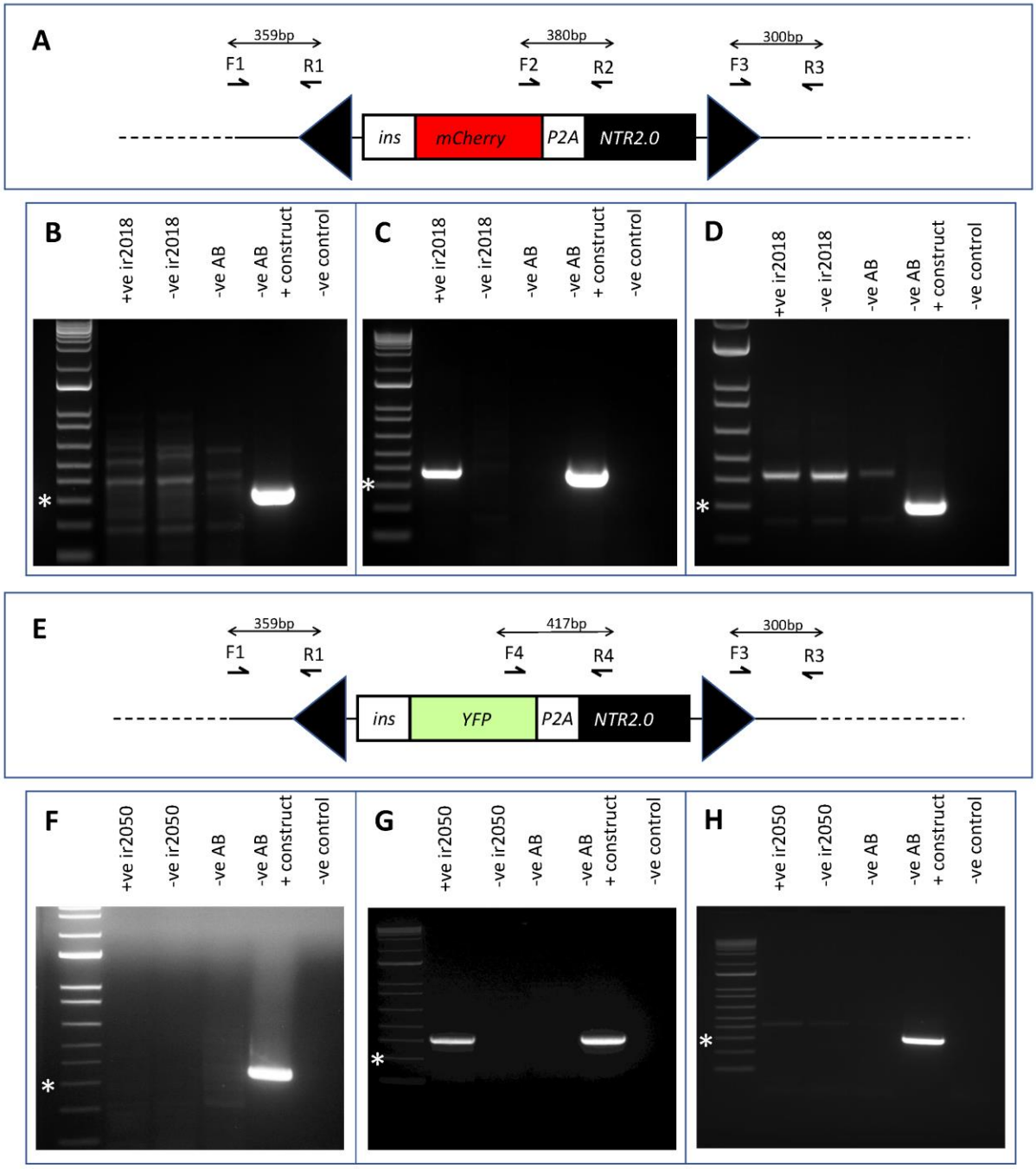

A
359bp
380bp
300bp
F1
R1
F2
R2
F3
R3
ins
mCherry
P2A
NTR2.0
B
C
D
+ve ir2018
-ve ir2018
-ve AB
-ve AB + construct
-ve control
E
359bp
417bp
300bp
F1
R1
F4
R4
F3
R3
ins
YFP
P2A
NTR2.0
F
G
H
+ve ir2050
-ve ir2050
-ve AB
-ve AB + construct
-ve control

Figure 4.2. Transgenes integrated via Tol2-mediated transgenesis.

(**A** and **E**) Schematics of the constructs used to generate the fish lines *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050}. Black triangles represent Tol2 arms. Position of insulin promoter is shown (white box, ins). Genes encoding mCherry (red), tagYFP (green), the P2A self-cleaving peptide (white, P2A) and NTR2.0 (black). The position of primers used in PCRs are also indicated (half arrows), as are the sizes of PCR products (double-headed arrows). Results of PCRs using primers: F1 and R1 in (**B)**, F2 and R2 in (**C**)**,** F3 and R3 in (**D**), F1 and R1 in (**F)**, F4 and R4 in (**C**)**,** F3 and R3 in (**H**). (**B-D**) Examination of genomic integration of *ins:mCherry NTR2.0*^{ir2018} 1st lane DNA template was extracted from a fluorescent positive *ins:mCherry NTR2.0*^{ir2018} larvae (+ve ir2018). 2nd lane from a fluorescent negative sibling (-ve ir2018). 3rd lane from a wildtype fish (-ve AB). 4th lane from same wildtype fish spiked with 80pg of construct shown in (**A**) (-ve AB + construct). 5th lane template was lysis buffer that went through whole DNA extraction protocol with other samples (-ve control). (**F-H**) Same set up but using DNA from *ins:YFP NTR2.0*^{ir2050} larvae and the construct shown in (**E**). DNA ladder is 1kb+ from Invitrogen and the 300bp band is marked by a ∗. Band above is 400 and the band below is 200.

PCRs using primers internal to the Tol2 arms only generate amplicons when fluorescent fish is the template or when construct DNA is added. PCRs using primers external to the Tol2 arms only generate amplicons when construct DNA is added. Together, this demonstrates that both *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} fish are transgenic curtesy of Tol2-mediated transposition. Non-specific amplification can be seen in several PCRs, but these products are weak, not the predicted size, occur in both wildtype and negative sibling DNA and, most importantly, are abolished by the addition of the construct (4th lanes).

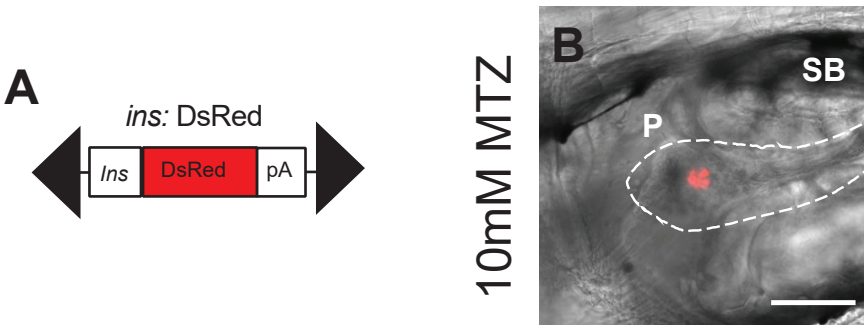

A
ins: DsRed
Ins
DsRed
pA
10mM MTZ
B
SB
P

Figure 5.2. Without NTR, MTZ does not lead to beta-cell death

(**A**) A schematic representation of the ins:DsRed transgene is shown. Tol2 arms are represented by black triangles. The position of the insulin promoter is shown by a white box and ins. The gene encoding dsRed is shown in red and is labeled. (**B**) Image showing a larva (5 dpf) expressing ins:DsRed after it was treated for 48hrs with 10 mM metronidazole (3-5 dpf). This image demonstrates that the prodrug does not kill beta cells when there is no NTR expression (n = 30). Image was taken via the confocal at 40× (scale bar = 100 uM).

Testing for transgene silencing

One issue when making a new transgenic line is encountering epigenetic silencing of the transgene. If silencing of the NTR transgene occurs, beta cells would escape MTZ-induced cell death. This could prevent fish from becoming hyperglycemic and would result in complicating the interpretation of beta-cell ablation experiments. From our transgene, NTR2.0 is co-expressed with either the mCherry or YFP fluorescent protein in order to report on and visualize the expression of NTR in beta cells. To ensure that NTR2.0 is expressed in all beta cells, I crossed by new NTR2.0 lines to well-characterized beta-cell reporter lines we have in our lab. I then used confocal microscopy to image the resulting double positive offspring to examine the co-localization of the two fluorescent proteins in all beta cells within the pancreatic islet. As can be seen, *ins:mCherry NTR2.0*^{ir2018}, *ins:hmgb1-GFP*^{jh10} (G. Wang et al., 2015) double transgenic larvae showed complete co-localization of mCherry (red) and nuclear GFP (green) in all beta cells looked at (**Figure 6.2A-B''**). This indicated to me that NTR2.0 is being correctly expressed in the beta cells of larval fish. Similarly, I also found that *ins:YFP NTR2.0*^{ir2050}, *ins:dsRed*^{m1018} (Shin et al., 2008) double transgenic larvae showed complete co-localization of YFP and dsRed (**Figure. 3.2B-B"**). To ensure that the transgene did not silence in adult fish, I performed immunofluorescent staining to confirm that NTR2.0 is correctly expressed in *ins:mCherry NTR2.0*^{ir2018} adults. An antibody against mCherry was used to indirectly detect the expression of NTR2.0 and an antibody against insulin was used to detect endogenous insulin produced (**Figure 6.2C-C''**). Here, I have shown that all insulin positive cells also contain the expression of mCherry. This indicated to me that the expression of NTR2.0 is being correctly expressed in the beta cells of adult NTR2.0 fish and that the transgene is not silencing into adulthood.

Figure 7.2 is an example of a different line of NTR2.0 fish where the beta cells were not labeled properly by the NTR2.0 transgene. As such, this line was not maintained and instead, the adult fish represented in **Figure 6.2** was selected for our new NTR2.0 line. This line of fish was used for all downstream experiments discussed below.

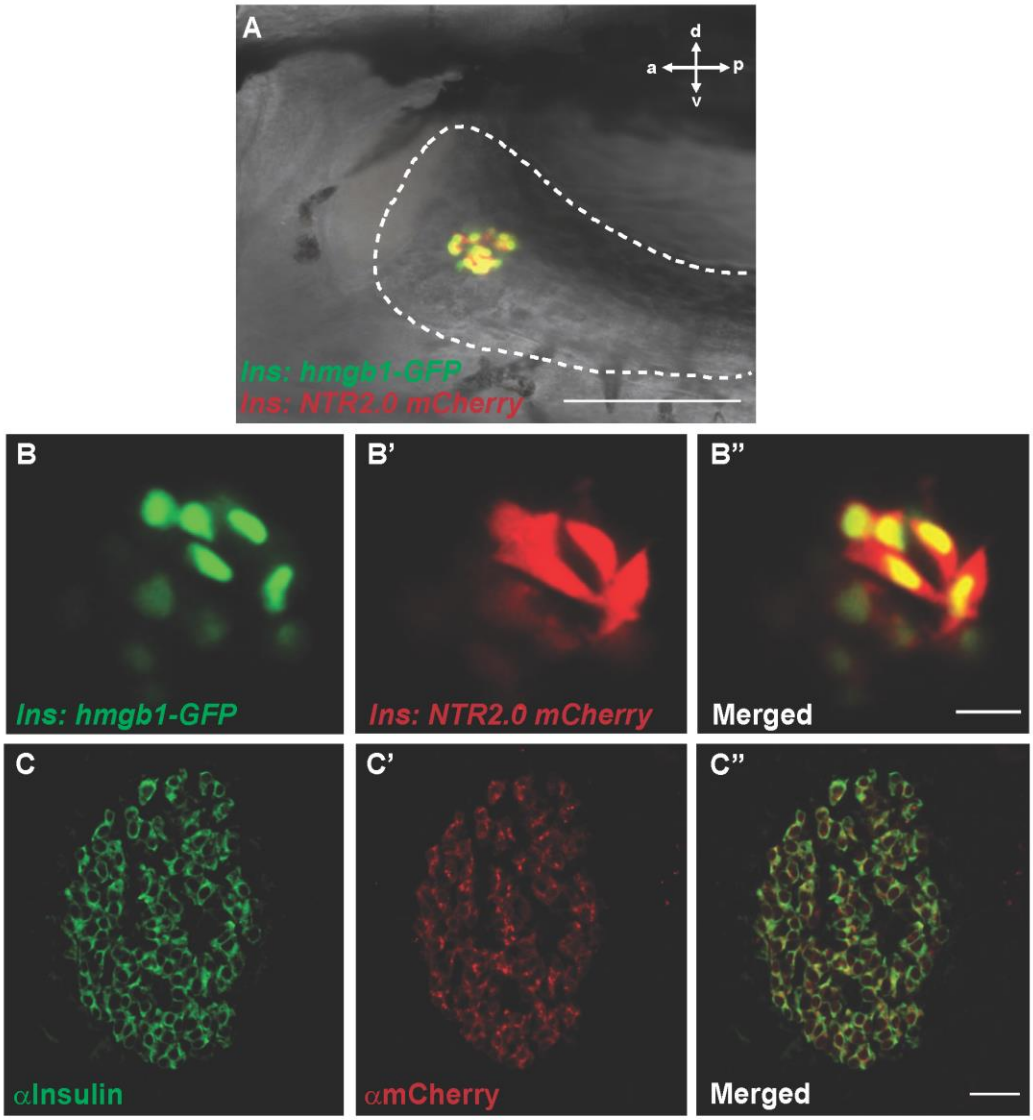

A
d
a
p
v
Ins: hmgb1-GFP
Ins: NTR2.0 mCherry
B
Ins: hmgb1-GFP
B'
Ins: NTR2.0 mCherry
B''
Merged
C
αInsulin
C'
αmCherry
C''
Merged

Figure 6.2. Testing for transgene silencing.

(**A-B”**) Live confocal images were taken of 5dpf larvae that carry two transgenes *ins:hmgb1-eGFP* (labels beta-cell nuclei green) and *ins:mCherry NTR2.0* (beta-cell expression of NTR2.0 and mCherry). (**A**) As can be seen, both transgenes are expressed in the head of the pancreas which marks islet cells (20×, scale bar = 100uM). Close up of green nuclei (**B**) and red cytoplasm (**B’**) and co-expression in beta cells (**B”**) Shows the correct beta-cell specific expression of NTR2.0 in the islet (40×, scale bar = 10 uM). Confocal images of sections from *ins:mCherry NTR2.0* fish with immunofluorescent detection of insulin (to detect beta cells, green in **C**) and mCherry (red - reports on NTR2.0 co-expression **C’**). As can be seen, there is complete co-localization throughout the islet (**C”**) (40x, scale bar = 10 uM).

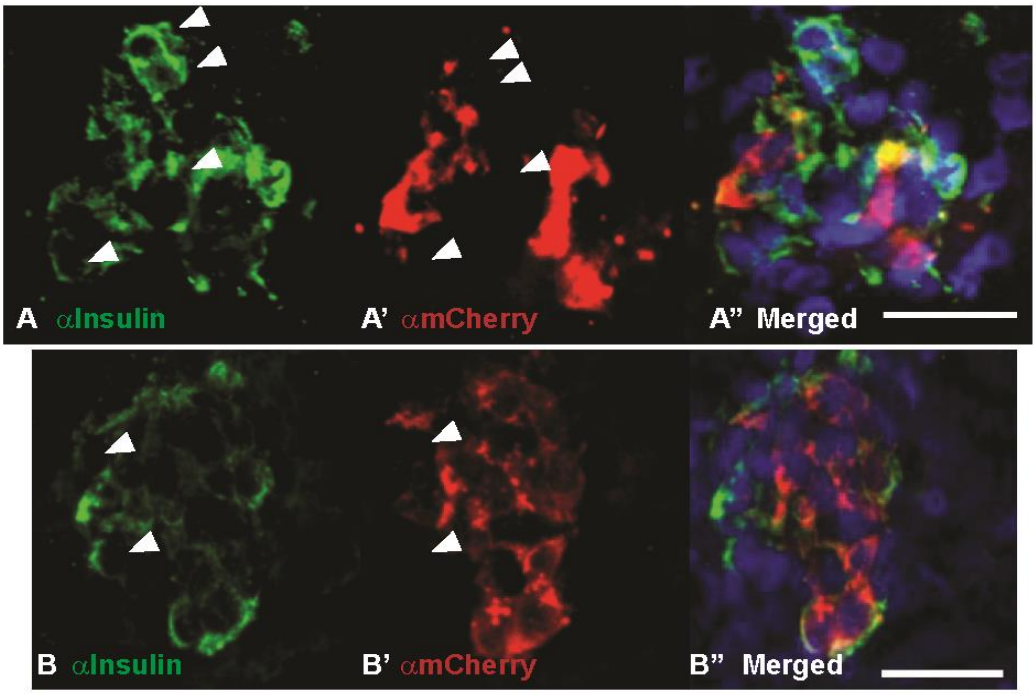

A αInsulin
A' αmCherry
A" Merged
B αInsulin
B' αmCherry
B" Merged

Figure 7.2. Indication of potential transgene silencing

Confocal images of sections from *ins:mCherry NTR2.0* fish with immunofluorescent detection of insulin (to detect beta cells, green in **A and B**) and mCherry (red -reports on NTR2.0 co-expression **A' and B'**). Arrows point to regions where insulin is detected but mCherry is not. **A''** and **B''** are merged images with nuclei stained with DAPI (blue).

Minimizing MTZ toxicity.

In order for my new NTR2.0 transgenic zebrafish lines to be a suitable model of hyperglycemia, it was important that I verify that the amount of MTZ required to ablate beta-cells does not affect the health and quality of life of the fish treated. To do this, I investigated the potential toxic side effects of MTZ on larval fish health. In initial pilot experiments I noticed that most larvae appeared unhealthy when I treated them with 10mM MTZ from 3 to 5dpf. These unhealthy appearances included some larval fish failing to inflate their swim bladder, having an abnormal gut morphology, and even resulted in the death of some larvae. Noticing these phenotypes in larval fish early on, I decided to do a larger experiment. During this larger scaled experiment, I documented larval survival, gross morphology, gut histology, and inflammation (without the NTR transgenes being expressed) when larvae were treated in a range of MTZ concentrations (0, 100 uM and 10 mM) from 3 to 5 dpf. To help me better visualize the morphology of the gut, I used non-pigmented *casper* larvae (White et al., 2008). The inability of larvae to inflate their swim bladder by 5dpf was used as a readout of abnormal development and ill health of the fish (Driever et al., 1996) (**Figure 8.2A-D**). To further investigate the health of the gut, I sent out larval samples to the UCI Histology Core Facility. At the core facility, transverse paraffin sections of larval fish were cut and stained with hematoxylin and eosin (H & E). Upon the return of these samples, I was able to visualize and blindly document intestinal morphology (**Figure 8.2E-G**). Differences between intestinal morphology were compared across the three different treatment groups. To investigate inflammation in the gut as a result of MTZ treatment, I observed the expression of GFP in a NFκB reporter line, *Tg*(*8xNFκB:eGFP)*^{ir2019} (**Figure 8.2H-O**).

Larvae that were incubated without MTZ (n=50) or 100 uM MTZ (n=50) all survived and had swim bladders that inflated normally (**Figure 8.2A, 8.2B**). I processed five fish from each

control group for histology. I found that all five fish from both groups had normal gut morphology, which was seen by the presence of normal intestinal folds (**Figure 8.2E, 8.2F**). Half the larvae that were immersed in 10 mM MTZ (25/50) died before reaching 5dpf. The remaining larvae that survived the 10mM MTZ treatment were unhealthy. These surviving fish had damaged digestive tracts, which was observed by looking at the level of gross morphology and seeing a lack of intestinal folds (**Figure 8.2C, 8.2D**). The surviving fish in the 10 mM MTZ treated group could also be separated further into two categories: i) 13 larvae that had inflated their swim bladders (**Figure 8.2C**) and had abnormal gut histology that lacked the presence of intestinal folds (5/5 taken from the 13 fish in group, **Figure 8.2G**); and ii) 12 larvae that had failed to inflate their swim bladders (**Figure 8.2D**). The fish that failed to inflate their swim bladders were very fragile. This led to my inability to process the tissue of these larvae for histology and is the reason why I was unable to acquire images for this group of fish. I attempted to process 5 fish out of the 12 fish in this group. To further characterize the intestinal phenotype I observed, I used *8xNFκB:eGFP*^{ir2019} larvae as negative controls (No MTZ treatment, **Figure 8.2H, 8.2L**)), and larvae treated with 100uM MTZ (**Figure 8.2I, 8.2M**) or 10mM MTZ (**Figure 8.2J, 8.2K, 8.2N, 8.2O**) from 3dpf to 5dpf. When I treated *8xNFκB:eGFP*^{ir2019} transgenic larvae without MTZ or with 100 uM MTZ I noticed that these fish were indistinguishable. Both fish had similar numbers of NFκB signaling cells around the gut (**Figure 8.2H, 8.2I, 8.2L, 8.2M**). From these results, I concluded that the 100 uM MTZ dose did not lead to inflammation in the intestine. Regardless of whether the 10mM MTZ treated fish had inflated (**Figure 8.2J, 8.2N**) or non-inflated swim bladders (**Figure 8.2K, 8.2O**), treating larvae with 10mM MTZ led to an increase in the numbers of cells having NFkB signaling. This observation is consistent with inflammation of the digestive tract. From these results, I concluded that 10mM MTZ is possibly toxic to the zebrafish intestine.

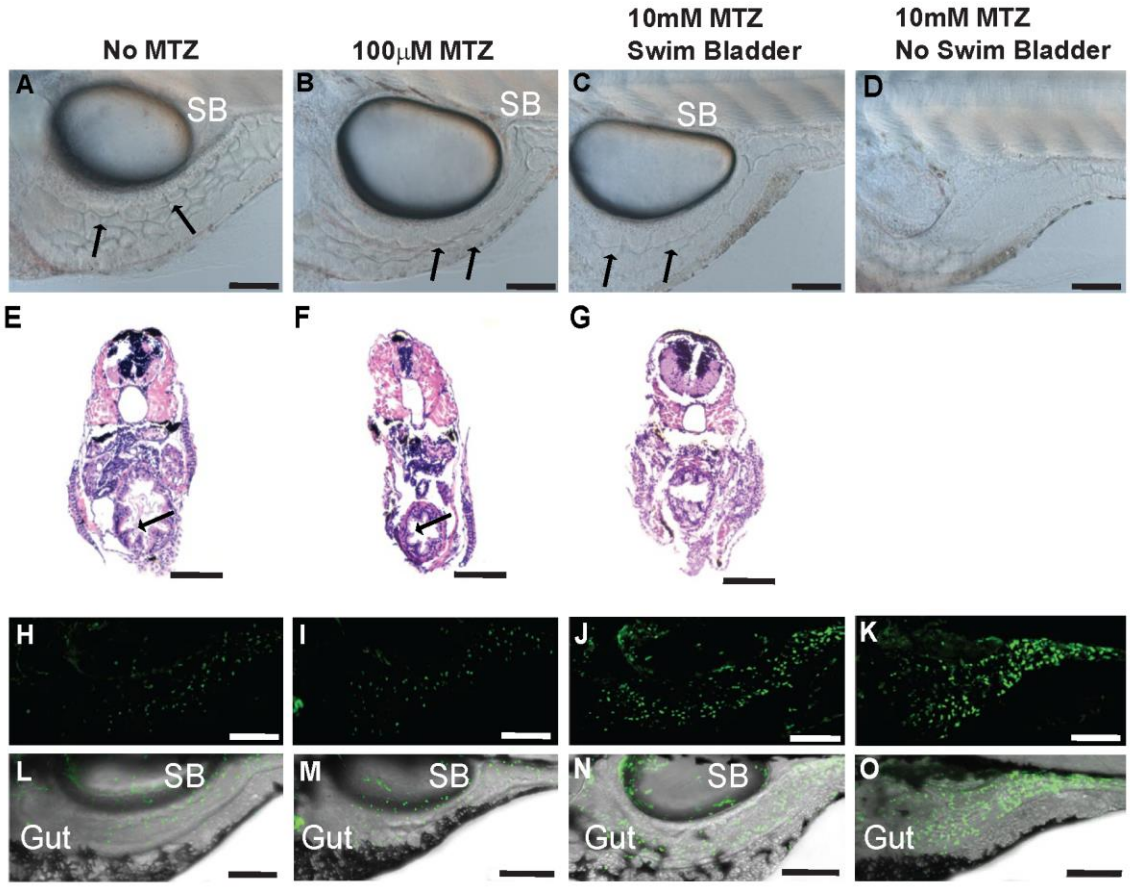

No MTZ
100μM MTZ
10mM MTZ
Swim Bladder
10mM MTZ
No Swim Bladder
A
SB
B
SB
C
SB
D
E
F
G
H
I
J
K
L
SB
Gut
M
SB
Gut
N
SB
Gut
O
Gut

Figure 8.2. Deleterious effects of 10mM MTZ on larval zebrafish

Images shown are 5dpf larvae treated with 0 mM MTZ (**A, E, H, L**), 100 uM MTZ (**B, F, I, M**), or 10 uM MTZ (**C, D, G, J, K, N, O**). (**A, B**). *casper* larvae were used to easily visualize the gut lumen. Images taken from the trunk region of *casper* larvae show that control and 100 uM MTZ treated larvae have visible gut lumens (normal intestinal folds). These *casper* larvae also have swim bladders that are inflated normally. Larvae that are capable of surviving the 10 mM MTZ treatment, have less visible gut lumens and intestinal folds are harder to see. Some of these treated larvae also have inflated swim bladders (**C**) where other larvae have no inflated swim bladder (**D**) (20×, scale bar = 100 uM). (**E-G**) Transverse sections through the trunk of 10mM MTZ treated larvae were stained with H&E to observe intestinal morphology. Larvae treated with 10mM MTZ and were capable of inflating their swim bladder had a lack of intestinal folds (**G**) (20×, scale bar = 100 uM). (**H-O**) Images taken of *8xNFκB:eGFP* 5dpf larvae treated with 10mM MTZ showed an increase in the number of cells with NFkB signaling (**J, K, N, O**) (20×, scale bar = 100 uM). SB-swim bladder, black arrowheads point to intestinal folds.

MTZ induced gut inflammation occurs without a microbiota.

In collaboration with the Wiles Lab, I next wanted to test whether lethality and tissue damage was due to direct toxic effects of MTZ or whether these results are due to changes in the microbiota of the fish. To do this, Courtney Knitter (a graduate student) from the Wiles Lab derived germ-free (GF) *8xNFκB:eGFP*^{ir2019} larvae. Half of the larvae that I provided to Courtney were maintained in GF conditions and half of them were conventionalized (CVZ). CVZ is a process that involves the reintroduction of conventional bacteria (Melancon et al., 2017). In each group the larvae were further divided by Courtney where half of the larvae were treated with 10mM MTZ from 3-5dpf and the other half were kept as a negative control (no MTZ treatment). Once larvae reached 5dpf, I obtained all larvae from the Wiles Lab and performed confocal imagining and analysis. I noticed that CVZ and GF fish treated with 10 mM MTZ from 3 to 5 dpf produced the same response in the gut as I saw previously in the conventional 'non-germ free' larvae (**Figure 8.2H-O**). Once again, I saw that about half of the 10 mM MTZ treated larvae did not inflate their swim bladders normally (8/20 CVZ, **Figure 9.2F**, 8/20 GF, **Figure 9.2L**). All treated CVZ fish (20/20, **Figure 9.2B, 9.2C**) and all treated GF fish (20/20, **Figure 9.2H, 9.2I**) had an increase in the number of cells expressing GFP in the gut when looking at the NFκB reporter. Here, I have shown that tissue damage occurs without the presence of micro-organisms. From this study, I was able to conclude that the 10 mM MTZ treatment is directly toxic to larval zebrafish tissues. Additionally, I also looked outside of the digestive tract and noticed that there was an increase in the levels of NFκB signaling in a few other structures. These structures include the neuromasts and skin, which can be seen in **Figures 9.2B**, **9.2C**, **9.2H**, **9.2I**.

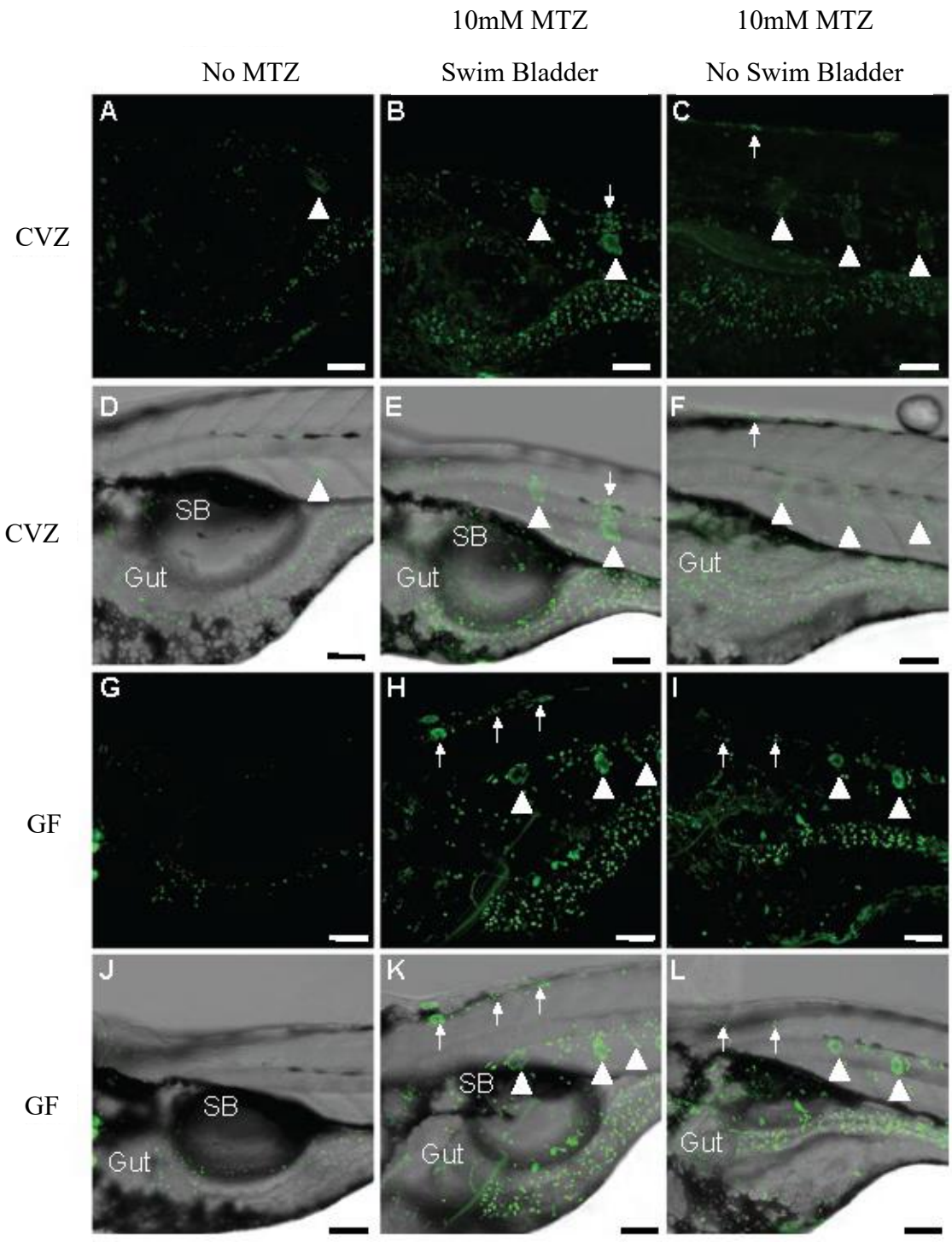

No MTZ
10mM MTZ
Swim Bladder
10mM MTZ
No Swim Bladder
CVZ
CVZ
GF
GF
A
B
C
D
E
F
G
H
I
J
K
L
SB
Gut
SB
Gut
Gut
SB
Gut
SB
Gut

Figure 9.2. 10mM MTZ has toxic effects on larvae tissues independent of microbiota

In collaboration with the Wiles' Lab we performed germ-free experiments to test MTZ toxicity. Confocal images are shown of conventionalized (CVZ) (**A-F**) and germ-free (GF) *8xNFκB:eGFP* 5dpf larvae (**G-L**) that were either kept as a negative control larvae (No MTZ, **A**, **D, G**, **J**) or were treated with 10mM MTZ from 3 – 5 dpf (10mM MTZ, **B**, **C**, **E**, **F**, **H**, **I, K, L**). Larvae that were treated with 10mM MTZ either inflated or failed to inflate their swim bladder (SB). There was no obvious difference between CVZ and GF larvae treated with 10mM MTZ. Both groups showed an increased response in the number of cells undergoing NFkB signaling in the gut, neuromasts (arrow heads), and skin (arrows). (**A-C**, **G-I**) 20x z-stacked fluorescent images. (**D-F**, **J-L**) same z-stack images imposed over brightfield image. Scale bar = 100 uM.

Acute beta-cell ablation in adult fish

In a previous study, it was reported that immersing adult fish for 20 hr in 10 mM MTZ could induce beta-cell ablation in *ins:NTR1.0-mCherry*^{jh4} fish (Ghaye et al., 2015). To test this approach, I immersed 20 wildtype adult fish (not expressing NTR) in 10 mM MTZ for 24 hrs. I found that half these fish died (n=10). During the course of treatment all of these fish displayed signs of both anorexia and lethargy. When looking at H&E sections of the gut, all surviving fish had some level of intestinal abnormalities (**Figure 10.2B,C**). From these observations, I concluded that immersing NTR2.0 fish in 10mM MTZ is not the best approach to ablate beta cells.

I next wanted to test whether a lower dose of MTZ could be used to ablate beta cells in NTR2.0 adult fish via immersion and whether this lower dose would be less toxic to the fish. To do this, I immersed *ins:mCherry NTR2.0*^{ir2018} fish in 5 mM (half the dose originally required to ablate beta cells) for either 24 hrs (**NTR2.0 5mM MTZ 24hrs**, n=10) or 48 hrs (**NTR2.0 5mM MTZ 48hrs**, n=10). I compared these treated fish to control fish which included untreated *ins:mCherry NTR2.0*^{ir2018} fish (**NTR2.0 control**, n=13), wildtype fish immersed in 5 mM MTZ for 24 hrs (**MTZ control**, n=10), and *ins:mCherry NTR2.0*^{ir2018} fish treated with 10 mM MTZ for 24hrs (**positive control**, n=10) (**Figure 11.2A**). For all groups of fish, I made sure to document fish survival and general health. When I completed this trial, I took fasting blood glucose (FBG) readings for all fish in each group to check that beta cell ablation was achieved via hyperglycemia readings. The maximum FBG measurement of the FreeStyle lite glucose meter I used in this study was 500 mg/dL. From our previous findings, it is possible that the actual levels are likely higher (Delaspre et al., 2015). I processed 5 fish from each experimental and control group for histology and used immunodetection to detect the presence of two islet markers. I used the anti-glucagon antibody to label alpha cells in the islet periphery and I used the anti-insulin antibody to label beta

cells – the target for ablation. I imaged 15 islets by confocal microscopy for each of the five fish I sampled from each group.

All MTZ (10/10) and NTR2.0 controls (13/13) survived my experiment. These fish also had normal FBG readings (**Figure 11.2A**) and they all appeared healthy. Looking at H&E sections of the gut, these fish also had normal intestinal epithelium (5/5, **Figure 11.2H, 11.2I**). Performing immunofluorescence staining showed that all fish had islets containing beta cells (5/5, **Figure 11.2C, 11.2D**). Like the control fish, **NTR2.0 5mM MTZ 24hrs** fish also survived. These fish also appeared healthy (10/10) and out of the five fish sampled for histology, they all had a normal gut morphology (5/5, **Figure 11.2J, 11.2J'**). Although all these fish became hyperglycemic there was a higher degree of variability compared to MTZ and NTR2.0 control fish groups (**Figure 11.2A**). When I performed immunostaining of islet markers in pancreatic samples taken from **NTR2.0 5mM MTZ 24hrs** fish, there was variability in beta-cell ablation which was consistent with FBG readings. I examined 75 islets (5 fish, 15 islets from each fish) and noted that 10 islets had normal proportions of beta cells, 15 islets had no beta cells, and 50 islets contained a combination of normal appearing beta cells along with immunoreactive cell debris which is consistent with ongoing but incomplete beta-cell ablation (a representative image of this can be found in **Figure 11.2E**).

Just like the other groups of fish, **NTR2.0 5mM MTZ 48hrs** fish survived and appeared healthy (10/10). I processed 5 fish for histology from this group and found that most of these fish showed signs of normal intestinal morphology (4/5, **Figure 11.2K, 11.2K'**). There was only one fish out of the 5 fish looked at that showed some signs of limited pathology in the intestine (a representative image is shown in **Figure 10.2E**). After immunostaining, there was very little insulin detected in these fish (5/5 fish, 75 islets in total) which was consistent with FBG readings.

Any signal that was detected was restricted to either cell debris or cells with fragmented nuclei, which suggests apoptotic cells (**Figure 11.2F**). Looking at my positive control fish, (*ins:mCherry NTR2.0*^{ir2018} fish in 10 mM MTZ for 24 hrs, n=10), only half of these fish survived (5/10). All 5 of the surviving fish became hyperglycemic (**Figure 11.2A**) and achieved the complete loss of beta cells (**Figure 11.2G**). However, there were significant signs of intestinal epithelium damage (**Figure 11.2L**). This damage was found to be scattered throughout the normal epithelium of the gut (**Figure 10.2C)**. In summary, I found that complete beta-cell loss can be achieved in *ins:mCherry NTR2.0*^{ir2018} adult fish immersed in 5 mM MTZ for 48 hrs. This treatment in NTR2.0 fish leads to the same level of hyperglycemia and beta-cell ablation when NTR1.0 fish are treated in 10 mM for 24 hrs however, NTR2.0 fish treated with a lower dose of MTZ do not have the associated gut pathology and lethality.

I have shown that my new model of acute adult beta-cell ablation uses a different enzyme (NTR2.0) and a new drug delivery method via the immersion of adult fish in 5 mM MTZ for 48 hrs. As a result of this, the duration of exposure to the prodrug, the rates of MTZ uptake, catalysis to active MTZ, and the cellular accumulation/retention are all most likely to be different compared to our lab's previous method where MTZ was injected into adult fish. Therefore, I decided to test whether these changes altered the ability of adult fish to regenerate beta cells as previously reported following acute beta-cell ablation using my newly adopted method of immersion. To do this, I immersed *ins:mCherry NTR2.0*^{ir2018} adult fish (n=16) in 5 mM MTZ for 48 hrs to ablate beta cells. I then removed the fish from MTZ treatment and allowed the fish to regenerate. I sacrificed fish and measured FBG levels at three different time points: 3, 7 and 17 days after fish were removed from 5 mM MTZ 48hr treatment. I chose to use these time points to sample blood glucose readings because they are the same time points that matched our lab's previous regeneration study (Delaspre

et al., 2015). Average (± SD) FBG readings for each group were as follows: 3 days ≥ 500 mg/dL (n=5), 7 days 249 mg/dL ± 140.2 (n=5), and 17 days 68.1 mg/dL ± 28.6 (n=6) (**Figure 11.2B**). The results I obtained for day17 were statistically comparable to samples from untreated NTR2.0 control fish (NTR2.0 control) – 50.2 mg/dL ± 22.0 (n =10). These results demonstrated a return to euglycemia. From this experiment, I concluded that the pattern of FBG recovery is identical to our previous study. This led me to also conclude that my new method of acute beta-cell ablation via immersion of NTR2.0 expressing fish in MTZ did not alter the dynamics of beta-cell regeneration.

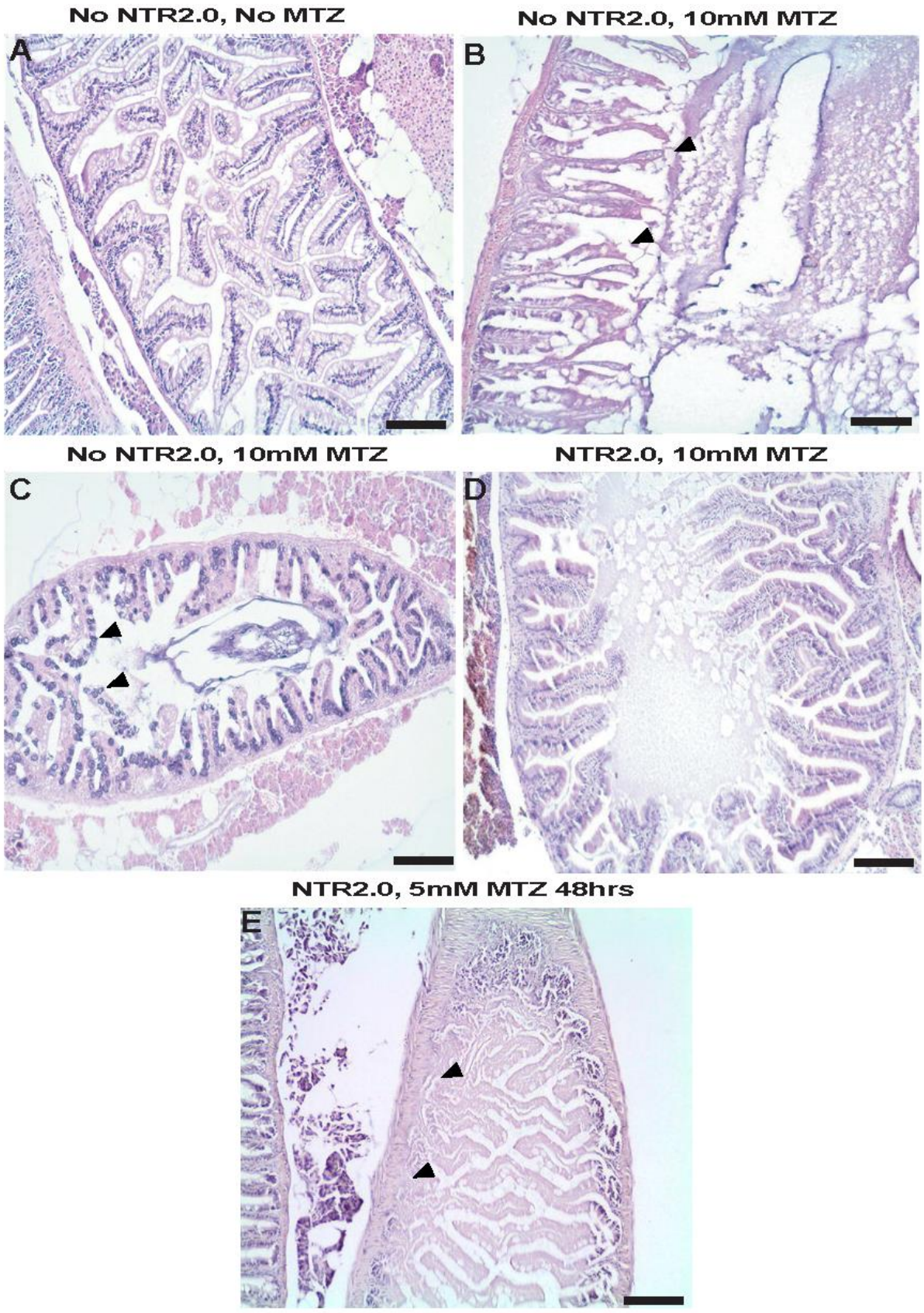

No NTR2.0, No MTZ
A
No NTR2.0, 10mM MTZ
B
No NTR2.0, 10mM MTZ
C
NTR2.0, 10mM MTZ
D
NTR2.0, 5mM MTZ 48hrs
E

Figure 10.2. Histological analysis of the gut in fish treated with MTZ

H&E staining was performed on adult gut sections of fish that were treated with and without MTZ. (**A**) Represents a region of the gut in wildtype fish that were not treated with MTZ (20×, scale bar = 100 uM). (**B**) Represents a region of the gut in wildtype fish that were immersed in 10mM MTZ for 24hrs. As can be seen, there is damage to the lining of the gut (black arrows) (20×, scale bar = 100 uM). This intestinal damage was consistently seen in all 5 fish looked at for this treatment group. (**C**) Shows a region of the gut where 1 out of the 5 fish in this treatment group (wildtype fish immersed for 24 hrs in 10mM MTZ) had an increase in the number of goblet cells present (black arrows) (20×, scale bar = 100 uM). (**D**) Shows a gut region that looked normal in NTR2.0 fish immersed for 24 hrs in 10mM MTZ. In all 5 fish looked at for both wildtype and NTR2.0 fish treated with 10mM MTZ for 24 hrs, there was consistent damage in the gut (**B**) that was interspersed throughout normal gut tissue represented in (**D**). (**E**) In all 5 fish looked at, only one fish (*ins:mCherry NTR2.0ir2018* fish immersed in 5mM MTZ for 48 hrs) had a single region of the gut that showed damage to the intestinal lining (black arrows) (20×, scale bar = 100 uM).

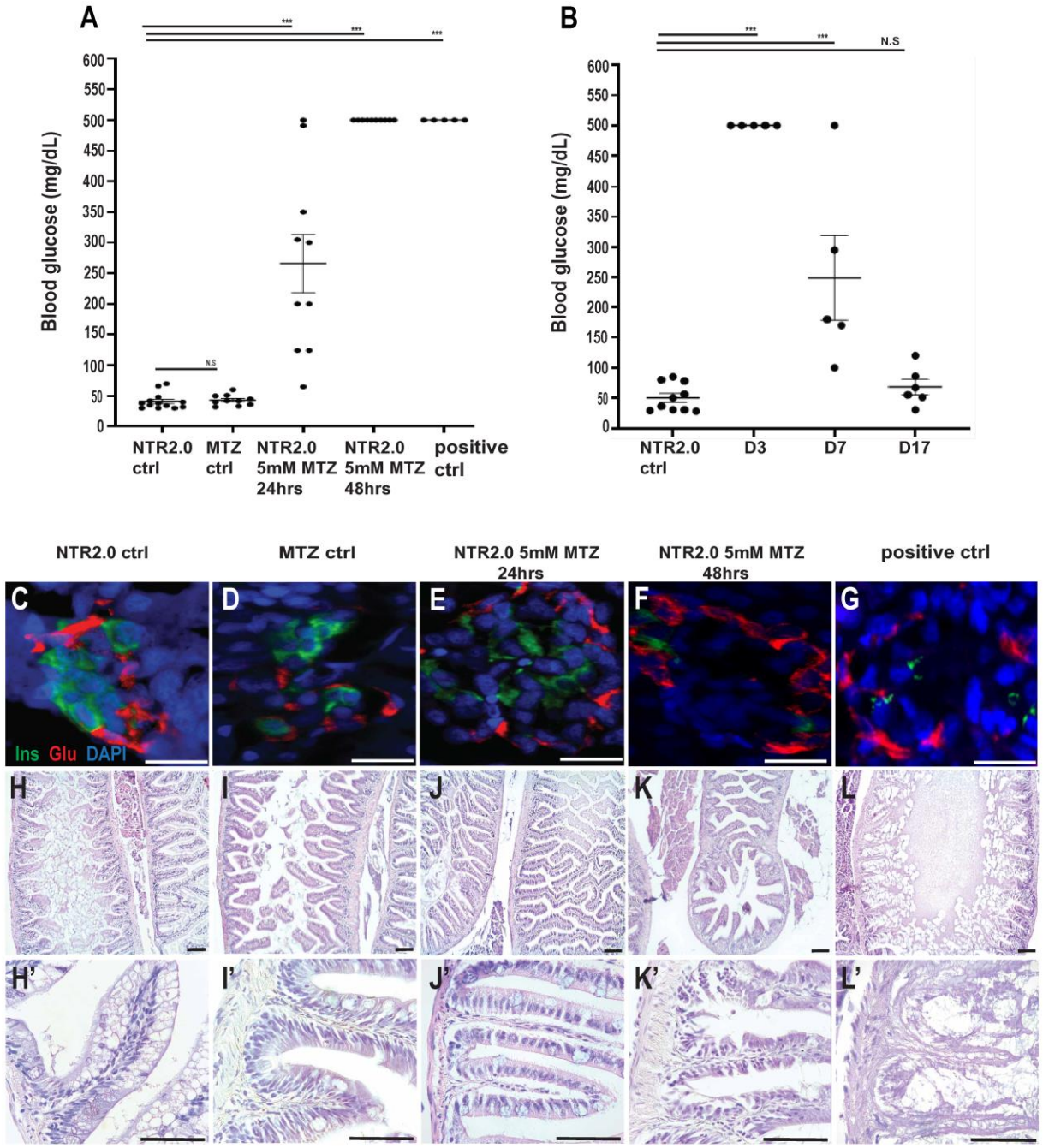

A
B
Blood glucose (mg/dL)
600
550
500
450
400
350
300
250
200
150
100
50
0
***
N.S
NTR2.0 ctrl
MTZ ctrl
NTR2.0 5mM MTZ 24hrs
NTR2.0 5mM MTZ 48hrs
positive ctrl
D3
D7
D17
NTR2.0 ctrl
MTZ ctrl
NTR2.0 5mM MTZ 24hrs
NTR2.0 5mM MTZ 48hrs
positive ctrl
C
D
E
F
G
Ins Glu DAPI
H
I
J
K
L
H'
I'
J'
K'
L'

Figure 11.2. Acute beta-cell ablation in adults by MTZ immersion

(**A**) Shows FBG readings from the 5 groups of adult fish: untreated *ins:mCherry NTR2.0*^{ir2018} fish (**NTR control**), wildtype fish immersed for 24 hrs in 5 mM MTZ (**MTZ control**), *ins:mCherry NTR2.0*^{ir2018} fish immersed for either 24 hrs or 48 hrs in 5 mM MTZ (**NTR2.0 5mM MTZ 24hrs** or **48hrs**) and same fish immersed for 24 hrs in 10 mM MTZ (**positive control**). The maximum reading of the blood glucose meter used in 500mg/dL and is therefore, the maximum FBG reading that can be captured. **(B)** Shows FBG readings of NTR2.0 control and *ins:mCherry NTR2.0*^{ir2018} fish. The NTR2.0 fish were treated with 5 mM MTZ for 48 hrs and were then allowed to recover their beta cells for either 3 (**D3**), 7 (**D7**) or 17 (**D17**) days. FBG measurements were taken following these periods of recovery. **N.S** = not significant, ******* = significant with a p value <0.05. (**C-G**) Confocal images were taken of paraffin sections through the pancreas. Immunofluorescence was performed to detect insulin (green) and glucagon (red) islet markers. DAPI (blue) was used to stain nuclei (40×, scale bar = 10 mM). (**H-L**) Shows H & E histological images of the intestines from fish in each treatment group (20×, scale bar = 100uM). (**H'-L**') Magnification of same images were taken at 60× (scale bar = 50 uM) to better show intestinal folds.

Maintaining hyperglycemia during development

Now that I have established a significantly improved model of acute beta-cell ablation, I next tested whether *ins:mCherry NTR2.0*^{ir2018} fish could be used to model chronic hyperglycemia during larval development. *ins:mCherry NTR2.0*^{ir2018} (n=30) and control *ins:dsRed*^{m1018} larvae (n=30) were incubated in either 0, 100 uM, or 10 mM MTZ for 10 days (3 dpf to 13 dpf). I plotted larvae survival over time (**Figure 12.2A**). Beta-cell ablation was monitored throughout the course of the experiment by checking daily for the presence or absence of beta cells using a fluorescent microscope. This was done by sampling 5 larvae from each group. Doing so, ensured that beta cells remained ablated throughout the course of the experiment. At the end of the experiment, I used confocal microscopy to document the presence or absence of beta cells within the pancreatic islet for each group of larvae (**Figure 12.2B-D**). Regardless of whether NTR was present, larval survival without MTZ treatment was about 75%. There was no effect on larval survival when they were incubated in 100uM MTZ, even with an NTR-dependent loss of beta cells (**Figure 12.2D**). However, when I incubated larvae in 10 mM MTZ, all larvae died within the first 5 days of MTZ treatment. These results tell me that: 1) the NTR2.0 transgene did not influence larvae survival, 2) larval fish can be maintained for 10 days without beta cells and it does not affect their survival; and 3) any models that require treatment with 10 mM MTZ will be incompatible with modeling chronic hyperglycemia during larval development.

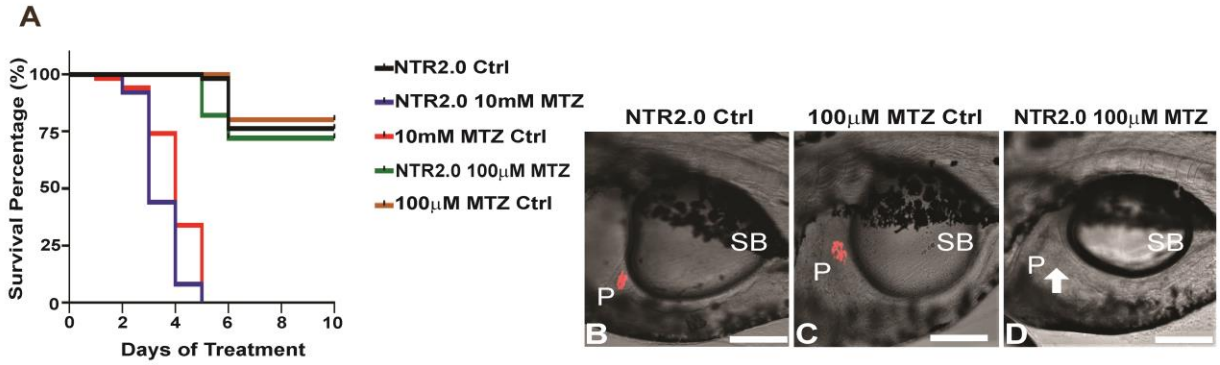

A
Survival Percentage (%)
100
75
50
25
0
0
2
4
6
8
10
Days of Treatment
NTR2.0 Ctrl
NTR2.0 10mM MTZ
10mM MTZ Ctrl
NTR2.0 100μM MTZ
100μM MTZ Ctrl
NTR2.0 Ctrl
100μM MTZ Ctrl
NTR2.0 100μM MTZ
SB
P
B
SB
P
C
SB
P
D

Figure 12.2. Chronic hyperglycemia can be achieved in larvae using NTR2.0 fish

(**A**) Survival curve showing *Ins:mCherry NTR2.0*^{ir2018} larvae treated for 10 days (3 dpf to 13 dpf) with either 0 MTZ (**NTR2.0 Ctrl**), 100 uM MTZ (**NTR2.0 100 uM MTZ**), or 10 mM MTZ (**NTR2.0 10mM MTZ**)**.** Other controls include *ins:dsRed*^{m1018} larvae treated with 10 mM MTZ (**10 mM MTZ Ctrl)** and 100 uM MTZ (**100 uM MTZ Ctrl**). (**B**, **C**) Single optical confocal images show the presence of fluorescently labeled beta cells in the trunk region of 13 dpf control larvae: **NTR2.0 Ctrl** (**B**) and **100 uM MTZ Ctrl** (**C**). (**D**) In **NTR2.0 100 uM MTZ** larvae, beta cells (white arrow) could not be detected. This indicated that this MTZ treatment was sufficient to ablate beta cells and prevent them from regenerating during the course of this experiment. Scale bar = 100 mM. Anterior to the left, dorsal at the top. SB- swim bladder and P- pancreas.

Modeling chronic diabetes in adult zebrafish

Next, I performed an 18 day MTZ trial experiment to test whether *ins:mCherry NTR2.0^{ir2018}* adult fish can be used as a new model to study chronic hyperglycemia (outlined in **Figure 13.2A**). I weighed each individual fish per group at the beginning (day 1) and towards the end of the trial (day 17) to determine any potential changes in body weight. Changes in body weight is an early symptom of diabetes and therefore was used as a readout of fish health. After weighing the fish on day 17, I photographed all the fish and then proceeded to fast each fish for 24 hrs for FBG reading the next day. On day 18, I euthanized the fish and took their FBG measurements. In this trial, I included two controls and three experimental groups. My negative control group (abbreviated to **-ve. Ctrl**) consisted of wildtype fish (n=20) that were treated in the same manner as the experimental groups but without any MTZ treatment. My second control group consisted of fish to account for any potential NTR-independent effects of MTZ (abbreviated to **MTZ ctrl**). This group included wildtype fish (i.e., no NTR2.0, n=20) that were maintained in the highest doses of MTZ being used in my trial. My three experimental groups of *ins:mCherry NTR2.0^{ir2018}* fish (abbreviated to **expt.1**, **expt. 2** and **expt. 3**) were at first immersed in 5 mM MTZ for 2 days at the beginning of the trial. Immersion in 5mM MTZ for 2 days was my 'ablation phase' in order to first remove all beta cells in these fish. I then designed an ablation 'maintenance phase' (days 3 to 16) as a way to stop the reappearance of new beta cells and keep fish hyperglycemic throughout the trial. This maintenance phase used a lower dose of MTZ to prevent fish from succumbing to the toxicity of MTZ and allow fish to be kept in MTZ for a long period. For the remaining 14 days of my trial, I kept fish in either **1 mM** MTZ (**expt.1**, n=12), **2 mM** MTZ (**expt. 2**, n=11) or **3 mM** MTZ (**expt. 3**, n=12). At the end of my trial, both control groups (**-ve. ctrl** and **MTZ ctrl**) had similar average (± SD) FBG levels of 35.9 mg/dL ± 3.94 and 34.8 mg/dL

± 3.67, respectively (**Figure 13.2B**). These control fish also maintained their body weight (**Figure 13.2C**) and appeared healthy (**Figure 13.2D-G**). These findings suggest that these treatments of MTZ alone do not have a detrimental effect on the weight and health of the fish.

In expt. groups 1, 2, and 3, all fish became hyperglycemic compared to control fish. Most of the fish in these three expt. groups had FBG measurement ≥500 mg/dL (**Figure 13.2B**). Additionally, these fish also lost a significant amount of body weight during the trial compared to control groups (**Figure 13.2C**). The loss in body weight was also independent of sex, as both male and female fish lost weight. For the fish in **Expt. 1**, fish had an average (± SD) FBG of 446.7 mg/dL ± 113 with 9/12 reaching ≥500 mg/dL and had an average weight loss of 27% ± 11.2. For the fish in **Expt. 2**, fish had an average FBG of 482.3 mg/dL ± 37.6 with 9/11 reaching ≥500 mg/dL and 31.8% ± 8.83 average weight loss. For the fish in **Expt. 3**, fish an average FBG of 495.8 mg/dL ± 13.8, with 11/12 reaching ≥500 mg/dL and 32.2% ± 9.66 average weight loss. I noticed that fish of both sexes in all experimental groups had a less colorful appearance and had abnormally curved body shapes. These phenotypes observed are consistent with severe weight loss (**Figure 13.2H-M**).

Together, my results demonstrated that fish treated for 2 days in 5 mM MTZ followed by treatment with either 1 mM, 2 mM, or 3 mM MTZ will keep NTR2.0 transgenic fish hyperglycemic for at least 16 days. Interestingly, I also observed significant NTR/MTZ dependent weight loss during the experimental trial. This observation in weight loss is consistent with the pathological effects associated with a prolonged diabetic state.

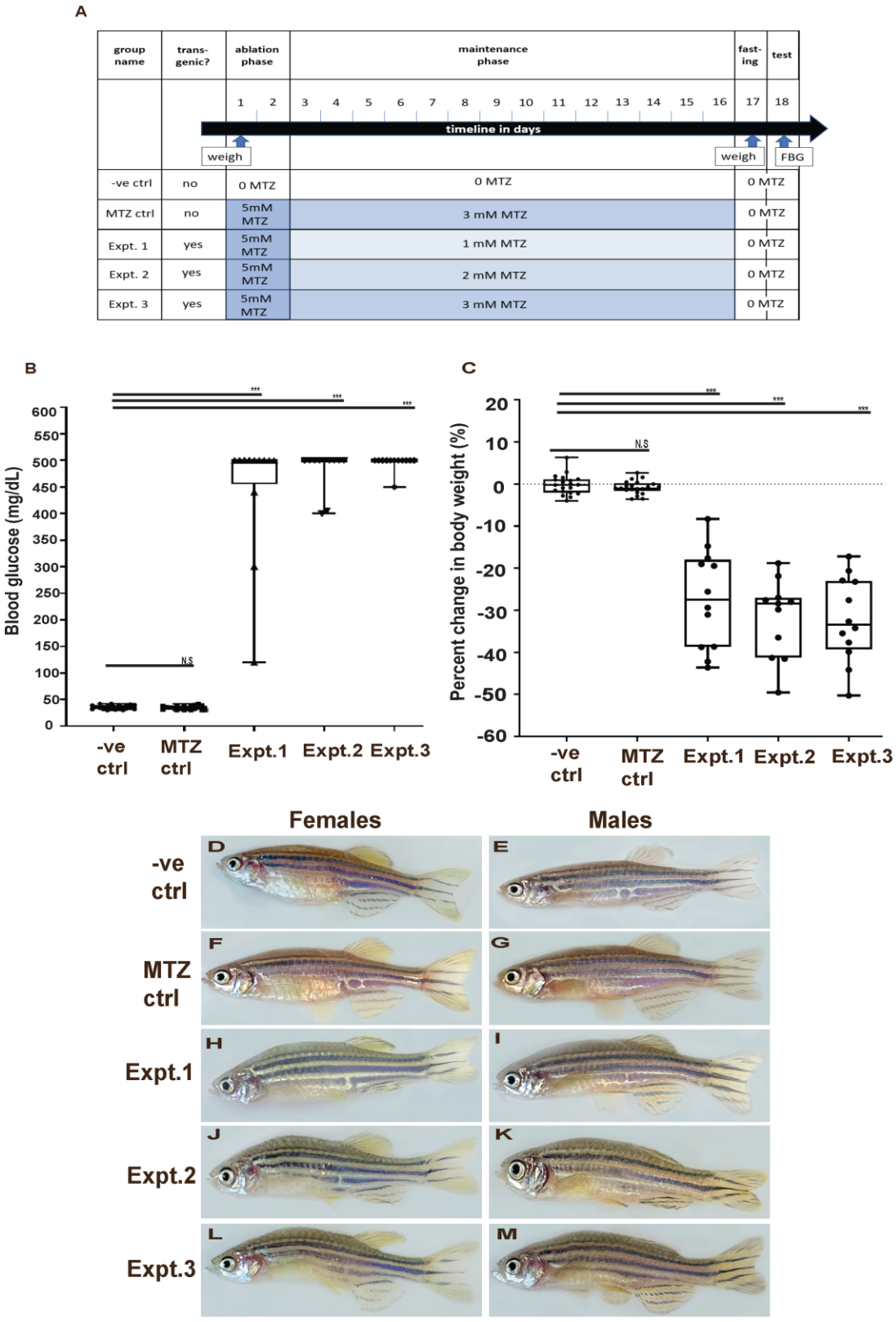

A
group name
trans-genic?
ablation phase
maintenance phase
fast-ing
test
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
timeline in days
weigh
weigh
FBG
-ve ctrl
no
0 MTZ
0 MTZ
0 MTZ
MTZ ctrl
no
5mM MTZ
3 mM MTZ
0 MTZ
Expt. 1
yes
5mM MTZ
1 mM MTZ
0 MTZ
Expt. 2
yes
5mM MTZ
2 mM MTZ
0 MTZ
Expt. 3
yes
5mM MTZ
3 mM MTZ
0 MTZ
B
Blood glucose (mg/dL)
600
550
500
450
400
350
300
250
200
150
100
50
0
***
***
***
N.S
-ve ctrl
MTZ ctrl
Expt.1
Expt.2
Expt.3
C
Percent change in body weight (%)
20
10
0
-10
-20
-30
-40
-50
-60
***
***
***
N.S
-ve ctrl
MTZ ctrl
Expt.1
Expt.2
Expt.3
Females
Males
-ve ctrl
MTZ ctrl
Expt.1
Expt.2
Expt.3
D
E
F
G
H
I
J
K
L
M

Figure 13.2. Chronic hyperglycemia can be achieved in adults using NTR2.0 fish

(**A**) Shows a timeline of the chronic MTZ treatment trial performed. (**B**) Blood glucose readings are plotted for each individual adult zebrafish from the following groups in the trial: negative controls (wildtype, no MTZ, **-ve. ctrl**), MTZ controls (wildtype with MTZ, **MTZ ctrl**) and the three experimental groups (*ins:mCherry NTR2.0*^{ir2018} fish treated with MTZ as outlined in **A**, **Expt. 1 – 3**). In all 3 expt groups, all fish became hyperglycemic as indicated. The blood glucose meter used for this trial can only read up to 500mg/dL and therefore, 500 is the only reading that can be captured. (**C**) Percent change in body weight over the course of the chronic MTZ trial for each fish in the control and expt. groups is shown. A dotted line is shown to represent no change in body weight. (**D-M**) Shows representative images of both female and male adult fish from controls and expt. groups. N.S. – non significant and *** is significant with a p value < 0.05.

d) Conclusion

In **Chapter 2**, I have described two new transgenic zebrafish tools that can be used to better facilitate beta-cell ablation and study regeneration. Additionally, these transgenic lines will also allow us and others in the field to temporally control the induction of chronic hyperglycemia in both larval and adult zebrafish for the first time using NTR as a model. Both of my new *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} transgenic fish will function as new model systems to study the pathologies associated with diabetes. Since NTR2.0 has a higher activity for MTZ, less MTZ is required to ablate beta cells. As I have shown, the importance of using a lower dose of MTZ reduces the toxic side effects and gut inflammation experienced by treated fish. I have also shown that the expression of NTR2.0 does not have any deleterious effects on both larval and adult health in the absence of MTZ treatment. Initially, I observed a loss in beta cells without the treatment of MTZ in transgenic lines that overexpressed the membrane-targeted tagYFP. It is believed that the membrane expression of this transgene is toxic to beta cells and led to their death without MTZ treatment. Our previous NTR1.0 transgenic lines of zebrafish required a 10mM MTZ dose to ablate beta cells. Here, I have shown that 10mM treatment is the LD_{50} for a 48 hr MTZ treatment in both larval and adult zebrafish. Fish that happened to survive this 10mM high dose treatment showed severe damage to the intestinal epithelium. Consistent with my result, others have shown that *Onchorhynchus mykiss* (rainbow trout) also displays an aberrant intestinal pathology when fish are treated with MTZ (Mitrowska, Pekala, & Posyniak, 2015). Although the mechanism of tissue damage in both fish is unknown, I have shown that in larval zebrafish the adverse effects of 10mM MTZ are independent of microbiome. Therefore, I have concluded that treating larval fish with 10mM MTZ has a direct toxic effect on some of the larval tissues observed.

In adult fish, it is likely that MTZ also has a direct effect on intestinal pathology. In an early collaboration with the Wiles Lab, I tried to test the effects MTZ had on the microbiome of adult fish. This was done by treating adult fish with various concentrations of MTZ and collecting feces over the course of MTZ treatment. Feces were then plated onto agar plates to colonize the resulting bacteria. In initial pilot experiments we observed a substantial difference in microbiome diversity between groups treated with 10mM MTZ versus lower MTZ doses and no MTZ treatment. However, numerous confounding variables (bacteria in existing system water and in our fish food) made it difficult to parse out these results. Therefore, these results were not included in this analysis. Further testing in adult fish is needed to rule out whether the microbiome plays a role in adult fish.

Since NTR2.0 requires a lower dose of MTZ to ablate beta cells and has a higher ablation efficacy, I was able to improve the way in which MTZ is delivered to adult fish. Previously, NTR1.0 adult fish had to be injected intracelomically with a single dose of 10 mM MTZ to effectively ablate beta cells (Delaspre et al., 2015; W. Huang et al., 2016). Having to inject fish with MTZ is technically challenging because the fish are very small. If the injections are not done properly, it can cause some beta cells to escape ablation and it can also lead to the accidental death of the fish. Having *ins:mCherry NTR2.0*^{ir2018} fish, I have shown that acute beta-cell ablation can now be achieved by simply immersing the adult fish in water treated with either 10 mM MTZ for 24 hrs or 5 mM MTZ for 48 hrs. Although both treatment doses induced hyperglycemia (FBG levels of 500 mg/dL), the fish treated with 5 mM MTZ for 48 hrs were much healthier and showed no signs of intestinal damage compared to those treated with the 10mM MTZ dose. I also want to point out that it is very likely that treating fish with lower concentrations of MTZ for longer periods over 48 hrs could also induce complete beta-cell ablation. In initial pilot experiments it is possible

that adult fish can be treated with 1mM MTZ for more than two days and achieve a hyperglycemic state. Treatment in 100uM MTZ did not achieve the same effect in adult fish as it did in larvae.

In addition to improving our model to study beta-cell regeneration, I have also shown that *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} fish are an appropriate new tool for modeling chronic hyperglycemia and the complications associated with hyperglycemia such as neuropathy, nephropathy, retinopathy, diabetic ketoacidosis and arterial disease. However, what I do want to point out is that depending on experimental goals, the following considerations should be carefully considered: 1) what age is appropriate for hyperglycemia induction, 2) how long fish should be kept hyperglycemic, and 3) what level of hyperglycemia is most relevant to the study planned? After much discussion with Dr. Parsons and with his help, we have outlined the following points below that should be considered when using my new NTR2.0 model to study chronic hyperglycemia:

1) Age of induction: When considering models of chronic hyperglycemia, transgenic methods have an advantage over genetic mutants because they provide us with the opportunity to incorporate inducibility. Having the ability to temporally control when beta cells are ablated allows the effects of chronic hyperglycemia on adult physiology to be separated from developmental effects. Here, we have shown that chronic hyperglycemia can be achieved in larval fish. This provides us with a potential model for studying gestational diabetes. Additionally, we have shown that chronic hyperglycemia can be achieved in adult fish. This provides us with the ability to mimic the onset of both T1D and T2D.

2) Duration of hyperglycemia: While designing my chronic hyperglycemic studies, we rationalized that 48 hr immersion in 5 mM MTZ would ablate all beta cells (as per our acute method) and then a lower dose would maintain hyperglycemia. Using a lower dose of MTZ was done to keep beta-cells ablated but to also maintain the health of the fish throughout the course of my experiment. During the first week of the 'maintenance phase', fish in the experimental groups (*ins:mCherry NTR2.0*^{ir2018} fish in MTZ) did not show any changes in their overall appearance and behavior. However, by the second week of treatment, these same fish showed a significant decline in their health. The fish had a decrease in their appetite and had visible weight loss. Since there was a rapid decline in fish health within two weeks of chronic MTZ treatment, it is believed that continuing my experimental conditions beyond 14 days would likely lead to lethality. From my experiments, I believe it is possible to extend the duration of hyperglycemia by lowering the MTZ dosing regimen in order to modulate the level of induced hyperglycemia.

3) Levels of hyperglycemia: In my trial to induce chronic hyperglycemia in adult fish, I had three experimental groups (**expt. 1**, **2**, and **3**). In all 3 groups a hyperglycemic state was achieved. I noticed that fish in **expt. 3** (treated with 3 mM MTZ) displayed the most extreme hyperglycemic outcome but they were also the sickest compared to the other two groups. These fish were more lethargic and were harder to exsanguinate for FBG measurements. If the goal of future studies is to study complications associated with diabetes, going to this extreme state of hyperglycemia may not be required or the most suitable to study diabetic complications. Fish in **expt. 1** (treated with 1 mM MTZ) were all hyperglycemic but they had more FBG variation than the other two groups. Blood glucose variation in this group is consistent with the possibility that beta cells are regenerating in some fish (3/12) to influence their FBG levels. From my chronic ablation studies,

I believe that maintaining fish in 1mM or 2Mm MTZ doses (or even lower) should be sufficient to elicit enough hyperglycemia to be able to model the pathology observed in patients who experience hyperglycemia (i.e. visible weight loss and lack of activity) (Roche, Menon, Gill, & Hoey, 2005).

It is also important to point out that in diabetic patients, the development of diabetic complications are not only due to constant levels of extremely high glucose concentrations. Instead, many of these complications arise from glucose fluctuations (Zhang et al., 2019). Therefore, a method that extends hyperglycemia and matches the correct physiology in diabetic patients would be to carry out cycles of ablation and recovery. An example of this would be placing adult fish in MTZ for 2 days to ablate beta cells and then allowing the fish to regenerate for 5 or more days and then repeating this experiment to induce glucose fluctuations.

In conclusion, my novel NTR2.0 expressing zebrafish lines will allow our lab and others in the field to interrogate beta-cell regenerative potential after acute or chronic beta-cell loss; and it will also help to expand chronic hyperglycemia disease modeling at both the pathological and molecular levels.

Chapter 3: Single-cell analysis identifies distinct subpopulations of centroacinar cells in the adult zebrafish pancreas

The results presented here are planned to be submitted as a paper.

a) Introduction

The average behavior of an entire population of cells may not reflect the behavior of numerous individual cells. Cellular heterogeneity is defined as cell-to-cell differences that exist in any cell population. These cellular differences are important in development, whole organism function, regeneration, and disease states (e.g., cancer) (Altschuler & Wu, 2010). Nearly all cellular systems within a single organism are considered heterogeneous including the following examples: the developing kidney (England et al., 2020), the liver (Aizarani et al., 2019), neuroepithelial cells (Eze, Bhaduri, Haeussler, Nowakowski, & Kriegstein, 2021), and the pancreas (Hendley et al., 2021). Understanding cellular heterogeneity in greater detail may help improve the way in which type one diabetes and pancreatic cancer can be treated.

The mammalian pancreas has two primary compartments, the exocrine and endocrine compartment. The exocrine compartment is involved in the digestion of food and is made up of both acinar and ductal cells. Acinar cells secrete digestive enzymes and ductal cells pass these enzymes into the gut. Cellular heterogeneity of the exocrine pancreas has been well studied in the context of pancreatic cancer since it is the primary compartment where pancreatic cancers arise, specifically pancreatic ductal adenocarcinoma (PDAC) (Espinet, Klein, Pure, & Singh, 2022; Orth et al., 2019; Peng et al., 2019). Although the primary cell source for PDAC is unknown, it is thought that both acinar and ductal cells could contribute (Kopp et al., 2012; Storz, 2017). A single-cell transcriptome study performed on patient derived PDAC organoid tumors revealed tumor heterogeneity across patient samples. This cellular heterogeneity was categorized into ‘classical’

and ‘basal’ subtypes where the latter consisted of cells with a poor drug response (Krieger et al., 2021). Understanding cellular heterogeneity that exists in a tumor can help clinicians screen for drugs that can specifically target the tumor and thus, can be used to treat patients with cancer. Although heterogeneity is mostly associated with tumors, it is important to first understand heterogeneity in the context of healthy tissue so that it can provide information that is required to interpret tumor results.

The endocrine compartment of the pancreas is responsible for glucose homeostasis and is organized into cell clusters called the islets of Langerhans, which are made up of five cell types: alpha, beta, delta, epsilon, and PP cells. Alpha cells secrete glucagon to increase blood glucose, beta cells secrete insulin to lower blood glucose, delta cells produce somatostatin to inhibit the secretion of endocrine hormones, epsilon cells produce ghrelin to induce hunger, and PP cells synthesize and release pancreatic polypeptide to regulate plasma glucose levels. Of all the cell types making up the islet, heterogeneity of mammalian beta cells have by far been the most well characterized (Dominguez-Gutierrez, Xin, & Gromada, 2019). During the production of insulin, beta cells can be functionally separated into states of high insulin production with signs of endoplasmic reticulum stress and unfolded protein response with low insulin expression (Xin et al., 2018). Changes in the composition of beta-cell heterogeneity are now being studied further in the context of type one diabetes (T1D) which will help aid in the understanding of the progression of disease onset and in the prevention of disease progression (Z. Fang et al., 2019; Y. J. Wang et al., 2016). T1D is characterized as an autoimmune condition that results in the loss of insulin-producing beta cells of the pancreas. Currently, there is an ongoing need for life-long treatments that can restore beta-cell mass in T1D patients.

Unfortunately, humans and other mammals have a somewhat limited beta-cell regenerative capacity and whether beta-cell neogenesis is involved is under debate (Meier et al., 2005; Suri et al., 2006). Beta-cell neogenesis is the process by which beta cells are replaced by either an existing endogenous stem cell or progenitor in the pancreas. Compared to rodents, zebrafish have the remarkable ability to regenerate pancreatic beta cells (Moss et al., 2009; Pisharath et al., 2007). The zebrafish pancreas is functionally and morphologically similar to the mammalian pancreas as they have both an endocrine and exocrine pancreas with the same cell types mentioned earlier (Kinkel & Prince, 2009). Given their remarkable ability to regenerate, zebrafish are an excellent model to understand the cellular and molecular mechanisms utilized in the fish to replace beta-cell mass. Gaining knowledge of these mechanisms in a highly regenerative animal model will reveal potential therapeutic treatments for patients with a loss in beta-cell mass including those with T1D.

Both the mammalian and zebrafish pancreas have specialized ductal cells called centroacinar cells (CACs) that are located at the ends of ductal cells and within the acinar lumen. CACs have long cytoplasmic extensions, they are Notch responsive, and they express high levels of Sox9 (W. Huang et al., 2016; Miyamoto et al., 2003; Parsons et al., 2009). Although CACs are abundantly found in the mammalian pancreas, there is little evidence of them behaving as a beta-cell progenitor (Inada et al., 2008; Kopp et al., 2011). Contrary to mammals, zebrafish CACs behave as a beta-cell progenitor during development (Parsons et al., 2009; Y. Wang et al., 2011) and in adult fish when beta-cell mass is damaged (Delaspre et al., 2015). To initially characterize the transcriptome of adult zebrafish CACs, bulk RNA-seq was performed (Delaspre et al., 2015; Ghaye et al., 2015). Delaspre et al. used a Notch-responsive transgenic line of fish called *tp1:eGFP* to label CACs and sort for CACs by FACS. Since blood vessels are also Notch-responsive cells of the pancreas two methods were carried out to deplete blood vessels. One method used the CD105

allophycocyanin-conjugated antibody to label and remove blood vessels. A second method used the *fli:dsRED* transgene to drive the expression of the red fluorescent protein in blood vessels. Using both methods separately, blood vessels were depleted and the cells that were left (CACs) were subject to bulk RNA-seq. The genes that were found to overlap in both methods were considered genes expressed by CACs. Bulk RNAseq identified *cftr, clcn1b, sox9b, epcam, nkx2.2a, nkx6.1* as CAC marker genes. In a separate study, it was shown that lower levels of *sox9b* causes beta cells to regenerate faster, however, the reason for this increased regenerative phenotype remains largely unknown (W. Huang et al., 2016). Outside of the behavior of CACs as a progenitor, CACs have also been considered as the cellular origin of pancreatic cancer (Kopp et al., 2012; Stanger et al., 2005). Much of this evidence relies on the fact that CACs express genes that are responsible for the progression of PDAC such as their high expression of *sox9* (Kopp et al., 2012).

Although the CAC transcriptome is well defined from bulk RNA-seq, the caveat with these studies is that they do not reveal cell-to-cell differences that may exist within the CAC population. These cell-to-cell differences are important because they could reveal the different biological functions of CACs such as those that are (**1**) more primed at regenerating beta cells which would allow us to uncover ways in which this subpopulation can be targeted to induce beta-cell regeneration better or (**2**) could function as the potential cellular origin for pancreatic cancer so that we have a better way of studying pancreatic cancer *in vivo*. The scientific premise that suggests that the CAC population is heterogeneous is based on several observations. One observation is that CACs have varying levels of Notch activity which contributes to their ability to differentiate into beta cells (Ninov, Borius, & Stainier, 2012). Higher Notch activity prevents CACs from differentiating into beta cells while lower Notch activity causes CACs to differentiate. It has also

been observed that during regeneration, not all CACs give rise to beta cells (Matsuda, Parsons, & Leach, 2013; Ninov et al., 2012; Parsons et al., 2009; Rovira et al., 2010). Together, these observations suggest that the CAC population is a heterogeneous population, but the details of these differences have yet to be uncovered.

Here I describe a genomics-based approach to uncover CAC heterogeneity in the adult zebrafish pancreas. This was accomplished by performing fluorescent activated cell sorting (FACS) to isolate GFP+ Notch-responsive cells from the zebrafish pancreas and then subjecting these sorted cells to single-cell RNA sequencing (scRNAseq). After quality control (QC) analysis, these Notch-responsive cell transcriptomes were then projected onto a Unifold Manifold Approximation Projection (UMAP) which followed downstream clustering analysis and differential gene (DE) expression. Here, I uncovered several populations of Notch-responsive cells of the pancreas including 2 blood vessel clusters, macrophages, and CACs that could be characterized into 7 subpopulations. Looking at these subpopulations of CACs in greater detail, I identified a potential population of transitioning acinar cells towards a CAC fate, a CAC subcluster with immune like characteristics, and a CAC subpopulation that may be primed for regenerating beta cells (hence, an endocrine precursor population).

b) Materials and Methods

Zebrafish Lines

Tg(tp1:eGFP)^{um10} transgenic adult zebrafish were used in whole pancreas scRNA-seq experiments and to collect Notch-responsive cells from the pancreas (including CACs). *Tg(tp1:eGFP)*^{um10} transgenic adult zebrafish were also crossed to *sox9b*^{fh313} heterozygous mutant fish to generate *tp1:eGFP*^{um10}*;sox9b*^{fh313} heterozygous fish. These fish were used to collect *sox9b* heterozygous pancreatic Notch-responsive cells for scRNA-seq experiments.

Genotyping

tp1:eGFP^{um10}*;sox9b*^{fh313} were genotyped to obtain sox9b heterozygous fish for scRNAseq. See **Chapter 1** for *sox9b* genotyping details.

Pancreas dissections, cell dissociation, and FACS purification

Adult zebrafish (5 months old) were euthanized on ice. The pancreas of 30 adult fish expressing *Tg(tp1:eGFP)*^{um10} were dissected to collect Notch-responsive cells from the pancreas. To obtain cells from the whole pancreas, 10 pancreata were dissected from *tg(tp1:eGFP)*^{um10} adult fish. Two replicate experiments were performed for the sorting and sequencing of Notch-responsive cells. During the dissections, Ringer's Solution (Fisher Science) was added to completely cover the exposed body cavity to prevent cell death. The gut was dissected from each fish first and then transferred to a 1% agarose plate that contained enough Ringer's Solution to cover the tissue. The pancreas was carefully dissected away from the gut and then transferred with

forceps to a 1.5mL tube containing 500uL of Ringer's Solution. To avoid excessive tissue clumping, no more than 5 dissected pancreata were placed into a single tube. To account for variation between sexes, all scRNA-seq experiments had an equal number of male and female fish.

Once all pancreata were dissected, tissue was spun down at 2,500 x g for 10 minutes at 4°C and the supernatant was removed. To perform cell dissociation, 50-100uL of Ringer's Solution containing 0.05% trypsin/EDTA was added to each tube and incubated at 37°C for 8 minutes. Rigorous agitation was performed to induce cell dissociation by pipetting up and down every 1 minute and ensuring that a cloudy solution was seen. The dissociation reaction was stopped by placing the samples on ice and adding an equal volume (50-100uL) of cold L-15 (Leibovitz) + 10% FBS. Each sample was spun down at 2,500 x g for 10 minutes at 4°C, the supernatant was removed, and the cells were resuspended in fresh 50-100uL of L-15 +10% FBS. Cells were stained with DAPI and then each sample tube was combined by filtering each sample into a single tube though a 100um cell strainer.

For the sorting of pancreatic Notch-responsive cells (PNCs), 5,000 cells were sorted for being *eGFP*+/DAPI-. For the whole pancreas, 19,000 cells were sorted for being DAPI-. All cells were sorted at 4°C using a BD FACS Aria Fusion Sorter and 100nm nozzle. PNCs were sorted into a single well of a 96-well plate containing 20uL of 10x Genomics RT buffer. No more than 46uL of cells (approximately < 10,000 cells required) were sorted into 20uL of RT buffer—bringing the total volume to 66uL. For the whole pancreas, cells were sorted similarly to PNCs but the 96-well plate contained 30uL of 10x Genomics RT Buffer and 70uL of cells were sorted into this buffer.

10x Library construction and sequencing

66uL of the single cell suspensions of sorted CACs (*eGFP*+/DAPI-) or whole pancreas (DAPI-) cells were loaded onto a 10x Chromium platform for single-cell library construction. A NovaSeq6000 was used to prepare each library using 400 million reads per lane, paired-ended sequencing run, 200 cycles, and approximately 200 million reads per sample.

Read mapping and pre-processing

FASTQ files were mapped to the zebrafish genome (GRCz11) (Lawson et al., 2020) using the 10x Genomics CellRanger software (10xgenomics.com). The two individual CAC data sets were mapped separately to generate two individual filtered featured matrix files. Both matrix files were loaded into R for integration. Cell transcriptomes were filtered by QC which excluded cells that have >5% of reads mapped to mitochondrial genes, less than the bottom 0.5% in gene counts, greater than 200 features, and greater than the top 0.5% in read counts. After QC, the data sets were integrated using Seurat v4 SCTransform and using Canonical Correlation Analysis (Hao et al., 2021). The SelectIntegrationsFeatures command was used to select features that are repeatedly variable across both sorted CAC datasets.

Computational analysis of single-cell RNA-seq data

Seurat v4 was used to perform variable features identification, principal component analysis (PCA), UMAP reduction, integration, and unbiased clustering using the functions FindVariableFeatures, RunPCA, RunUMAP, FindNeighbors, and FindClusters. For

dimensionality reduction and unbiased clustering 28 PCAs were used in the analysis of CAC heterogeneity/subclustering and whole pancreas datasets. The ElbowPlot function was used to determine statistically significant PCAs. Clustertree analysis and looping through different cluster resolutions was performed to choose the highest clustering resolution without cluster destabilization or mixing (Zappia & Oshlack, 2018). The cluster resolution was set to res < - 0.3 to cluster cell transcriptomes for both the sorted CAC dataset and whole pancreas dataset. Differential expression analysis in Seurat v4 was used to identify cluster markers by the Wilcoxon rank sum test.

Identification of cell type-specific genes

To identify marker genes in each cluster against all other cells, the FindAllMarkers function (only.pos = true, min.pct = 0.25, logfc.threshold = 0.25) was used. GO term enrichment analysis was performed using both Enrichr and Fish Enrichr. The top 50 genes were used for GO term analysis based on a ranking of their P value.

Pseudo-time analysis by PCA

Pseudo-time analysis on the sorted CAC dataset was based on PCA selection using Monocle 3 (Cao et al., 2019). We selected Cluster 4 as our starting point and Cluster 0 as our end point in scenario 1.

Whole Mount *In situ* hybridization

Riboprobes were designed against *spint2*, *cldn7b*, *tm4sf4* transcripts. The riboprobe forward primer used for *spint2* was 5'- GCGGTGGCAACAAGAACAAT -3' and the reverse primer used was 5'- GTGTTTTGAACCCGACCGTG -3'. The corresponding riboprobe was cloned into TOPO pCR2.1 (Thermo Fisher). The restriction enzyme required for linearization was NotI and the polymerase needed for transcription was sp6. The riboprobe forward primer used for *cldn7b* was 5'- GCCAGACACTGAGGTGCTTA -3' and the reverse primer used was 5'- GGATCCATTTAGGTGACACTATAGGGTTCAAGCACAGACCAAAGC -3'. The riboprobe forward primer used for *tm4sf4* was 5'- CGGAGATATCGTCACCTGGC -3' and the reverse primer used was 5'- GGATCCATTTAGGTGACACTATAGTGCACGTTCCCTTCCATTCT -3'. Both riboprobe reverse primers used for *cldn7b* and *tm4sf4* contain a GGATCC adapter sequence followed by the sp6 polymerase binding sequence ATTTAGGTGACACTATAG.

For details regarding whole mount *in situ* hybridization staining procedure on 5dpf dissected pancreata, see **Chapter 1**.

c) Results

Identifying Notch responsive cell populations

Two replicate scRNA-seq experiments were performed on Pancreatic Notch-responsive cells (PNCs) isolated from the pancreas of 30 *Tg(tp1:eGFP)*^{um14} transgenic fish at 5 months old. *tp1:eGFP* is a Notch responsive transgene used to label and isolate all GFP+ PNCs including CACs (Delaspre et al., 2015). **Figure 1.3A** is a schematic representation of my experimental approach. The pancreas was carefully dissected from adult fish and dissociated into a single-cell suspension. All GFP+ cells were sorted into a single well of a 96-well plate by FACs and subject to scRNA-seq.

In sample 1, 2,699 GFP+ cell transcriptomes were obtained and 2,542 were obtained in sample 2. After QC analysis was performed on the two scRNA-seq datasets, a total of 4,233 high-quality single-cell transcriptomes were left for further downstream analysis. The two data sets were integrated into a single data set and projected onto the two dimensions of the UMAP plot (**Figure 1.3B**). UMAP has been proven superior to t-distributed stochastic neighborhood embedding (t-SNE) since it can resolve subtle differences in cell populations and provides a population relationship based on cluster proximity (Armstrong et al., 2021; Becht et al., 2018; Butler, Hoffman, Smibert, Papalexi, & Satija, 2018). A heat map of all 11 clusters clearly shows a separation in gene expression between all clusters (**Figure 1.3C**). The heat map was generated using the top 10 genes for each cluster, however, only 2 out of the 10 top genes are listed to the left of the heat map plot. Since the *tp1:eGFP*^{um14} transgene is Notch responsive and was used to sort cells expressing GFP, I confirmed that all clusters represented in my UMAP (**Figure 1.3B**)

are Notch responsive based on the expression of at least one of the following four Notch receptors: *notch1a*, *notch1b*, *notch2*, or *notch3* (**Figure 2.3A-D**).

Looking at the clusters represented in my UMAP (**Figure 1.3B**), I then identified the cells represented in each of my 11 clusters by performing DE gene analysis and looking at a list of all DE genes for each cluster. CAC clusters were identified based on the expression of 6 known CAC markers (*cftr, clcn1b, sox9b, epcam, nkx2.2a,* and *nkx6.1*) (**Figure 1.3D-O**) (Delaspre et al., 2015). I found a total of 6 putative CAC subclusters (Clusters 0-5). Cluster 9 expressed 2 out of the 6 CAC marker genes, *sox9b* and *epcam* (**Figure 1.3F, 1.3G**). Performing GO term analysis on the top 50 genes of Cluster 9 revealed that Cluster 9 was not a subpopulation of CACs. Terms associated with Cluster 9 included 'intrahepatic bile duct development', 'pancreatic progenitor cell', exocrine pancreas such as 'acinar cell' and 'ductal cell', and 'hepatocyte' (**Figure 3.3A**). The top two DE genes of Cluster 9 were *cldn15lb* – a tight junction protein expressed in the intrahepatic bile duct connecting the liver and pancreas (**Figure 3.3B**) (Cheung et al., 2012) and *hpda*—an enzyme highly expressed in the liver (Y. Wang & Zhang, 2011) (**Figure 3.3C**). Based on these gene expression profiles, only Clusters 0-5 are subpopulations of CACs. From this initial analysis, having multiple CAC subclusters supports my hypothesis that the CAC population is heterogeneous.

Since Clusters 6, 7, 8, and 10 did not express any of the CAC marker genes tested, these clusters were considered non-CAC clusters. Looking at the list of DE genes making up these 3 clusters, I performed a literature search to identify the origins of these cell types. Cluster 6 was verified as vascular smooth muscle based on the expression of *vim* (R. Wang, Li, & Tang, 2006) and *tagln* (Tsuji-Tamura, Morino-Koga, Suzuki, & Ogawa, 2021) (**Figure 2.3E**). Cluster 7 was verified as blood vessels based on the expression of *fli1a* and *kdr* (**Figure 2.3F**) (Brown et al.,

2000; Ziegler et al., 1999). It was no surprise to find blood vessels in my scRNA-seq analysis since blood vessels are Notch-responsive cells of the pancreas and were not depleted from my analysis as they were in our previous bulk study (Delaspre et al., 2015). Cluster 8 was verified as macrophages based on the expression of *marco* and *mpeg1.1* (**Figure 2.3G**) (Kanno et al., 2020; Spilsbury et al., 1995). It has been found that macrophages can be Notch-responsive (Keewan & Naser, 2020). Cluster 10 was the only cluster found to be contaminating Notch-responsive cells captured since these cells were identified as testicular cells and were not part of the pancreas. This cluster was identified as cells residing in the testis based on the expression of *piwil*, *pprc1*, and *ddx4* which are known testicular cell markers (i.e. spermatocytes) (Qian, Kang, Liu, & Xie, 2022; D. Ye et al., 2023) (**Figure 2.3H**). These cells were accidentally taken up with the pancreas dissections and ended up in this analysis because the testis reside above the pancreas in male fish.

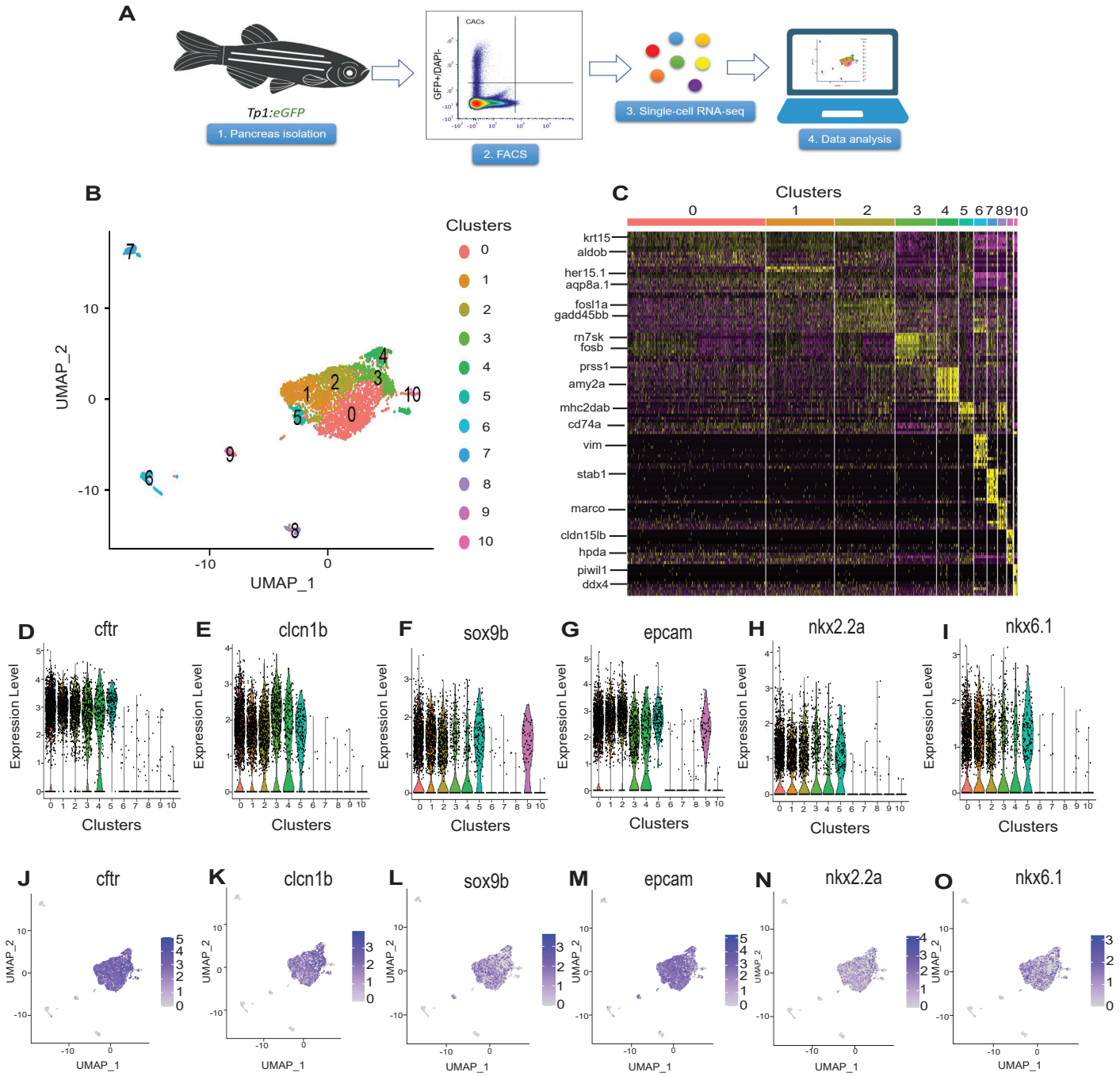

A
Tp1:eGFP
1. Pancreas isolation
CACs
GFP+/DAPI-
2. FACS
3. Single-cell RNA-seq
4. Data analysis
B
Clusters
UMAP_2
UMAP_1
C
Clusters
krt15
aldob
her15.1
aqp8a.1
fosl1a
gadd45bb
rn7sk
fosb
prss1
amy2a
mhc2dab
cd74a
vim
stab1
marco
cldn15lb
hpda
piwil1
ddx4
D cftr
E clcn1b
F sox9b
G epcam
H nkx2.2a
I nkx6.1
Expression Level
Clusters
J cftr
K clcn1b
L sox9b
M epcam
N nkx2.2a
O nkx6.1
UMAP_1
UMAP_2

Figure 1.3. Identification of CAC subpopulations

(**A**) Schematic representation of my experimental approach. (**B**) Projection of 4,233 GFP+ single-cell transcriptomes onto a UMAP plot showing 11 total clusters (**C**) Heat map showing 2 of the top 10 DE genes for all 11 Clusters identified. (**D-I**) Violin plots showing the expression of 6 known CAC marker genes. (**J-O**) Feature plots showing the expression of 6 known CAC marker genes. Clusters 0-5 were found to be subclusters of CACs. Clusters 6, 7, 8, and 10 were found to be non-CAC clusters.

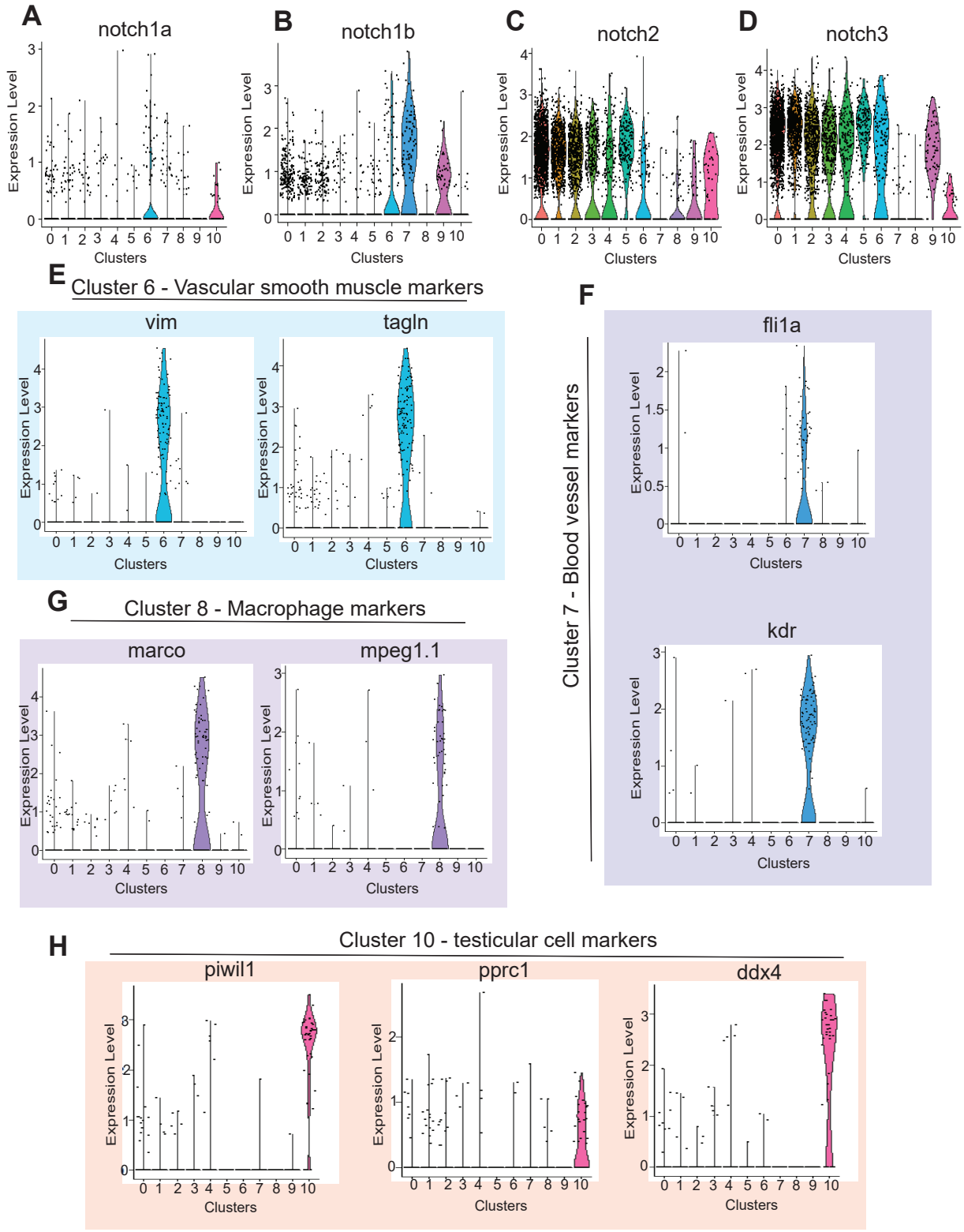
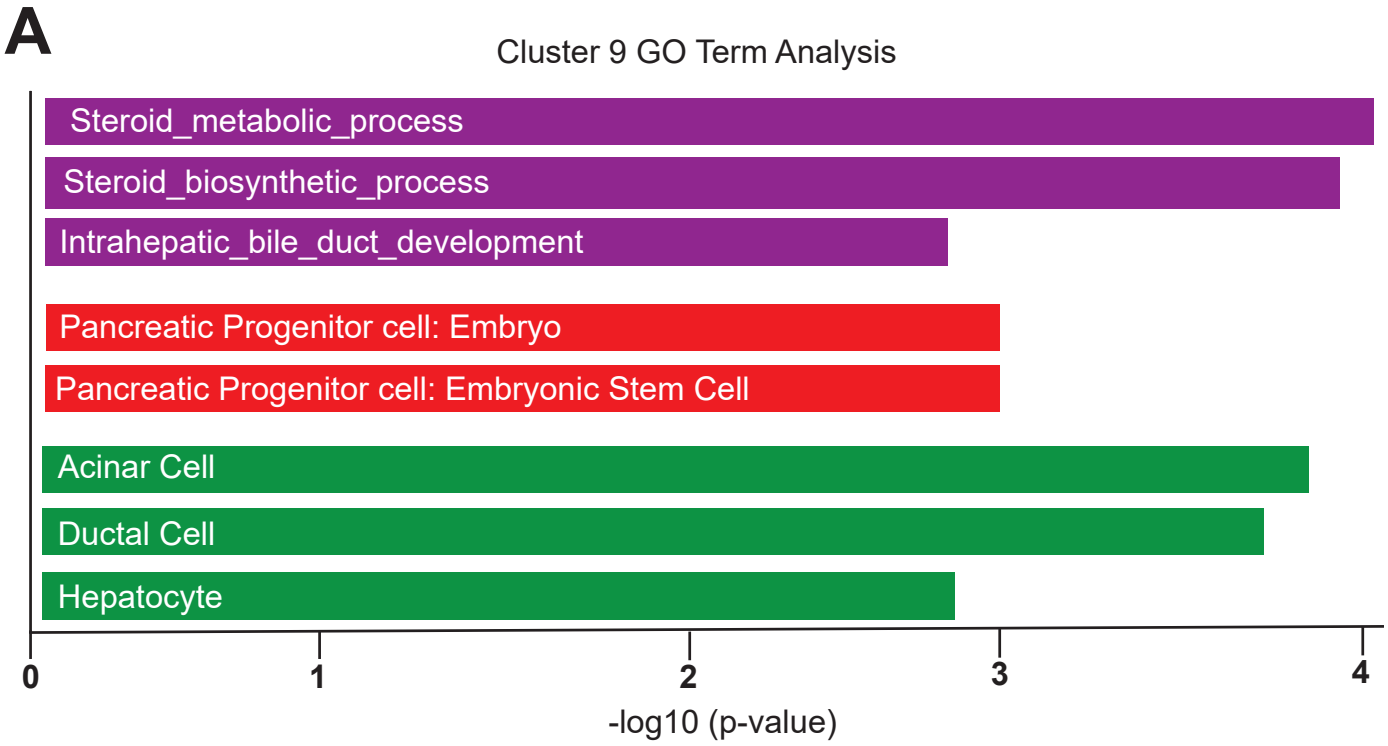
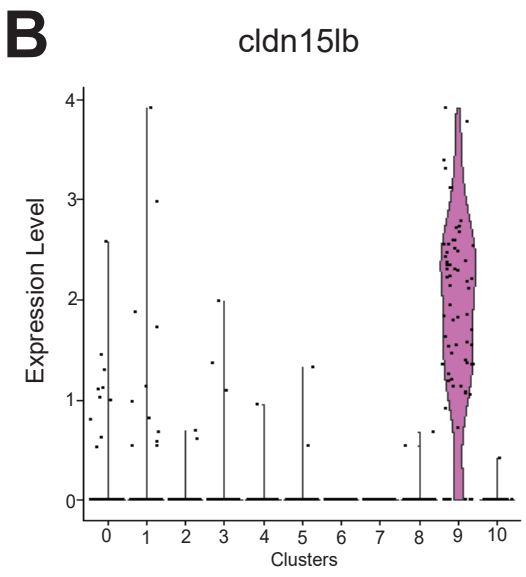

A
notch1a
B
notch1b
C
notch2
D
notch3
Expression Level
Clusters
E
Cluster 6 - Vascular smooth muscle markers
vim
tagln
F
Cluster 7 - Blood vessel markers
fli1a
kdr
G
Cluster 8 - Macrophage markers
marco
mpeg1.1
H
Cluster 10 - testicular cell markers
piwil1
pprc1
ddx4

Figure 2.3. Verification of Notch responsive clusters and identification of non-CAC clusters

(**A-D**) Violin plots showing that all clusters represented in my UMAP expressed at least one out of the four Notch receptors which confirms that all cells sorted for and sequenced are Notch responsive. (**E**) Violin plots showing the expression of *vim* and *tagln* to confirm Cluster 6 as single-cell transcriptomes from vascular smooth muscle. (**F**) Violin plots showing the expression of *fli1a* and *kdr* to confirm Cluster 7 as single-cell transcriptomes from blood vessels. (**G**) Violin plots showing the expression of *marco* and *mpeg1.1* to confirm Cluster 8 as single-cell transcriptomes from macrophages. (**H**) Violin plots showing the expression of *piwil1*, *pprc1*, *ddx4* to confirm Cluster 10 as single-cell transcriptomes from testicular cells.


A
Cluster 9 GO Term Analysis
Steroid_metabolic_process
Steroid_biosynthetic_process
Intrahepatic_bile_duct_development
Pancreatic Progenitor cell: Embryo
Pancreatic Progenitor cell: Embryonic Stem Cell
Acinar Cell
Ductal Cell
Hepatocyte
0
1
2
3
4
-log10 (p-value)


B
cldn15lb
Expression Level
Clusters

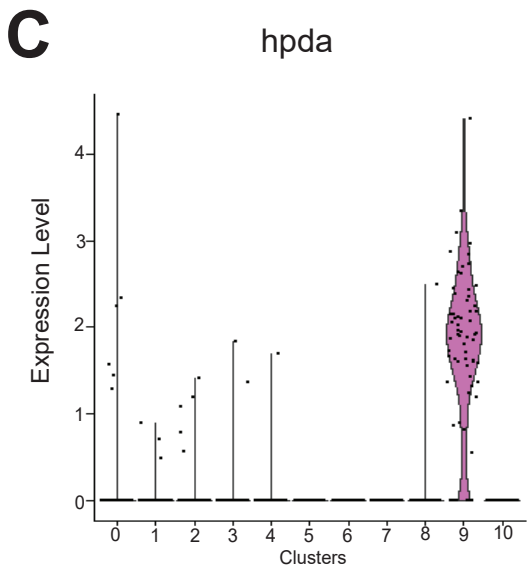

C
hpda
Expression Level
Clusters

Figure 3.3. Identification of CAC subcluster 9

(**A**) GO term analysis using the top 50 DE genes making up Cluster 9. (**B**) Violin plot showing the expression of one of the top DE genes of Cluster 9, *cldn15lb*. (**C**) Violin plot showing the expression of a second top DE gene of Cluster 9, *hpda*. Both *cldn15lb* and *hpda* are two genes associated with the GO terms in (**A**).

Identification of new CAC marker genes

Looking at genes that are broadly expressed across all CAC subpopulations (Clusters 0-5), 5 new potential marker genes were identified *in silico* (Figure **4.3A-C**, **4.3G, 4.3H**) some of which have been implicated in pancreas biology. Violin plots show the expression of *spint2*- a serine peptidase inhibitor (Faller, Gautschi, & Schild, 2014) (**Figure 4.3A**), *cldn7b*- a tight junction protein (Xing et al., 2020), *tm4sf4*- a negative regulator of pancreas differentiation (Anderson et al., 2011) (**Figure 4.3C**), *onecut1*- a DNA-binding transcription factor (Jacquemin, Lemaigre, & Rousseau, 2003) (**Figure 4.3G**), and *id2a*- a regulator of the Notch signaling pathway (Uribe, Kwon, Marcotte, & Gross, 2012) (**Figure 4.3H**) across all CAC subclusters (Clusters 0-5). Out of all the clusters, *tm4sf4* is the most highly expressed in Cluster 9 (**Figure 4.3C**). Whole mount *in situ* hybridization was performed on the dissected pancreas of 5dpf wildtype larvae to verify the *in vivo* expression of *spint2*, *cldn7b*, and *tm4sf4* in CACs. The expression of *spint2* (20/20 pancreata), *cldn7b* (20/20 pancreata), and *tm4sf4* (10/20 pancreata) is restricted to CACs (**Figure 4.3D-F**). Interestingly, the expression of *tm4sf4* appears to be the most obvious in marking the tail of the pancreas and within common bile duct connecting the pancreas and the liver (**Figure 4.3F**). The detection of *tm4sf4* in the common bile duct connecting the liver and pancreas suggests that *tm4sf4* is also a marker of Cluster 9 (**Figure 3.3A**).

Since CACs have been suggested as the potential origin for pancreatic cancer, I looked for genes in my DE gene analysis that are associated with pancreatic cancer. I then looked to see if these genes were expressed in CACs *in silico*. I found six genes that were associated with pancreatic cancer and enriched in my CAC subclusters (**Figure 4.3I-N**). *basal cell adhesion molecule (bcam)* has been suggested as a potential biomarker for pancreatic cancer (K. H. Yu et al., 2009). More recently, *BCAM* expression has been linked to basal cell carcinoma with *EPCAM*

(Guerrero-Juarez et al., 2022). Although lowly expressed in CAC subclusters, Cluster 2 appears to have the greatest number of CACs expressing *bcam* (**Figure 4.3I**). Previous work has shown that pancreatic cancer can be separated into two subtypes: classical and basal where the latter contributes to a poor prognosis because basal cells are primarily made up of proliferating cancerous cells (Krieger et al., 2021). Out of the 6 genes I identified as markers of pancreatic cancer, 3 of them were well known basal cell markers of pancreatic cancer. These 3 genes include *dlg2* – a member of the guanylate kinase family (Juiz et al., 2020) (**Figure 4.3J**), *pde3a*—a phosphodiesterase (Juiz et al., 2020; Nicolle et al., 2017) (**Figure 4.3K**), and *krt15*—a keratin protein that promotes cancer cell migration and invasion (Krieger et al., 2021; Monberg et al., 2022) (**Figure 4.3L**). Although *pde3a* is expressed in only a few number of CACs, it has been suggested as one of the most strongly related basal-like markers in pancreatic cancer when overexpressed (Juiz et al., 2020; Nicolle et al., 2017). The expression of these basal-like genes in CACs suggests that CACs may behave similarly to basal cells found in pancreatic cancer. However, further investigation is needed to confirm this possibility. Two additional cancer genes that were enriched in CAC subclusters includes the expression of *nrp2a* (**Figure 4.3M**) and *cav1* (**Figure 4.3N**). *nrp2a* encodes a neuropilin-2 protein that enhances the progression of pancreatic cancer by promoting vascular endothelia growth factor signaling (L. Wang et al., 2021). *cav1* encodes a structural protein that forms vesicles at the plasma membrane. In humans, the high expression of CAV-1 has been found as a potential prognostic biomarker in metastatic pancreatic cancer and its overexpression has been implicated in chemoresistance (Demirci et al., 2017). Once again, the expression of these cancer genes by CACs suggests that CACs could serve as the potential origin of pancreatic cancer. Perhaps when these pathways become disrupted in CACs it leads to the formation and progression of pancreatic cancer.

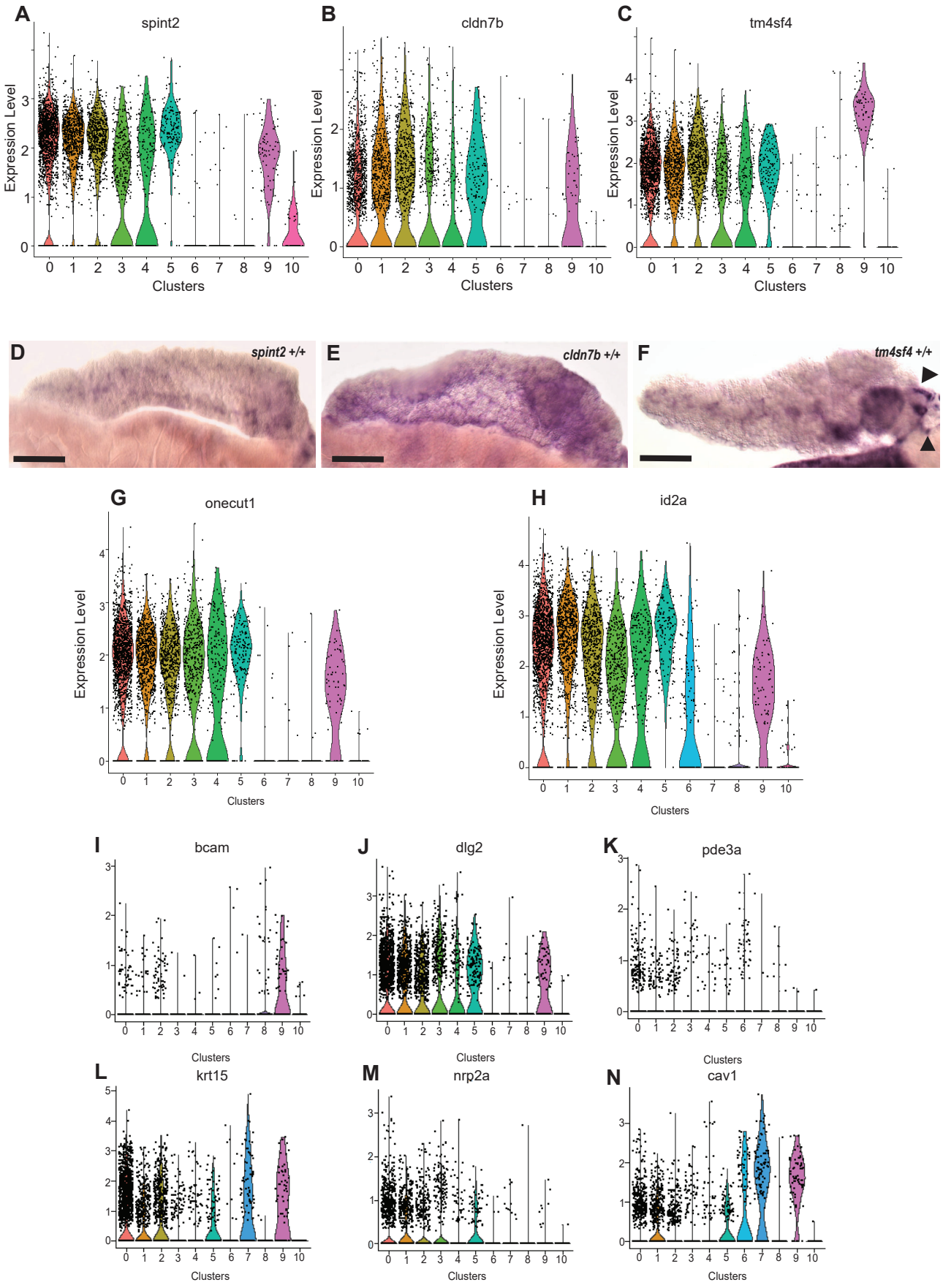

A
spint2
B
cldn7b
C
tm4sf4
Expression Level
Clusters
D
spint2 +/+
E
cldn7b +/+
F
tm4sf4 +/+
G
onecut1
H
id2a
I
bcam
J
dlg2
K
pde3a
L
krt15
M
nrp2a
N
cav1

Figure 4.3. Newly identified marker genes of CACs (**A-C**) Violin plots showing the expression of *spint2*, *cldn7b*, and *tm4sf4* in CAC subclusters 0-5. (**D-F**) Whole mount *in situ* hybridization was performed on dissected pancreata from 5dpf larvae which shows the expression of *spint2*, *cldn7b*, and *tm4sf4* in CACs. (**F**) Black arrows point at the expression of *tm4sf4* in the duct connecting the pancreas and liver. (scale bar = 100uM, 20X). (**G, H**) Violin plots showing the expression of *onecut1* and *id2a* in CAC subclusters 0-5. (**I-N**) Violin plots showing the expression of marker genes (*bcam*, *dlg2*, *pde3a*, *krt15*, *nrp2a*, and *cav1*) that have been affiliated with pancreatic cancer. (**I**) *bcam* is primarily expressed in CAC subclusters 0-2. (**J-N**) *dlg2*, *pde3a*, *krt15*, *nrp2a*, and *cav1* are expressed in all CAC subclusters 0-5.

Identification of a subpopulation of transitioning acinar cells

Given that the CAC population appears to be heterogeneous based on the multiple subclusters of CACs found, I investigated these subpopulations further. Performing GO term analysis on Cluster 4 revealed terms associated with acinar cells of the pancreas (**Figure 5.3A**). Interestingly, one of the terms associated with this cluster was 'pancreatic ductal stem cells'. **Table 1.3** shows the top 22 genes of Cluster 4 and their corresponding p-value (a lower p-value corresponds with a gene significantly enriched in this cluster). The four genes associated with the 'acinar cell' term in **Figure 5.3A** includes *prss1*, *amy2a*, *cpa5*, and *ela2*. Interestingly, *ptf1a* expression could not be detected within this "acinar-like" cluster. Expression of *prss1*, *amy2a*, *cpa5*, and *ela2* are highly enriched Cluster 4 compared to all other clusters (**Figures 5.3B-E**). Cells in Cluster 4 not only expresses acinar cell markers, but these cells are Notch responsive (**Figure 2.3**) and expresses CAC marker genes namely, *cftr*, *clcn1b*, *sox9b*, *epcam*, *nkx2.2a*, and *nkx6.1* (**Figure 1.3D-O**).

Given that Cluster 4 co-expresses both acinar and CAC marker genes, I first hypothesized that this cluster may represent doublets in my analysis that were not initially removed during QC. To rule out Cluster 4 as a population of doublets, Changhan (a postdoc from Dr. Qing Nie's Lab) helped me project total gene expression (UMI counts) onto my UMAP (**Figure 6.3A**). Although the UMAP flips in this analysis, Cluster 4 can be found at the bottom of the UMAP. As can be seen, Cluster 4 expresses the same level of genes as all other clusters. This bioinformatically rules out Cluster 4 as a population consisting of doublets. Next, I hypothesized that Cluster 4 is a real population of cells co-expressing acinar and CAC markers. Cluster 4 could represent a population of transitioning acinar cells to CACs. The scientific premise for this hypothesis comes from what we know about pancreatic cancer (Chuvin et al., 2017). Acinar cells becoming ductal cells is a

characteristic associated with pancreatic cancer and in pancreatic cancer, this process is known as metaplasia (Chuvin et al., 2017; Kopp et al., 2012). During this process, acinar cells can be found to co-express both acinar and ductal genes. To resolve Cluster 4 as a potential transitioning state of cells, I used Monocle3 to arrange cell transcriptomes in an unbiased pseudo temporal manner. A UMAP was generated in Monocle3 but only the 'acinar-like' cells (Cluster 4) and Clusters 0-3, 5, and 9 were included in the Pseudotime analysis (**Figure 6.3B**). Cluster 4 was made the starting point and Cluster 0 (CACs) was the end point for the Pseudotime trajectory (**Figure 6.3C**). From this analysis, the 'acinar-like cells' transition first into CAC subcluster 3, and from this subcluster there are two branching points from which acinar cells transition next (**Figure 6.C**). In one branch, 'acinar-like' cells from Cluster 3 end in the CAC subcluster 0. In the second branch, 'acinar-like' cells from Cluster 3 end in CAC subcluster 1. **Figure 6.3D** represents the numbered Pseudotime trajectory, which corresponds to changes in CAC marker genes represented in **Figure 6.3E**. From the Pseudotime trajectory, the 6 CAC marker genes (*epcam*, *sox9b*, *cftr*, *clcn1b*, *nkx2.2a*, and *nkx6.1*) appear to increase along the Pseudotime trajectory (**Figure 6.3E**).

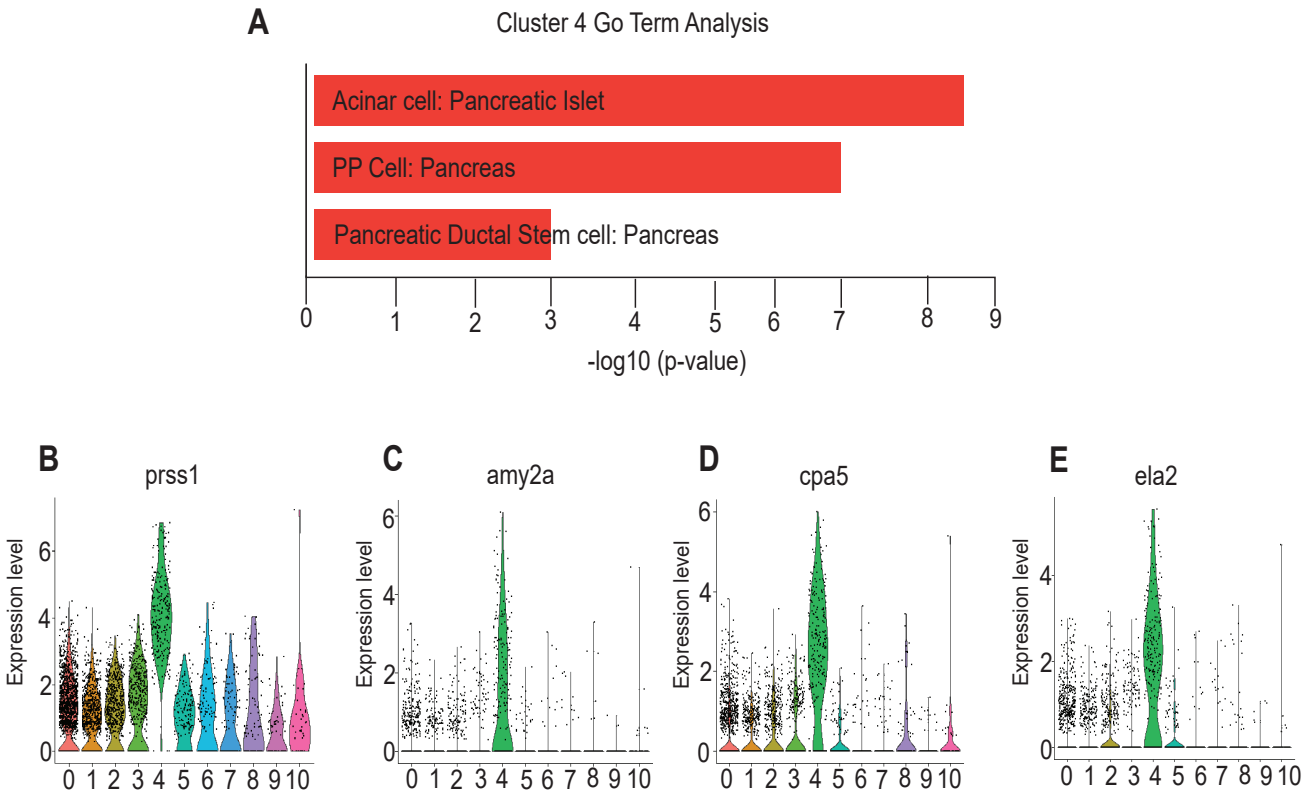

A
Cluster 4 Go Term Analysis
Acinar cell: Pancreatic Islet
PP Cell: Pancreas
Pancreatic Ductal Stem cell: Pancreas
0 1 2 3 4 5 6 7 8 9
-log10 (p-value)
B
prss1
C
amy2a
D
cpa5
E
ela2
Expression level
0 1 2 3 4 5 6 7 8 9 10

Figure 5.3. Identification of an acinar like subpopulation that co-expresses CAC marker genes

(**A**) GO term analysis using the top 50 DE genes of Cluster 4 predicts an acinar cell profile. (**B-E**) Violin plots showing the expression of 4 known acinar marker genes: *prss1*, *amy2a*, *cpa5*, and *ela2*.

Table 1.3 Top 22 DE Genes of Cluster 4

Gene	avg_log2FC	p_val_adj
CELA1	4.46561004	3.64E-130
cel.2	3.43699437	5.31E-130
prss1	4.43053306	2.60E-129
prss59.2	4.31822706	1.17E-127
ctrb1	4.4390472	2.93E-126
cpa5	3.98453587	9.19E-121
zgc:92590	3.60358054	1.15E-120
pla2g1b	1.903603	2.53E-120
prss59.1	4.46564236	2.74E-119
sycn.2	3.04706716	1.05E-118
ela3l	4.15609906	1.20E-118
cpb1	3.69312711	1.58E-114
ctrl	3.89347808	6.71E-114
si:ch211-240l19.6	2.8180256	2.36E-113
cel.1	3.54866784	4.71E-110
sycn.1	2.41164341	1.63E-107
zgc:112160	3.69502999	1.15E-106
fep15	1.12224385	4.57E-102
ela2l	4.11365149	6.59E-100
ela2	3.57348087	1.62E-97
c6ast3	2.74784822	1.76E-95
amy2a	3.70242319	1.29E-90

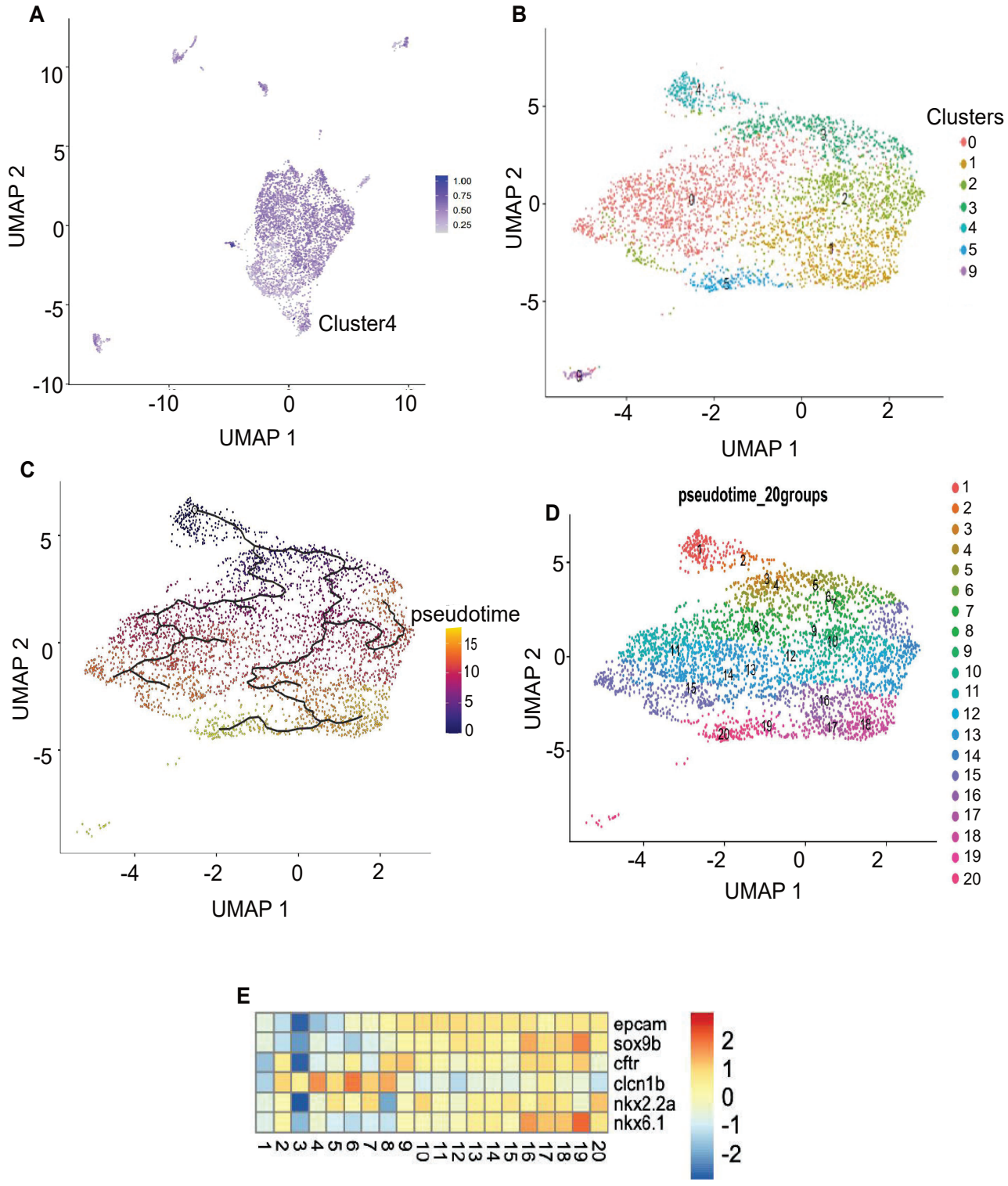

A
UMAP 2
UMAP 1
Cluster4
1.00
0.75
0.50
0.25
B
Clusters
0
1
2
3
4
5
9
C
pseudotime
15
10
5
0
D
pseudotime_20groups
E
epcam
sox9b
cftr
clcn1b
nkx2.2a
nkx6.1
2
1
0
-1
-2

Figure 6.3. Pseudotime analysis demonstrating a potential transition between acinar cells and CACs

(**A**) UMAP showing gene expression (UMI) plotted (**B**) UMAP showing clustering using Moncle3 and including Clusters 0-5 and 9 in the pseudotime analysis. (**C**) Pseudotime analysis using Cluster 4 as the starting point and Cluster 0 as the end point. The heat map shows purple as the starting point and yellow as the end point along the pseudotime trajectory (**D**) Pseudotime analysis showing the numbered points along the trajectory that corresponds to gene expression shown in (**E**). (**E**) Shows heat map of 6 CAC specific marker genes that become upregulated along the pseudotime trajectory.

To better resolve this hypothesized transition state of these 'acinar-like cells', I performed scRNA-seq on the whole pancreas of the adult zebrafish. By looking at the whole pancreas, I predicted to find more intermediate cell identities between acinar cells and CACs including mature acinar cells (hence, non-transitioning acinar cells), transitioning acinar cells, and CACs. **Figure 7.3A** shows a representative UMAP of the whole pancreas. Once again, known CAC marker genes including *cftr*, *clcn1b*, *sox9b*, *epcam*, *nkx2.2a*, and *nkx6.1* were used to identify the CAC cluster represented in the whole pancreas UMAP (**Figures 7.3B-G**). Cluster 1 was identified as CACs based on the expression of these markers. Here, CACs form a single cluster and heterogeneity is lost at this level of cellular resolution. To identify acinar cells in the whole pancreas UMAP, known acinar marker genes were used namely, *ptf1a*, *prss1*, *amy2a*, *cpa5*, and *ela2* (**Figures 7.3H-K**). Cluster 0 was found to be acinar cells based on the expression of these markers. Surprisingly, I found fewer than expected cells expressing these acinar markers in Cluster 0. I believe this is a result of these cells dying during the FACS process and leaking ambient RNA, which is a known phenomenon that can happen as cells are dying (Wolfien, David, & Galow, 2021). *Ptf1a* expression was also not detected in this cluster. Since *Ptf1a* is a transcription factor, it will not be highly expressed in cells. Just as lower than expected expression was seen for the other acinar marker genes, it is likely that *ptf1a* expression is not detected in Cluster 0 because of RNA leakage. It is also possible that the acinar cells captured here are not mature acinar cells which normally express *ptf1a*. Mature acinar cells (which are much larger cells) were found to die during the FAC sorting process compared to smaller and less mature acinar cells. Therefore, the acinar cells captured in this analysis are most likely less mature acinar cells with lower levels of *ptf1a* expression that go undetected. Regardless, Cluster 1 expresses little to no acinar marker genes which suggests an absence of the 'acinar-like' cluster found in the genomic analysis of sorted

CACs (**Figure 5.3**). Although performing scRNA-seq on the whole pancreas did not reveal a transition state of acinar cells, it did clearly distinguish a separation between acinar cells and ductal cells. This is seen by the two clusters being further apart on the UMAP for whole pancreas compared to the UMAP presented in **Figure 1.3**. This finding suggests that the 'acinar like' Cluster in **Figure 1.3B** may be different than the acinar cluster found in **Figure 6.3A**. One explanation as to why the 'acinar like' cluster is not found in **Figure 6.3A** could be a result of its small population size which makes it less likely to get captured and sequenced during scRNAseq compared to the rest of the pancreas. This is seen for the CAC cluster where subpopulations of CACs are lost when the whole pancreas is sequenced.

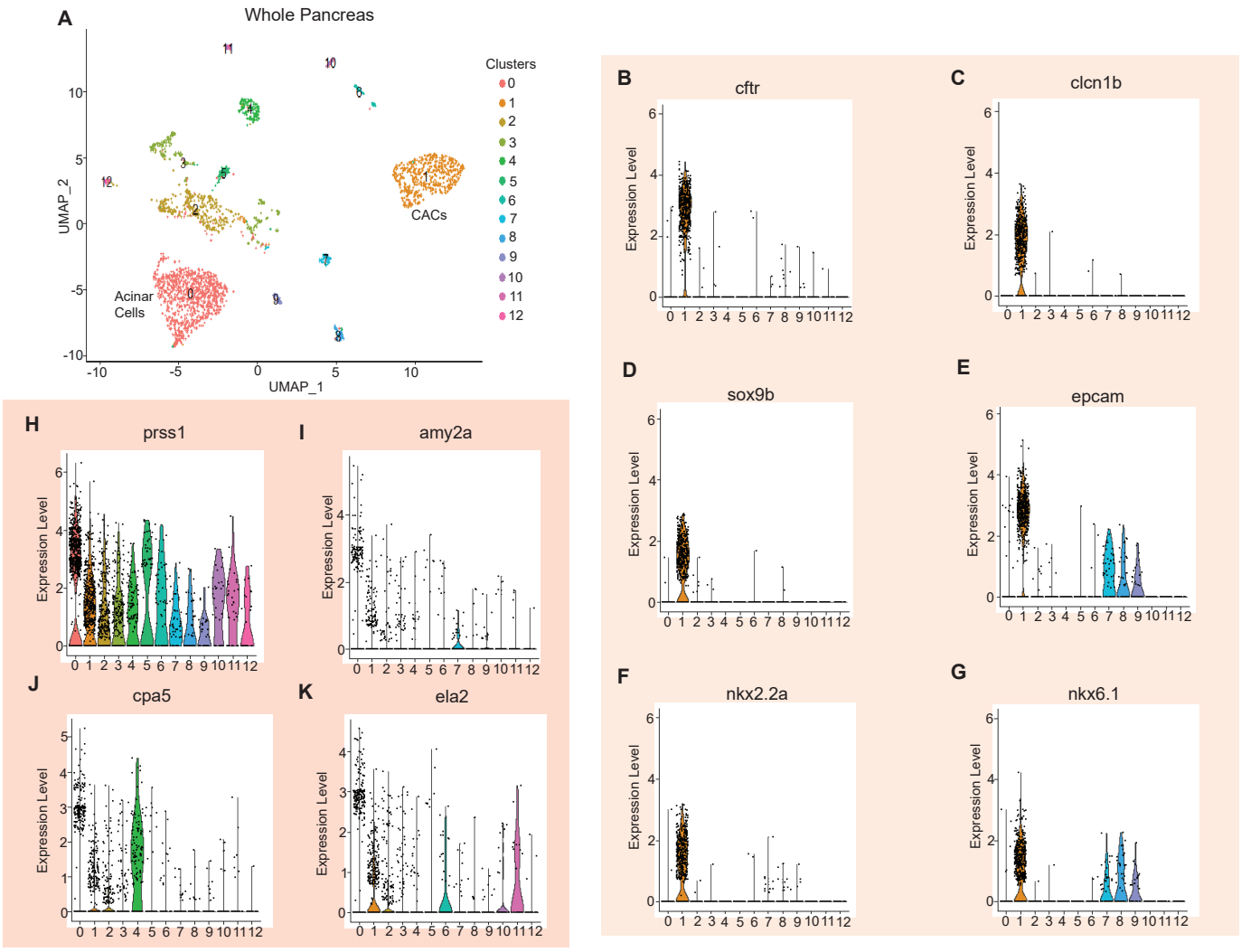

A
Whole Pancreas
UMAP_2
UMAP_1
Clusters
0
1
2
3
4
5
6
7
8
9
10
11
12
Acinar Cells
CACs
B
cftr
C
clcn1b
D
sox9b
E
epcam
F
nkx2.2a
G
nkx6.1
H
prss1
I
amy2a
J
cpa5
K
ela2
Expression Level

Figure 7.3. Gene expression of CAC and acinar gene markers in the whole pancreas

(**A**) Whole pancreas single-cell transcriptomes projected onto a UMAP. (**B-G**) Violin plots looking at gene expression of specific CAC marker genes including *cftr*, *clcn1b*, *sox9b*, *epcam*, *nkx2.2a*, and *nkx6.1*. Cluster 1 was identified as CACs based on the expression of these 6 CAC marker genes. (**H-K**) Violin plots looking at gene expression of specific acinar marker genes including *prss1*, *amy2a*, *cpa5*, and *ela2*. Cluster 0 was identified as acinar cells based on the expression of these 4 acinar marker genes.

Identification of a subpopulation of CACs with immune-like expression patterns

Performing GO term analysis on Cluster 5 using the top 50 DE genes revealed terms associated with immune-like characteristics such as 'antigen processing and presentation', 'immunoregulator interactions', 'antigen processing: cross presentation', and 'interferon-gamma signaling pathway' (**Figure 8.3A**). The genes associated with the terms 'antigen presentation' and 'immunoregulator interactions' were *mhc2dab*- a major histocompatibility complex predicted to be involved in antigen presenting and processing (Lewis, Del Cid, & Traver, 2014), *cd74a* and *cd74b*- two protein chains that are part of MHCII (Schroder, 2016), and *cd99*- which is involved in T Cell activation (Wingett, Forcier, & Nielson, 1999). A Violin and Feature Plot confirms that these genes are expressed the highest in Cluster 5 out of all other CAC subpopulations (**Figure 8.3B-I**). *mhc2dab, cd74a,* and *cd74b* are also expressed in the macrophage cluster (Cluster 8). From the Violin plot *cd99* appears to be highly expressed in Cluster 5 (**Figure 8.3E**), however, upon closer examination using a Feature Plot (**Figure 8.3I**), *cd99* is expressed lowly in Cluster 5 and the other CAC subclusters. From the Feature Plots, *mhc2dab*, *cd74a*, and *cd74b* are clear marker genes of Cluster 5 compared to all other CAC subclusters (**Figure 8.3F-H**). In conclusion, Cluster 5 supports my hypothesis that the CAC population is heterogeneous. Cluster 5 stands out on its own amongst the other CAC subclusters because Cluster 5 has distinct marker genes that separate it from all other CAC subclusters.

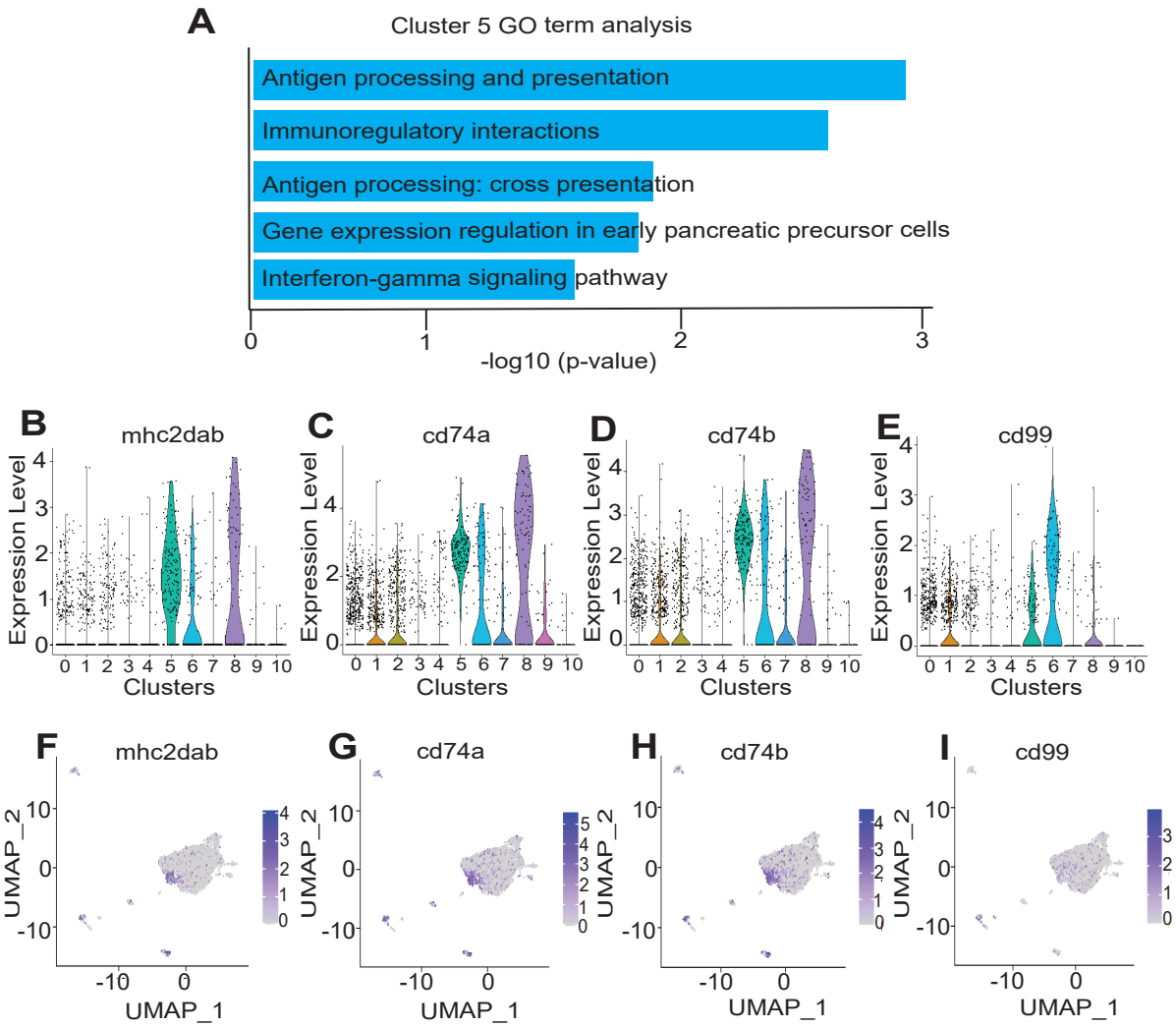

A
Cluster 5 GO term analysis
Antigen processing and presentation
Immunoregulatory interactions
Antigen processing: cross presentation
Gene expression regulation in early pancreatic precursor cells
Interferon-gamma signaling pathway
0
1
2
3
-log10 (p-value)
B
mhc2dab
C
cd74a
D
cd74b
E
cd99
Expression Level
Clusters
F
mhc2dab
G
cd74a
H
cd74b
I
cd99
UMAP_2
UMAP_1

Figure 8.3. Identification of an immune-like subcluster of CACs

(**A**) GO term analysis of the top DE genes of Cluster 5 reveals a subcluster of CACs with an immune cell role. (**B-E**) Violin plot showing the expression of the 4 genes (*mhc2dab, cd74a*, *cd74b*, and *cd99*) associated with immune GO terms for Cluster 5. (**F-I**) Feature plots showing the expression of the same 4 genes from (**B-E**).

CACs could serve as a potential source of ins/sst1.1 double positive cells involved in beta-cell neogenesis

A very small population of double positive cells expressing both insulin (*ins*) and somatostatin 1.1 (*sst1.1*) were found on my UMAP (**Figure 9.3A, 9B,**). The expression of *ins* and *sst1.1* across my scRNA-seq dataset was visualized using a Feature Plot (**Figure 9.3A, 9.3B**). These double positive cells were not originally separated into their own cluster (**Figure 1.3B**). Further clustering was performed which separated this cluster into what is now called Cluster 11 (**Figure 9.3C**). This finding is interesting because these double positive cells could represent an endocrine precursor population. The cellular origin of these double positive cells remains largely unknown although delta cells are thought to be the cellular origin because they are the primary cell type that expresses *sst1.1*. In a recent genomics study by Singh et al., it was shown that that *ins/sst1.1* double positive cells were found within the islet of the adult zebrafish pancreas (S. P. Singh et al., 2022). These double positive cells also co-express *dkk3b* and *pyyb* which were specific markers of this double positive population residing in the islet. I hypothesized that these double positive cells are an endocrine precursor and that these cells found by Singh et al. originate from CACs. To test whether CACs could be the origin of these endocrine precursor cells, I used a Feature Plot to look at the expression of known CAC marker genes in this double positive population of cells. I found that Cluster 11 does indeed express 5 out of 6 of these known markers (**Figures 1.3J, 1.3K, 1.3M-1.3O**). The only CAC marker gene that was absent in Cluster 11 was *sox9b* (**Figure 1.3L**). Finding that the expression of *sox9b* is lower in these cells is interesting because lower levels are needed to regulate endocrine differentiation (W. Huang et al., 2016). I also found that the expression of *dkk3b* and *pyyb* was absent in Cluster 11 (**Figures 10.3A, 10.3B**). The absence of *dkk3b* and *pyyb* suggests that the endocrine precursor cells I found in my analysis

are a different population of cells compared to those found by Singh et al. It is likely that Singh et al. is looking at a later stage of double positive cells residing in the islet compartment where I am looking at an earlier stage of these endocrine precursors arising from CACs that will eventually give rise to the cells identified by Singh et al.. Therefore, the expression of *dkk3b* and *pyyb* may be islet specific markers marking this population of endocrine precursor cells residing in the islet where the endocrine precursor cells in my analysis are still marked by CAC marker genes instead. To test whether *dkk3b* and *pyyb* expression is specific to the islet compartment, I looked at my whole pancreas scRNA-seq data set and identified endocrine cells as Cluster 7 (**Figure 10.3C**) based on the expression of *sst1.1*, *ins*, and *gcga* (**Figures 10.3D-F**). I then looked at the expression of *dkk3b* (**Figure 10.3G**) and *pyyb* (**Figure 10.3H**) and found that these two genes are expressed in the endocrine cell cluster. These findings further suggest that the *ins/sst1.1* cells identified by Singh et al. were a later stage of endocrine precursor cells found within the islet compared to the those identified in my genomics study.

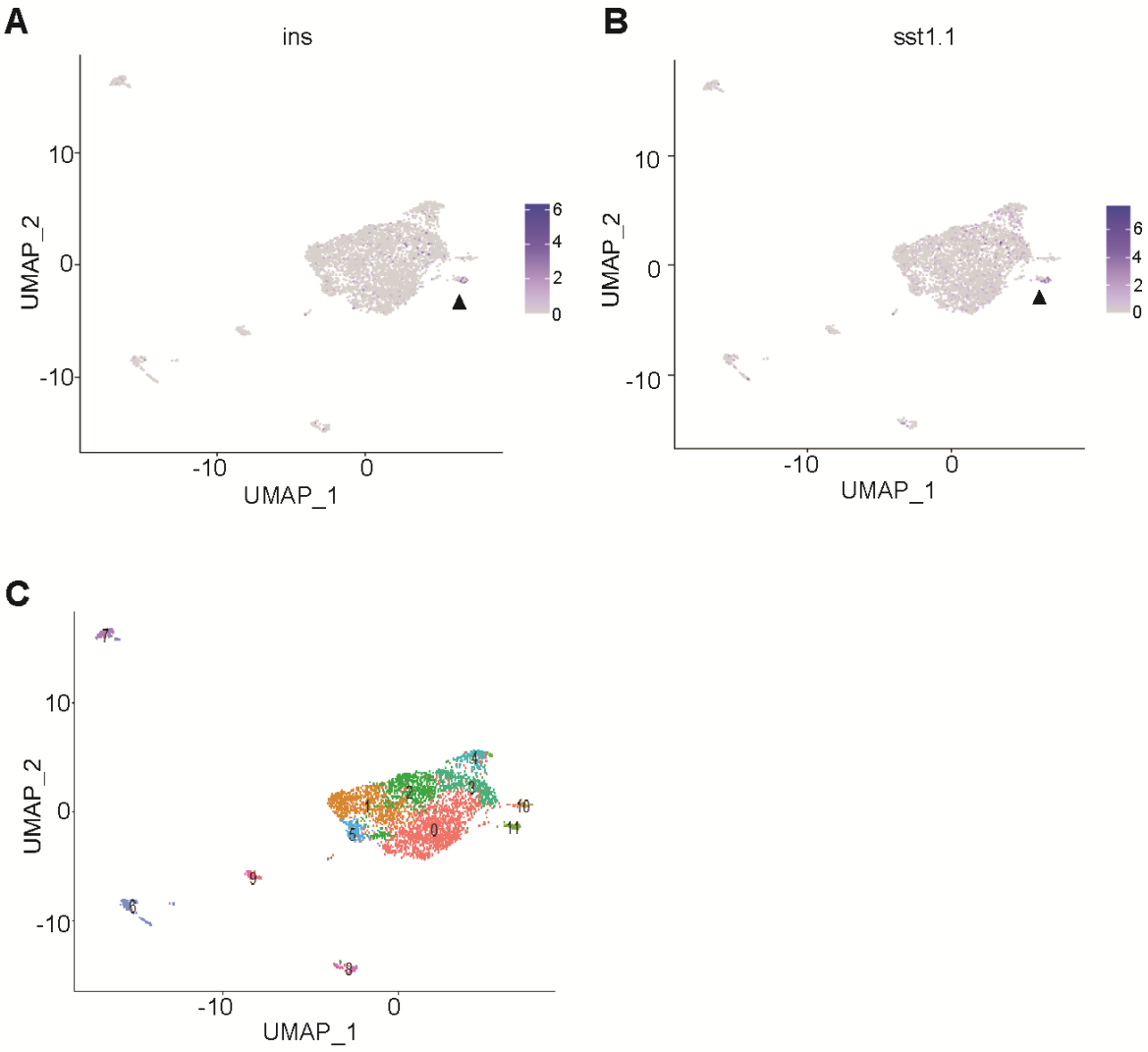

A
ins
B
sst1.1
C
UMAP_1
UMAP_2
10
0
-10
6
4
2
0

Figure 9.3. Identification of a bihormonal population of CACs expressing *sst1.1* and *ins*

(**A-B**) Feature plots showing the expression of *ins* and *sst1.1* in the genomics data generated from the transcriptomes of GFP+ cells. A black arrow points to an identified bihormonal subpopulation expressing both *ins* and *sst1.1*. (**C**) UMAP showing further clustering into 12 clusters and demonstrating that the bihormonal subpopulation identified can cluster into its own cluster (now called Cluster 11).

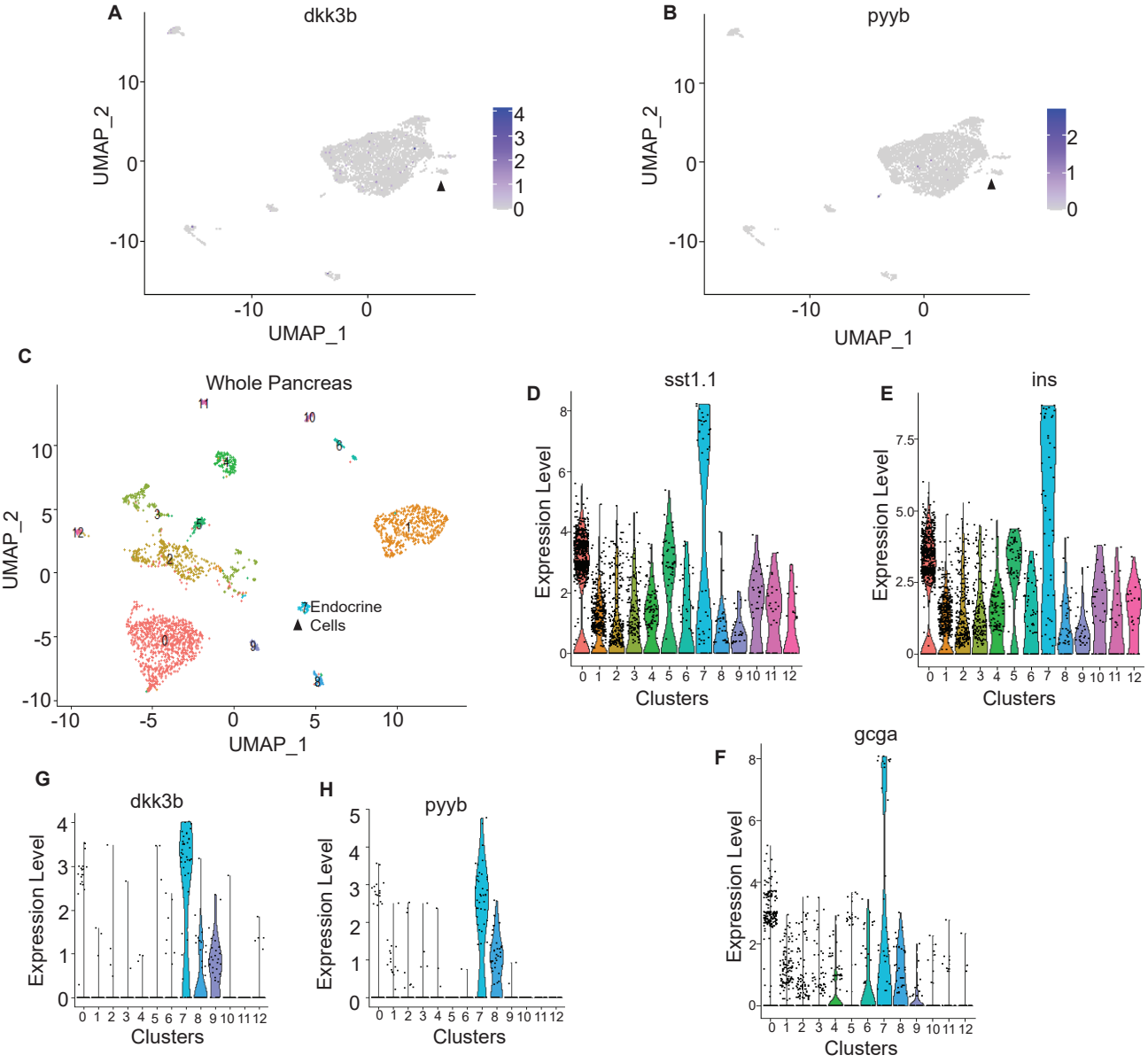

A
dkk3b
UMAP_2
UMAP_1
B
pyyb
C
Whole Pancreas
Endocrine
Cells
D
sst1.1
Expression Level
Clusters
E
ins
F
gcga
G
dkk3b
H
pyyb

Figure 10.3. ScRNA-seq of the whole pancreas predicts an early state of bihormonal cells identified from the scRNA-seq of CACs.

(**A-B**) Feature plots show the absence of the expression of *dkk3b* and *pyyb* in the bihormonal cells identified from sorted CACs. (**C**) The single-cell transcriptomes of the whole pancreas were projected onto a UMAP plot. (**D-F**) Violin plots show the expression of *sst1.1*, *ins*, and *gcga* across the single-cell transcriptomes of the whole pancreas. Black arrows point to Cluster 7 which was identified as a small cluster of endocrine cells. (**G, H**) Violin plots show the expression of *dkk3b* and *pyyb* in clusters from the whole pancreas.

SOX9 is a transcription factor that maintains pancreatic progenitors during pancreas organogenesis and has a role in endocrine cell differentiation (Seymour et al., 2007). During pancreas development, SOX9 expression is restricted to a population of Notch-responsive pancreatic progenitors. The expression of SOX9 is absent in both endocrine precursors and differentiated cells. In mice, the inactivation of Sox9 causes pancreatic hypoplasia that results from the progenitor pool of the pancreas becoming depleted (Seymour et al., 2007). In zebrafish, there is a haploinsufficient phenotype where beta-cell regeneration is accelerated in *sox9b* heterozygous mutant adult fish (W. Huang et al., 2016). To better understand why *sox9b* heterozygous mutants have an increased regenerative phenotype, I crossed *sox9b* heterozygous mutant fish to *tp1:eGFP* and performed scRNA-seq on sorted PNCs expressing heterozygous levels of *sox9b* (**Figure 11.3**). The data generated from these mutants was integrated with the wildtype PNCs from **Figure 1.3** to determine differences in gene expression or clustering between the two data sets. **Figure 11.3A** is the UMAP plot generated after integration. As done previously, CAC subclusters were identified by looking at the expression of known CAC marker genes (**Figures 12.3A-F**). I identified Clusters 0-5 as CAC subclusters (**Figure 11.3A**). These CAC subclusters were like those found in **Figure 1.3B**. This finding indicates the reproducibility of the data sets and suggests that there are no changes in the composition of the CAC subclusters present between the wildtype and *sox9b* heterozygous mutant data set. A list of all DE genes was obtained to determine which genes had a change in their expression between the wildtype and *sox9b* heterozygous mutant datasets. Interestingly, one of the genes that was found in this list was *sst1.1*. The expression of *sst1.1* was plotted for both the wildtype and *sox9b* heterozygous mutant data set and it was found that more cells in the *sox9b* heterozygous mutant data set expressed *sst1.1* compared to cells in the wildtype dataset (**Figure 11.3B**). A Feature Plot was used to better examine the expression of *sst1.1* across

CAC subclusters. This finding demonstrates an increase in the number of CACs expressing *sst1.1* with heterozygous levels of *sox9b* (**Figure 11.3D**) compared to CACs with wildtype levels of *sox9b* (**Figure 11.3C**).

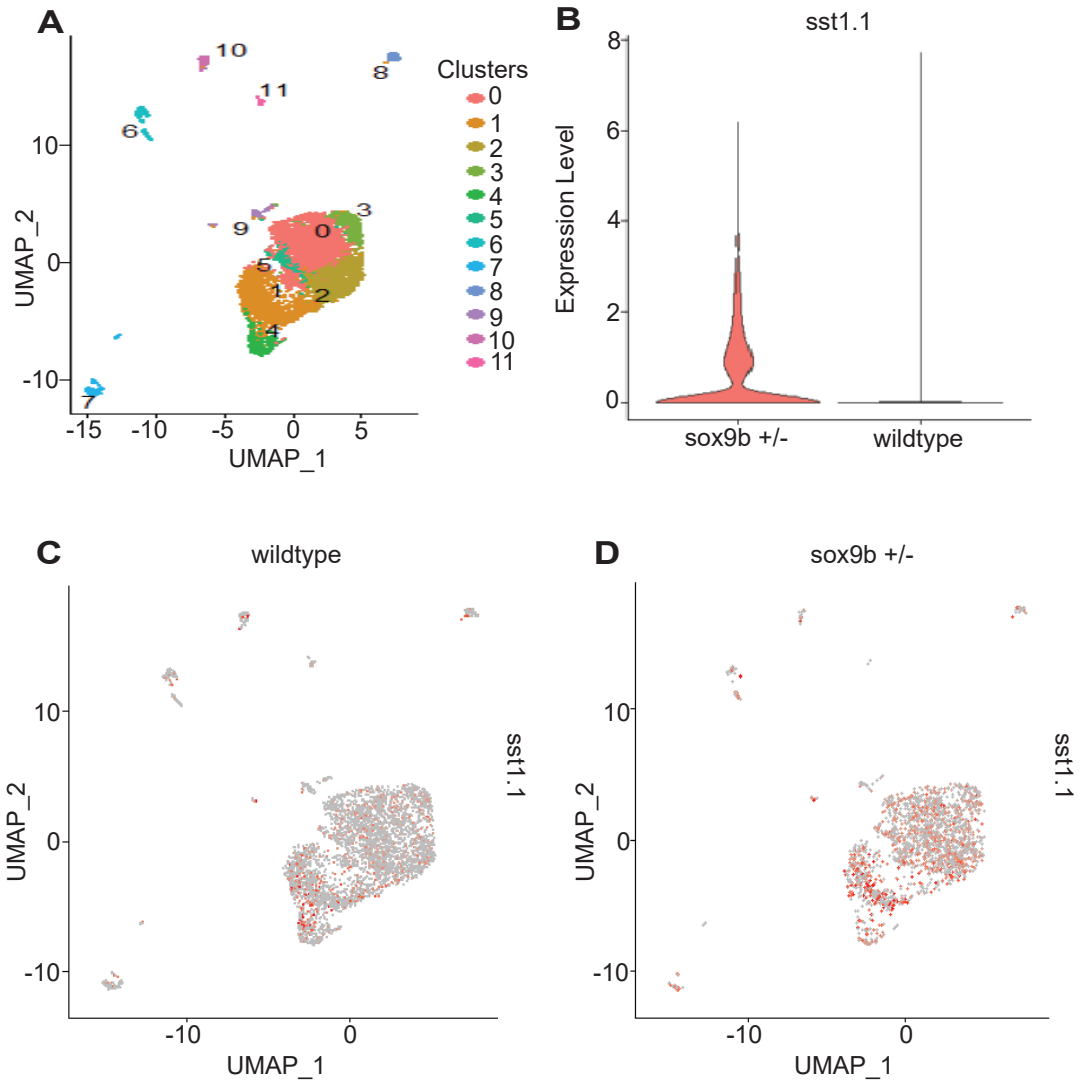

A
B
C
D
Clusters
0
1
2
3
4
5
6
7
8
9
10
11
UMAP_1
UMAP_2
sst1.1
Expression Level
sox9b +/-
wildtype

Figure 11.3. sst1.1 expression increases in the CACs of *sox9b* heterozygous mutant zebrafish

(**A**) The CAC transcriptomes from both wildtype and *sox9b* heterozygous mutants were integrated and projected onto a UMAP plot. (**B**) A violin plot shows the expression of *sst1.1* in *sox9b* heterozygous mutants versus wildtype. (**C, D**) Feature plots show the expression of *sst1.1* in both the CAC subclusters (Clusters 0-5,9) of wildtype and *sox9b* heterozygous mutants. There is a clear increase in the expression of *sst1.1* in the CAC subclusters represented in the *sox9b* heterozygous mutants compared to the CAC subclusters represented in the wildtype fish.

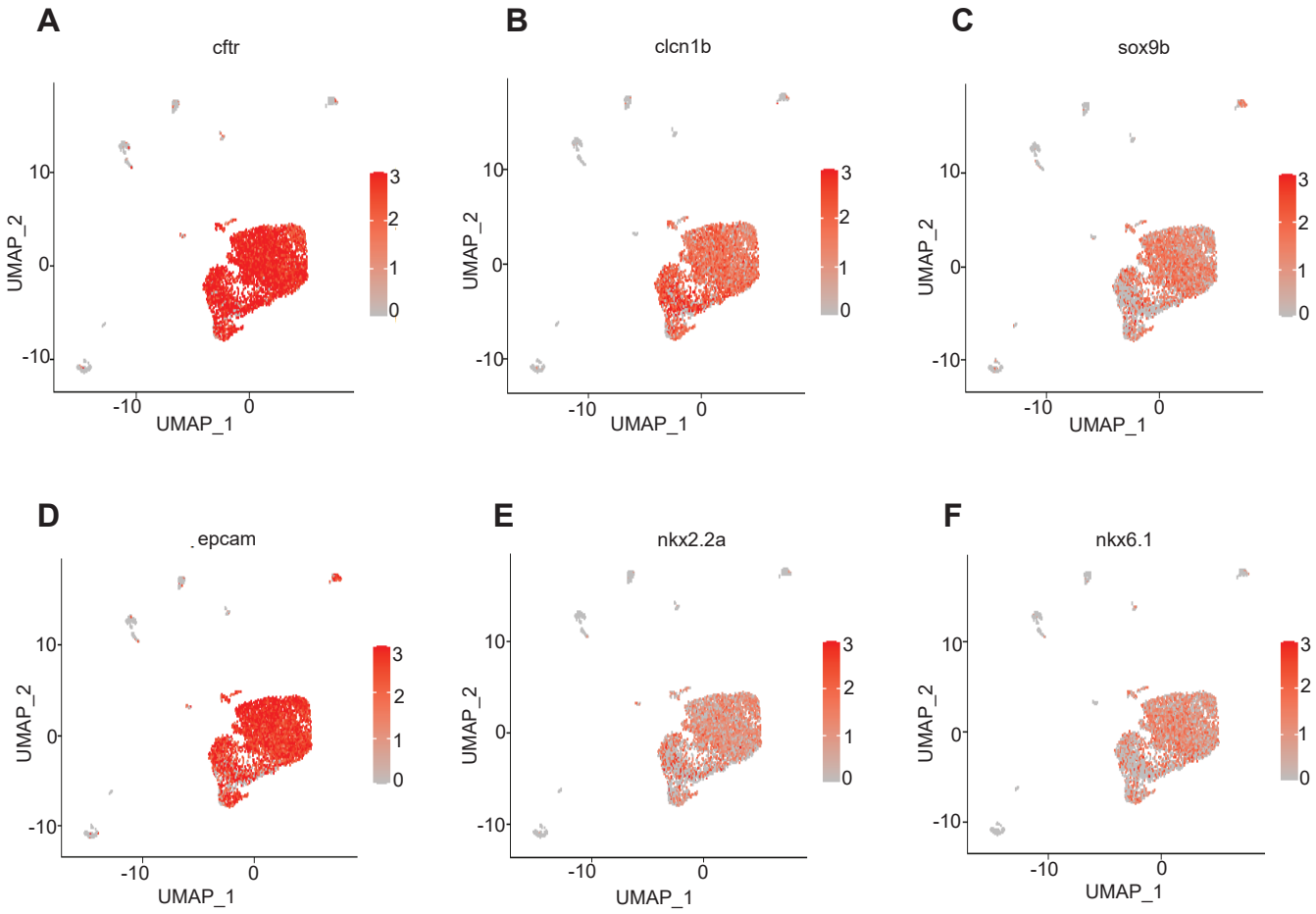

A
cftr
B
clcn1b
C
sox9b
D
epcam
E
nkx2.2a
F
nkx6.1
UMAP_1
UMAP_2
10
0
-10
3
2
1
0

Figure 12.3 Identification of CAC subclusters from the wildtype and *sox9b* heterozygous mutant integrated scRNA-seq data

(**A-F**) Feature plots show the expression of 6 known CAC marker genes (*cftr*, *clcn1b*, *sox9b*, *epcam*, *nkx2.2a*, *nkx6.1*) across the wildtype and *sox9b* heterozygous mutant integrated data set. The highest expression of these genes is indicated in red with no expression being in grey. Clusters 0-5, and 9 were identified as CAC subpopulations based on the expression of these known CAC marker genes.

c) Conclusion

Previously, ductal cells/CACs were isolated from the adult zebrafish pancreas to characterize their transcriptome (Delaspre et al., 2015; Ghaye et al., 2015; Tarifeno-Saldivia et al., 2017). For the first time, I analyzed adult zebrafish CACs at the single-cell transcriptome level and revealed CAC heterogeneity. I identified various CAC subpopulations including an acinar to ductal cell transitioning subpopulation, a subpopulation of CACs with an immune cell profile, and a potential beta-cell precursor subpopulation. I also identified new CAC marker genes that may play a role in both pancreatic cancer and the progenitor capacity of CACs. Overall, my scRNA-seq data revealed novel CAC subpopulations that could further explain the role CACs have as a source for pancreatic cancer and as a beta-cell progenitor.

Cluster 4 was identified as a potential transitioning subpopulation of acinar cells to CACs. The transition of acinar cells to a more ductal phenotype is a common process found in pancreatic cancer called ADM. It is not clear of the cell source for pancreatic cancer but both CACs and acinar cells could be a potential source. Evidence of this comes from looking at early stages of PDAC formation where it has been seen that acinar cells lose their characteristics and take on a more ductal cell profile such as the expression of ductal marker genes and ductal cell morphology (Kopp et al., 2012). These observations suggest that acinar cells could be the source of PDAC but must first transition through a ductal-like state during the early stages of transformation to give rise to pancreatic cancer. Although ADM is most associated with pancreatic cancer, a genomics study on human pancreatic ductal cells reveals that this transitioning process may be a normal process of the pancreas under non-cancerous conditions. Qadir et al. performed scRNA-seq on isolated human ALK3+ ductal cells and not only did they reveal that the ductal cell population is

heterogeneous, but they also identified a transition state of ductal cells becoming acinar cells (Qadir et al., 2020). Both my genomics study of CACs and Qadir et al.'s genomics study on human ductal cells uses Pseudotime analysis to predict the directionality of this acinar or ductal/CAC transition state. One of the pitfalls with Pseudotime analysis is that one must know the starting and end point to predict the directionality of a cell transition. If the starting and end point are not known, Pseudotime analysis can force transition state predictions based on any given starting or end point. Therefore, either *in vivo* methods are needed to validate the directionality of this transition state or better bioinformatics tools are required to resolve cell transition states. One bioinformatics tools that can better predict the direction of cell transition states is RNA velocity (La Manno et al., 2018). RNA velocity does not require a known starting point or end point because it predicts cell transition states based on changes in spliced (mature) transcripts versus unspliced (immature) transcripts. Once a script has been worked out for using RNA velocity on my dataset, our lab aims to use this bioinformatics tool to validate our Pseudotime predictions.

There are already some *in vivo* lineage tracing studies done in the zebrafish that may already confirm the directionality of this 'acinar-like' transition state seen in my analysis. During development acinar cells have been found to directly transition into CACs (Y. J. Wang, Park, Parsons, & Leach, 2015). Lineage tracing studies in CACs have demonstrated that CACs contribute very little to acinar cells during early development and do not appear contribute to acinar cells at later stages of development (Y. Wang et al., 2011). The transition of acinar cells into CACs can be enhanced when the levels of *ptf1a* are lowered (Y. J. Wang et al., 2015). Ptf1a is a master regulator of both pancreatic development and acinar cell fate specification. The expression of Ptf1a specifically becomes restricted to mature acinar cells (Hesselson, Anderson, & Stainier, 2011). Surprisingly, I found that the acinar-like cells of Cluster 4 did not express *ptf1a* but express other

acinar marker genes (*prss1, amy2a, cpa5*) and CAC marker genes (*epcam, sox9b, cftr, cldn7b, nkx2.2a, nkx6.1*) concurrently. Looking at gene expression of Cluster 4, this finding could suggest that these acinar-like cells are decreasing acinar marker genes and upregulating CAC marker genes as they transition into becoming CACs. Looking at the transcriptome of the whole pancreas, the acinar cells I identified as Cluster 1 also do not express *ptf1a*. One possible explanation for this is that because *ptf1a* is a transcription factor its expression is low and may go undetected in the scRNA-seq analysis.

The accumulating *in vivo* evidence of acinar to ductal cell transition strongly supports that the transition state I am seeing in my genomics data is a transition of acinar cells to CACs. Regardless, *in vivo* studies that lineage trace acinar cells in the adult zebrafish are required to validate transition directionality. Our lab is currently developing a transgenic line that can carefully lineage trace acinar cells in the adult fish.

From my genomics study, I have also identified a subpopulation of CACs with a potential role in the immune system. By DE gene analysis, Cluster 5 revealed the expression of genes involved in the regulation of the immune system such as the expression of *mhc2dab, cd74a, cd74b,* and *cd99*. More recently, it has been found that ductal cells of the human pancreas may have an immune function based on the finding that they express low levels of *HLA-DPB1*, an MHC class II gene associated with T1D risk (Fasolino et al., 2022). *HLA-DPB1* is also the human equivalent to *mhc2dab* expressed in zebrafish. Surprisingly, Fasolino et al. found that T1D human donors had a higher number of ductal cells expressing *HLA-DPB1* and assumed a transcriptional identity resembling dendritic cells— immune cells involved in antigen presenting. Since this new role of ductal cells was revealed by genomics-based studies, the biological function of these cells has yet to be determined. It is intriguing to wonder whether CACs have a role in the autoimmunity of T1D

based on these findings in T1D human donors. Additionally, it is unknown what causes ductal cells to switch to a more dendritic cell profile in T1D. Therefore, it would be interesting to confirm whether zebrafish CACs express these similar immune cell properties *in vivo* because if they do, zebrafish could function as an excellent model system to uncover the role this subpopulation of CACs has in both the normal pancreas and in a T1D setting. Our lab is currently developing a mhc2dab transgenic zebrafish line to test whether CACs express *mhc2dab*.

As mentioned, previous work by Singh et al. identified a hybrid cell population in adult zebrafish pancreatic islets that co-express *sst1.1* and *ins* (S. P. Singh et al., 2022). The source of these hybrid cells is unknow, however, one possibility is that these cells originate from pre-existing delta cells in the islet since delta cells are the primary cell type that express *sst1.1*. A second possibility is that these cells arise from CACs based on the expression of endocrine-progenitor markers including the expression of Her genes, low levels of Notch, Fgf, and Wnt (S. P. Singh et al., 2022). From my genomics analysis of CACs in the adult zebrafish pancreas, I identified a small population of cells co-expressing *sst1.1* and *ins*. This subpopulation also expressed known CAC marker genes which rules out the possibility of these cells being contaminating endocrine cells. Contrary to Singh et al., the *sst1.1*/*ins* double positive cells I identified lacked the expression of *pyyb* and *dkk3b*. One potential explanation for this lack of expression is that these cells are an earlier population of an endocrine precursor captured in my scRNA-seq analysis compared to the population found later within the islet and captured by Singh et al.. I confirmed this possibility by looking at the expression of *pyyb* and *dkk3b* in my whole pancreas genomics data set where *pyyb* and *dkk3b* are indeed expressed in the endocrine cell cluster. From my genomics analysis of zebrafish CACs, it is possible that CACs are the source of these endocrine precursor cells that give

rise to beta cells upon regeneration. However, further *in vivo* work is needed to validate whether this is true.

My genomics data from the adults of *sox9b* heterozygous mutants showed an increase in the expression of *sst1.1* in all CAC subpopulations compared to CACs from wildtype fish. Previous work in our lab found that *sox9b* heterozygous adults have an increased regenerative phenotype compared to wildtype fish (W. Huang et al., 2016). For the first time, my genomics findings may explain why *sox9b* heterozygous adult fish have an increased regenerative phenotype. This may be due to the upregulation of *sst1.1* in the CACs of *sox9b* heterozygous fish which may make them more primed at regenerating beta cells. However, our lab needs to confirm *in vivo* whether the CACs of *sox9b* mutants have increased *sst1.1* expression compared to wildtype fish. Our lab is working on confirming this by incrossing adult *sox9b* mutant fish to generate wildtype, hets, and homozygous mutant larvae. We will then use an RNA probe against *sst1.1* and perform whole mount *in situ* hybridization using my optimized protocol on the pancreata of the *sox9b* wildtype, hets, and homozygous mutants to validate whether *sst1.1* expression increases when the levels of *sox9b* are lowered. Due to the limitations of detecting lowly expressed genes by whole mount *in situ* hybridization, we have also obtained an sst1.1 transgenic line as an alternative approach. These fish will be crossed to our *sox9b* mutant fish to generate hets which will then be incrossed to generate all subsequent *sox9b* genotypes so that *sst1.1* expression can be observed via the corresponding transgene.

Lastly, using an *in silico* approach, I have identified 5 new CAC marker genes and out of those 5 genes, I have verified the expression of three of these genes in the CACs of 5dpf larval zebrafish. I identified two genes expressed in all CAC subpopulations that may be involved in pancreatic cancer, namely *spint2* and *cldn7b*. *spint2* encodes a cell membrane associated Kunitz type 2 serine

protease inhibitor that regulates the activity of numerous proteases such as matriptase (Szabo et al., 2009). *cldn7b* (claudin-7) encodes a tight junction membrane protein. The role of these genes in pancreatic cancer and the 6 genes (*bcam, dlg2, pde3a, krt15, nrp2a,* and *cav1*) I found to be directly involved in pancreatic cancer will be described in the **Discussion** section of this thesis as it ties together **Chapters 1** and **3**.

tm4sf4 is another gene I found to be highly expressed in all CAC subpopulations *in silco*. Tm4sf4 is a tetraspanin protein that is involved in signaling processes such as cell adhesion, migration, and differentiation. Previous work in mice found that Tm4sf4 is a transcriptional downstream target of Nkx2.2a (Anderson et al., 2011). In mammals, Nkx2.2a is a transcription factor that is only expressed in mature endocrine cells, however, in the adult zebrafish pancreas CACs retain the developmental expression of Nkx2.2a (Beer et al., 2016; Delaspre et al., 2015; Doyle & Sussel, 2007; Sussel et al., 1998). The retention of Nkx2.2a expression in zebrafish CACs may explain why they are able to maintain their progenitor capacity in adult fish compared to mammalian CACs. Loss-of-function studies in zebrafish revealed that *tm4sf4* inhibits the specification of both alpha and beta cells (Anderson et al., 2011). This finding suggests that there may be an important link between Nkx2.2a inhibiting Tm4sf4 in progenitor cells to prevent them from delaminating and differentiating into endocrine cells. Anderson et al. showed that *tm4sf4* is expressed in the zebrafish pancreas, however, I have showed for the first time that *tm4sf4* is specifically expressed in the CACs of the pancreas. Further studies are needed to determine whether Tm4sf4 could be a potential target for inducing CACs to regenerate beta cells. Our lab is currently working on making a Tm4sf4 transgenic line to further study the role of Tm4sf4 in CACs. Our lab also has several allelic series *nkx2.2a* mutants that we aim to use to understand whether *tm4sf4* is regulated in a *nkx2.2a* dependent manner. This can be done by whole mount *in situ*

hybridization using an RNA probe targeted to *tm4sf4* or can be done by using a Tm4sf4 transgenic line and looking at the expression of tm4sf4 in subsequent *nkx2.2a* mutants.

The expression of *tm4sf4* was found to be the highest in Cluster 9 which had GO terms associated with both the pancreas and liver. The expression of *cldn15lb* suggests that Cluster 9 may be a type of extrahepatopancreatic ductal cell residing in the common bile duct. In my data, the highest expression of *tm4sf4* is found in the common bile duct when looking at the *in situ* hybridization performed on the pancreas. This result suggests that these cells being marked by *tm4sf4* in the common bile duct could be cells that make up Cluster 9. Out of the 6 CAC marker genes from bulk seq, only two (*sox9b* and *epcam*) are expressed in Cluster 9.

Although I was unable to identify *id2a* and *onecut1* as *in vivo* markers of CACs, I have shown that they are markers of all CAC subclusters *in silico*. This is consistent with previous bulk RNA seq findings that found CACs to express both *onecut1* and *id2a* (Ghaye et al., 2015; Mi & Andersson, 2023; Tarifeno-Saldivia et al., 2017). From previous bulk RNA-seq on CACs, Ghaye et al. showed that *id2a* co-expresses with *sox9b* in the pancreas of 3dpf larvae. In the retina, Id2a has been found to be involved in the Notch signaling pathway and has a role in progenitor biology (Uribe et al., 2012). In the mouse pancreas, it has been found that BMP4 regulates pancreatic progenitor cell expansion through Id2 (Hua et al., 2006). Thus, it would be interesting to further uncover the role of Id2a in CACs given its role in the Notch signaling pathway and progenitor biology. The expression of *onecut1* in CACs is fascinating because ONECUT1 is a crucial regulator in pancreatic progenitor differentiation (Heller et al., 2021) and it is also involved in the SOX9 pathway (Lynn et al., 2007). Therefore, just like Id2a, it would be interesting to uncover the role Onecut1 plays in the progenitor status of CACs and try to link *onecut1* to the Sox9b pathway in zebrafish CACs.

Although much of the work presented here is genomics data, it is clear that the data I have generated points to important aspects of CAC biology that have not been fully uncovered. Much of my findings build upon numerous *in vivo* studies from the literature and draw upon previous work to make a connection to CACs. As stated throughout, future *in vivo* work will need to be done to validate my *in silico* findings presented here. It is with high hopes that much of this future work will be carried out by both current and future members of the Parsons Lab.

Discussion

As was described in **Chapter 1**, our lab is interested in uncovering pathways that have an implication in pancreatic cancer. One of those pathways was the SOX9 pathway because its dysregulation in the adult pancreas can lead to the formation and progression of PDAC (Kopp et al., 2012). CACs have been hypothesized as a source for pancreatic cancer because they express high levels of Sox9. In **Chapter 1**, I was able to use zebrafish as an animal model to further elucidate the downstream target genes of *sox9b* and found that *epcam* is regulated in a *sox9b* dependent manner in CACs. Since EpCAM is known to have a role in pancreatic cancer, our lab is interested in using zebrafish as an animal model to further elucidate its pathway.

As was mentioned in **Chapter 3**, I uncovered several new marker genes of CACs and two of those genes identified were found to be involved in the *epcam* signaling pathway, namely *spint2* and *cldn7b*. Previous work in the intestinal epithelium revealed a pathway link between SPINT2, matriptase, EpCAM, and claudin-7 (Wu, Feng, Lu, Morimura, & Udey, 2017). SPINT2 normally inhibits matriptase from cleaving EpCAM at the membrane of the cell. Interactions between EpCAM and claudin have been shown to be extremely important in the integrity of the intestine (Ding et al., 2012; Maghzal, Kayali, Rohani, Kajava, & Fagotto, 2013; Tanaka et al., 2015). Mutations in SPINT2 were found to inactivate the ability of SPINT2 to inhibit matriptase which led to EpCAM cleavage and the dissociation of EpCAM and claudin-7 at the cell membrane (Szabo et al., 2009). Like EpCAM, SPINT2 has also been found to be highly expressed in pancreatic cancer, particularly PDAC (Muller-Pillasch et al., 1998; Roversi, Olalla Saad, & Machado-Neto, 2018). However, there has been no link made between EpCAM and SPINT2 with regards to these pathways going hand in hand with the initiation and progression of pancreatic cancer. Due to our interest in the EpCAM pathway, it would be interesting to determine whether there is a link

between Epcam/Spint2/Cldn7b in zebrafish CACs and uncover whether this pathway is important in pancreatic cancer.

Besides our interest in the SOX9 pathway and EpCAM as a marker of pancreatic cancer, our lab is also interested in uncovering other ways in which CACs could be a source for pancreatic cancer. One of those ways is understanding other gene expression similarities shared between cells in PDAC and CACs. In **Chapter 3**, I found 6 new genes expressed by CACs and associated with pancreatic cancer. These genes include *bcam, dlg2, pde3a, krt15, nrp2a*, and *cav1*. Only 3 out of the 6 genes have been implicated in basal cells of PDAC. These three genes include *dlg2, pde3a*, and *krt15*. We hypothesize that because CACs express similar pathways involved in pancreatic cancer that CACs may be the cellular origin of PDAC. As mentioned before, perhaps when these pathways become dysregulated it leads to the initiation and progression of PDAC. It is also intriguing to find that CACs share similar properties of gene expression with basal cells involved in pancreatic cancer. Our lab aims to follow up on cancerous genes expressed by CACs to understand how the dysregulation of these genes may lead to pancreatic cancer.

Second, we are not only interested in understanding cancerous genes expressed by CACs but CAC subpopulations that may be more primed at contributing to pancreatic cancer. As mentioned in **Chapter 1**, it is not clear whether CACs are the main source of pancreatic cancer. Acinar cells have also been described as a source but may have to first transition into ductal cells or CACs through a process called ADM (Kopp et al., 2012). ADM is a process primarily associated with pancreatic cancer; however, the downfall of many pancreatic cancer studies is that they fail to understand what the normal pancreas looks like. A genomics study done on healthy human pancreatic ductal cells found a transitioning population of ductal to acinar cells (Qadir et al., 2020). Similarly in **Chapter 3**, I uncovered a potential transitioning population of acinar cells to CACs

which looks like ADM but is seen in the pancreas of healthy adult zebrafish. My findings and Qadir et al.'s findings beg the question as to whether transitioning acinar to ductal or ductal to acinar cells is a characteristic novel to pancreatic cancer or is a normal process of the pancreas. Perhaps, when this normal transitioning process becomes disrupted it then leads to pancreatic cancer.

If our lab can first verify that this acinar to CAC transition holds true in the pancreas of healthy zebrafish, it would be interesting to test ways in which this transitioning process can be disrupted to mimic a disease state. The best way to study this transitioning population of 'acinar-like' cells is to have a model at hand that can induce acinar cells to transition into ductal cells. One example of initiating ADM was mentioned in **Chapter 1** where Kopp et al. used a transgenic mouse model to overexpress Sox9 in acinar cells. Overexpression of Sox9 caused acinar cells to decrease their expression of acinar gene markers and upregulate the expression of ductal markers (Kopp et al., 2012). It would be interesting to see whether this 'acinar-like' transition state increases if we overexpress *sox9b*. There is already a transgenic zebrafish line published that our lab has used in a previous study to successfully overexpress *sox9b* (W. Huang et al., 2016). Inducing pancreatitis is another way of initiating ADM and studying this 'acinar-like' transition state. As mentioned in my **introduction** and in **Chapter 1**, pancreatitis is one of the leading causes of ADM which can make patients at an increased risk for pancreatic cancer. One study in zebrafish has shown that *cftr* mutants could be a potential model of pancreatitis due to their severe loss of exocrine pancreatic tissue (Navis & Bagnat, 2015). Our lab has *cftr* mutants that could function as an *in vivo* way of studying the transition of acinar cells into CACs/ductal cells. Another model of pancreatitis that can be used to stimulate ADM in the zebrafish pancreas is the use of cerulein— an oligopeptide that causes increased digestive secretions and therefore, the loss of pancreatic exocrine tissue.

Cerulein has been used successfully in the mouse to cause the development of chronic pancreatitis (Ahmadi et al., 2016). This method has yet to be fully developed in the zebrafish but could function as a method to study this transitioning population of 'acinar-like' cells further. In conclusion, my findings in **Chapter 1** and **3** suggest that zebrafish CACs are an excellent *in vivo* model for studying pathways involved in pancreatic cancer and ADM.

Other than our interest in studying pancreatic cancer, our lab is also interested in treating diseases where beta-cell mass is lost in humans (i.e., pancreatic cancer, pancreatitis, diabetes, etc.). As mentioned in my **introduction**, our lab's primary interest and goal is to determine ways in which endogenous stem cells or progenitors can be induced to restore beta-cell mass. This can ideally be accomplished via drug treatment which is less invasive and more cost effective than treating patients with cell transplants (Shapiro et al., 2006) or stem cell/iPSC (Kroon et al., 2008; Path et al., 2019) based approaches. To accomplish such a goal, one must first understand the cellular composition (e.g., heterogeneity) of a cell population before perturbations (e.g., beta-cell ablation) are induced. If we can understand the cellular composition of a cell population before beta-cell regeneration, we can potentially identify cellular populations that can be targeted to induce beta-cell regeneration. Once the first goal has been met, we can then induce beta-cell regeneration and understand the cellular and molecular mechanisms that allow a beta-cell progenitor to contribute to the restoration of beta-cell mass. As was discussed throughout the entirety of this thesis, zebrafish have proven an excellent model system that can be used to uncover such mechanisms since beta-cell neogenesis can be studied throughout the lifespan of the fish (Delaspre et al., 2015; Ghaye et al., 2015).

The work I have accomplished in **Chapter 2** and **Chapter 3** sets the foundation for our lab's future work which is to uncover the cellular and molecular mechanisms permitting CACs to

transition into beta cells. Uncovering such mechanisms can be accomplished by performing scRNA-seq and applying Pseudotime analysis to investigate transcriptome changes as CACs transition into beta cells during regeneration.

The goal of **Chapter 2** was to first improve our NTR/MTZ model so that our lab can use it to induce beta-cell regeneration more easily. This improved NTR line can then be crossed to *tp1:eGFP* fish so that beta-cells, CACs, and CAC-derivatives can be isolated during regeneration and subject to scRNA-seq to uncover mechanisms allowing CACs to become beta cells. Although uncovering such mechanisms could be done with our old NTR1.0 line (Pisharath & Parsons, 2009; Pisharath et al., 2007), my improved NTR2.0 transgenic line is less technically challenging and adult fish no longer need to be screened for a functional NTR. As described in **Chapter 2**, NTR2.0 adult fish can achieve complete beta-cell loss via simply immersing fish into MTZ treated system water. This avoids having to inject adult fish with MTZ which can lead to inconsistent beta-cell ablation and inadvertent morbidity. Additionally, NTR1.0 transgenic adults had to be screened for a functional NTR before they can be used for any downstream regeneration experiments (Pisharath & Parsons, 2009; Pisharath et al., 2007). Screening was required because our NTR1.0 line was multi copy which means that some fish have an NTR copy that works better than others. Using NTR2.0, screening for a functional NTR is no longer required because NTR2.0 is more active than NTR1.0 and fish expressing NTR2.0 have a single NTR copy. By not having to screen fish, multiple fish do not have to be maintained for a scRNA-seq experiment, time is saved having to screen numerous fish, and it avoids wrongful interpretation of beta-cell ablation that can affect the analysis of scRNA-seq experiments. Therefore, my work in **Chapter 2** has provided the Parsons Lab with a better transgenic fish to study beta-cell regeneration in the future work of the lab.

The goal of **Chapter 3** was to establish methods for (**1**) sorting CACs, (**2**) performing scRNA-seq, and (**3**) generating scripts that can be used to analyze and understand CAC heterogeneity before regeneration is induced. As mentioned, the idea that the CAC population is heterogeneous comes from the observation that not all CACs contribute to beta-cell regeneration (Delaspre et al., 2015; Parsons et al., 2009) and not all CACs express the same levels of Notch activity (Ninov et al., 2012). Dissecting cellular heterogeneity is a prerequisite required to understand how a population of cells responds to external perturbations, such as beta-cell ablation. Understanding how heterogeneity relates to differentiation should reveal pathways that could be targeted to induce a subpopulation to differentiate into beta cells. Also, identifying subpopulations in zebrafish could further explain the difference in regenerative capacity between zebrafish and mammals. Finding these differences are beyond the scope of my thesis, but as mentioned in the **introduction**, others in the field can use my data to interrogate these differences between mammals and zebrafish.

Looking at CAC heterogeneity in **Chapter 3**, I have identified two potential subpopulations of CACs that could be a source for beta-cell regeneration. One of those subpopulations identified is the *ins/sst1.1* endocrine precursor cells. Sing et al. not only found that these cells are located within the islet, but observed an increase in this cell population when beta-cell regeneration was induced (S. P. Singh et al., 2022). The cell source of these endocrine precursor cells is largely unknown, however, recent *in vivo* work in the zebrafish demonstrates that these cells may arise from CACs. A separate study done by Carril Pardo et al. found that CACs increase the expression of *sst1.1* and *ins* during regeneration in the adult fish (Carril Pardo et al., 2022). This *in vivo* evidence along with my genomics findings strongly suggests that these endocrine precursor cells may have originated from CACs. Regardless of these findings, we still do not understand how these endocrine precursor cells fit into the trajectory of CACs becoming beta cells. A second

potential source of cells for beta-cell regeneration is the 'acinar-like' population I identified which lacked the expression of *ptf1a*. It has been shown that lower levels of *ptf1a* can cause acinar cells to transition into endocrine cells (Dong et al., 2008). During beta-cell regeneration, it would be interesting to determine whether (**1**) this transitioning population of 'acinar-like' cells contributes directly to beta-cell regeneration, (**2**) if this transitioning population of 'acinar-like' cells contributes indirectly to regeneration via the CAC subcluster 3 it transitions into or (**3**) if these 'acinar-like' cells are a potential cell source for the repopulation of CACs after they have contributed to beta-cell regeneration.

After accomplishing our lab's first goal in **Chapter 3** – which was to define the cellular composition of CACs before regeneration, our lab is now taking *tp1:eGFP* adult fish used in my scRNA-seq experiments and crossing them to my *ins:NTR2.0*^{ir2018} fish from **Chapter 2**. Using *tp1:eGFP; ins:NTR2.0*^{ir2018} double transgenic fish our lab aims to accomplish our second goal—identify the cellular and molecular mechanisms that allow CACs to become beta cells. Using these fish, we can sort for beta cells, CACs and their derivatives from the adult pancreas following beta-cell ablation. These cells will then be subject to scRNA-seq as was done in **Chapter 3**. Due to GFP perdurance, CACs and their descendants will remain fluorescently labeled for several days (Delaspre et al., 2015; Ghaye et al., 2015). This future work not only aims to identify various transition states of CACs but will also place CAC heterogeneity (**Chapter 3**) in the context of regeneration.

Pseudotime analysis (Monocle3) has been used successfully to order cell transcriptomes from start to end to capture various transition states (Cao et al., 2019; Durruthy-Durruthy & Heller, 2015; R. D. Lee et al., 2021; Trapnell et al., 2014). After scRNA-seq and applying my analysis outlined in **Chapter 3**, our lab aims to use Pseudotime analysis to order GFP positive cell

transcriptomes (clusters) in our trajectory from CACs all the way to beta cells (Trapnell et al., 2014; Zeng et al., 2017). Our lab can take the results I generated in **Chapter 3** and can place CAC heterogeneity in the context of beta-cell regeneration. For example, during regeneration I would hypothesize that the *sst1.1/ins* cluster would increase in cell number if these cells were important for beta-cell regeneration. I would also expect to identify the position of these endocrine precursor cells in the regeneration Pseudotime trajectory. Additionally, by inducing beta-cell regeneration, our lab can also determine whether the transitioning 'acinar-like' cells have a role in the regeneration of beta-cells. Whether these acinar-like cells contribute to beta-cell regeneration directly or indirectly can be determined by applying a Pseudotime trajectory and looking at where these cells fall within the trajectory.

Besides *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} being used to study chronic hyperglycemia and acute beta-cell ablation, I believe another utility of these fish would be to further characterize beta-cell regeneration under chronic ablation conditions. Studies using acute beta-cell ablation in adult zebrafish have identified several cellular origins of regenerated beta cells including: ductal associated CACs (Delaspre et al., 2015; Ghaye et al., 2015), as well as, the islet localized alpha cells (L. Ye et al., 2015), delta cells (Carril Pardo et al., 2022) and epsilon cells (J. Yu et al., 2023). With the new ability to cause continuous beta-cell ablation we can expand this work and ask the following questions about these cellular origins: can these sources be depleted? Are these sources replenished and, if so, how? And are there other unknown origins of new beta cells still to be discovered? In **Chapter 2**, I showed that NTR2.0 transgenic adults can achieve a hyperglycemic state for up to 2 weeks. What has yet to be tested is whether these fish are capable of regenerating beta-cells and if regeneration takes the same amount of time as it does in an acute setting (Delaspre et al., 2015). One unanswered question that remains is whether CACs

can become depleted if fish are under chronic beta-cell ablation conditions. Under acute ablation conditions, it has been shown that CACs are capable of repopulating by increasing proliferation (Delaspre et al., 2015). Using *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} fish we now have the ability for the first time to antagonize the CAC population by either inducing a chronic hyperglycemic state for a long period of time or inducing fish to regenerate repeatedly by taking them in and out of prodrug over a period of time. Antagonizing the CAC population can also be applied to other mutants we are interested in such as the *sox9b* mutants which have been found to have an increased regenerative phenotype (W. Huang et al., 2016). Thus, using *ins:mCherry NTR2.0*^{ir2018} and *ins:YFP NTR2.0*^{ir2050} it would be interesting to determine whether mutants with an increased or decreased regenerative phenotype have a different outcome of regeneration capacity compared to wildtype fish under chronic ablation conditions. Furthermore, I would also be curious to understand whether the 'acinar-like' cells identified in **Chapter 3** could serve as a potential cell source for the replenishment of CACs under chronic ablation conditions. Perhaps these 'acinar-like' cells do not contribute to beta-cell regeneration under acute conditions but contribute to beta-cell regeneration when CACs are chronically depleted.

Provided the work that I have accomplished in my thesis, I am excited to see the future work of the Parsons Lab and how they can utilize my work to generate and answer numerous hypotheses.

References

Acharjee, S., Ghosh, B., Al-Dhubiab, B. E., & Nair, A. B. (2013). Understanding type 1 diabetes: etiology and models. *Can J Diabetes, 37*(4), 269-276. doi:10.1016/j.jcjd.2013.05.001

Adamska, A., Elaskalani, O., Emmanouilidi, A., Kim, M., Abdol Razak, N. B., Metharom, P., & Falasca, M. (2018). Molecular and cellular mechanisms of chemoresistance in pancreatic cancer. *Adv Biol Regul, 68*, 77-87. doi:10.1016/j.jbior.2017.11.007

Afelik, S., Qu, X., Hasrouni, E., Bukys, M. A., Deering, T., Nieuwoudt, S., . . . Jensen, J. (2012). Notch-mediated patterning and cell fate allocation of pancreatic progenitor cells. *Development, 139*(10), 1744-1753. doi:10.1242/dev.075804

Ahmadi, A., Nikkhoo, B., Mokarizadeh, A., Rahmani, M. R., Fakhari, S., Mohammadi, M., & Jalili, A. (2016). An optimised mouse model of chronic pancreatitis with a combination of ethanol and cerulein. *Cent Eur J Immunol, 41*(1), 54-63. doi:10.5114/ceji.2016.58816

Aizarani, N., Saviano, A., Sagar, Mailly, L., Durand, S., Herman, J. S., . . . Grun, D. (2019). A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature, 572*(7768), 199-204. doi:10.1038/s41586-019-1373-2

Al-Hasani, K., Pfeifer, A., Courtney, M., Ben-Othman, N., Gjernes, E., Vieira, A., . . . Collombat, P. (2013). Adult duct-lining cells can reprogram into beta-like cells able to counter repeated cycles of toxin-induced diabetes. *Dev Cell, 26*(1), 86-100. doi:10.1016/j.devcel.2013.05.018

Al-Shehri, A., Silverman, S., & King, K. M. (2008). Squamous cell carcinoma of the pancreas. *Curr Oncol, 15*(6), 293-297. doi:10.3747/co.v15i6.265

Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N., & Perucho, M. (1988). Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell, 53*(4), 549-554. doi:10.1016/0092-8674(88)90571-5

Altschuler, S. J., & Wu, L. F. (2010). Cellular heterogeneity: do differences make a difference? *Cell, 141*(4), 559-563. doi:10.1016/j.cell.2010.04.033

AmericasHealthRankings.org. Retrieved from https://www.americashealthrankings.org/explore/annual/measure/diabetes

Anderson, K. R., Singer, R. A., Balderes, D. A., Hernandez-Lagunas, L., Johnson, C. W., Artinger, K. B., & Sussel, L. (2011). The L6 domain tetraspanin Tm4sf4 regulates endocrine pancreas differentiation and directed cell migration. *Development, 138*(15), 3213-3224. doi:10.1242/dev.058693

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., . . . Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature, 400*(6747), 877-881. doi:10.1038/23716

Armstrong, G., Martino, C., Rahman, G., Gonzalez, A., Vazquez-Baeza, Y., Mishne, G., & Knight, R. (2021). Uniform Manifold Approximation and Projection (UMAP) Reveals Composite Patterns and Resolves Visualization Artifacts in Microbiome Data. *mSystems, 6*(5), e0069121. doi:10.1128/mSystems.00691-21

Arnes, L., Hill, J. T., Gross, S., Magnuson, M. A., & Sussel, L. (2012). Ghrelin expression in the mouse pancreas defines a unique multipotent progenitor population. *PLoS One, 7*(12), e52026. doi:10.1371/journal.pone.0052026

Ashizawa, N., Sakai, T., Yoneyama, T., Naora, H., & Kinoshita, Y. (2005). Three-dimensional structure of peripheral exocrine gland in rat pancreas: reconstruction using transmission electron microscopic examination of serial sections. *Pancreas, 31*(4), 401-404. doi:10.1097/01.mpa.0000181488.27399.dd

Ashraf, H., Colombo, J. P., Marcucci, V., Rhoton, J., & Olowoyo, O. (2021). A Clinical Overview of Acute and Chronic Pancreatitis: The Medical and Surgical Management. *Cureus, 13*(11), e19764. doi:10.7759/cureus.19764

Bailey, J. M., Hendley, A. M., Lafaro, K. J., Pruski, M. A., Jones, N. C., Alsina, J., . . . Leach, S. D. (2016). p53 mutations cooperate with oncogenic Kras to promote adenocarcinoma from pancreatic ductal cells. *Oncogene, 35*(32), 4282-4288. doi:10.1038/onc.2015.441

Bailey, P., Chang, D. K., Nones, K., Johns, A. L., Patch, A. M., Gingras, M. C., . . . Grimmond, S. M. (2016). Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature, 531*(7592), 47-52. doi:10.1038/nature16965

Bakker, J., & de la Garza, M. A. (2022). Naturally Occurring Endocrine Disorders in Non-Human Primates: A Comprehensive Review. *Animals (Basel), 12*(4). doi:10.3390/ani12040407

Becht, E., McInnes, L., Healy, J., Dutertre, C. A., Kwok, I. W. H., Ng, L. G., . . . Newell, E. W. (2018). Dimensionality reduction for visualizing single-cell data using UMAP. *Nat Biotechnol*. doi:10.1038/nbt.4314

Beer, R. L., Parsons, M. J., & Rovira, M. (2016). Centroacinar cells: At the center of pancreas regeneration. *Dev Biol, 413*(1), 8-15. doi:10.1016/j.ydbio.2016.02.027

Belhiba, O., Aadam, Z., Jeddane, L., Saile, R., Salih Alj, H., Bousfiha, A. A., & Jennane, F. (2020). Research of anti-GAD and anti-IA2 autoantibodies by ELISA test in a series of Moroccan pediatric patients with diabetes type 1. *Afr Health Sci, 20*(3), 1337-1343. doi:10.4314/ahs.v20i3.40

Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M., & MacDonald, R. J. (2006). PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Mol Cell Biol, 26*(1), 117-130. doi:10.1128/MCB.26.1.117-130.2006

Bernard, P., Tang, P., Liu, S., Dewing, P., Harley, V. R., & Vilain, E. (2003). Dimerization of SOX9 is required for chondrogenesis, but not for sex determination. *Hum Mol Genet, 12*(14), 1755-1765. doi:10.1093/hmg/ddg182

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., & de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nat Genet, 22*(1), 85-89. doi:10.1038/8792

Binot, A. C., Manfroid, I., Flasse, L., Winandy, M., Motte, P., Martial, J. A., . . . Voz, M. L. (2010). Nkx6.1 and nkx6.2 regulate alpha- and beta-cell formation in zebrafish by acting on pancreatic endocrine progenitor cells. *Dev Biol, 340*(2), 397-407. doi:10.1016/j.ydbio.2010.01.025

Borazanci, E., Millis, S. Z., Korn, R., Han, H., Whatcott, C. J., Gatalica, Z., . . . Von Hoff, D. D. (2015). Adenosquamous carcinoma of the pancreas: Molecular characterization of 23 patients along with a literature review. *World J Gastrointest Oncol, 7*(9), 132-140. doi:10.4251/wjgo.v7.i9.132

Bosco, D., Armanet, M., Morel, P., Niclauss, N., Sgroi, A., Muller, Y. D., . . . Berney, T. (2010). Unique arrangement of alpha- and beta-cells in human islets of Langerhans. *Diabetes, 59*(5), 1202-1210. doi:10.2337/db09-1177

Brock, C., Nielsen, L. M., Lelic, D., & Drewes, A. M. (2013). Pathophysiology of chronic pancreatitis. *World J Gastroenterol, 19*(42), 7231-7240. doi:10.3748/wjg.v19.i42.7231

Brown, L. A., Rodaway, A. R., Schilling, T. F., Jowett, T., Ingham, P. W., Patient, R. K., & Sharrocks, A. D. (2000). Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech Dev, 90*(2), 237-252. doi:10.1016/s0925-4773(99)00256-7

Brunner, M., Wu, Z., Krautz, C., Pilarsky, C., Grutzmann, R., & Weber, G. F. (2019). Current Clinical Strategies of Pancreatic Cancer Treatment and Open Molecular Questions. *Int J Mol Sci, 20*(18). doi:10.3390/ijms20184543

Burghardt, B., Elkaer, M. L., Kwon, T. H., Racz, G. Z., Varga, G., Steward, M. C., & Nielsen, S. (2003). Distribution of aquaporin water channels AQP1 and AQP5 in the ductal system of the human pancreas. *Gut, 52*(7), 1008-1016. doi:10.1136/gut.52.7.1008

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., & Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol, 36*(5), 411-420. doi:10.1038/nbt.4096

Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D. M., Hill, A. J., . . . Shendure, J. (2019). The single-cell transcriptional landscape of mammalian organogenesis. *Nature, 566*(7745), 496-502. doi:10.1038/s41586-019-0969-x

Capiotti, K. M., Antonioli, R., Jr., Kist, L. W., Bogo, M. R., Bonan, C. D., & Da Silva, R. S. (2014). Persistent impaired glucose metabolism in a zebrafish hyperglycemia model. *Comp Biochem Physiol B Biochem Mol Biol, 171*, 58-65. doi:10.1016/j.cbpb.2014.03.005

Carril Pardo, C. A., Massoz, L., Dupont, M. A., Bergemann, D., Bourdouxhe, J., Lavergne, A., . . . Manfroid, I. (2022). A delta-cell subpopulation with a pro-beta-cell identity contributes to efficient age-independent recovery in a zebrafish model of diabetes. *Elife, 11*. doi:10.7554/eLife.67576

cdc.gov. Retrieved from https://www.cdc.gov/diabetes/prevention/about-prediabetes.html#print

Chatenoud, L. (2008). Chemical immunosuppression in islet transplantation--friend or foe? *N Engl J Med, 358*(11), 1192-1193. doi:10.1056/NEJMcibr0708067

Chen, D., Thayer, T. C., Wen, L., & Wong, F. S. (2020). Mouse Models of Autoimmune Diabetes: The Nonobese Diabetic (NOD) Mouse. *Methods Mol Biol, 2128*, 87-92. doi:10.1007/978-1-0716-0385-7_6

Chen, S., Li, C., Yuan, G., & Xie, F. (2007). Anatomical and histological observation on the pancreas in adult zebrafish. *Pancreas, 34*(1), 120-125. doi:10.1097/01.mpa.0000246661.23128.8c

Chera, S., Baronnier, D., Ghila, L., Cigliola, V., Jensen, J. N., Gu, G., . . . Herrera, P. L. (2014). Diabetes recovery by age-dependent conversion of pancreatic delta-cells into insulin producers. *Nature, 514*(7523), 503-507. doi:10.1038/nature13633

Cheung, I. D., Bagnat, M., Ma, T. P., Datta, A., Evason, K., Moore, J. C., . . . Stainier, D. Y. (2012). Regulation of intrahepatic biliary duct morphogenesis by Claudin 15-like b. *Dev Biol, 361*(1), 68-78. doi:10.1016/j.ydbio.2011.10.004

Chiang, E. F., Pai, C. I., Wyatt, M., Yan, Y. L., Postlethwait, J., & Chung, B. (2001). Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev Biol, 231*(1), 149-163. doi:10.1006/dbio.2000.0129

Chin, T., Toy, C., Vandeven, C., & Cairo, M. S. (1989). Lymphokine-activated killer cytotoxicity in neonatal mononuclear cells: in vitro responses to tumor cell lines from pediatric solid tumors. *Pediatr Res, 25*(2), 156-160. doi:10.1203/00006450-198902000-00016

Chuvin, N., Vincent, D. F., Pommier, R. M., Alcaraz, L. B., Gout, J., Caligaris, C., . . . Bartholin, L. (2017). Acinar-to-Ductal Metaplasia Induced by Transforming Growth Factor Beta Facilitates KRAS(G12D)-driven Pancreatic Tumorigenesis. *Cell Mol Gastroenterol Hepatol, 4*(2), 263-282. doi:10.1016/j.jcmgh.2017.05.005

Cirulli, V., Crisa, L., Beattie, G. M., Mally, M. I., Lopez, A. D., Fannon, A., . . . Hayek, A. (1998). KSA antigen Ep-CAM mediates cell-cell adhesion of pancreatic epithelial cells: morphoregulatory roles in pancreatic islet development. *J Cell Biol, 140*(6), 1519-1534. doi:10.1083/jcb.140.6.1519

Connaughton, V. P., Baker, C., Fonde, L., Gerardi, E., & Slack, C. (2016). Alternate Immersion in an External Glucose Solution Differentially Affects Blood Sugar Values in Older Versus Younger Zebrafish Adults. *Zebrafish, 13*(2), 87-94. doi:10.1089/zeb.2015.1155

Courtney, M., Gjernes, E., Druelle, N., Ravaud, C., Vieira, A., Ben-Othman, N., . . . Collombat, P. (2013). The inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion into functional beta-like cells. *PLoS Genet, 9*(10), e1003934. doi:10.1371/journal.pgen.1003934

Cui, X. B., Luan, J. N., Ye, J., & Chen, S. Y. (2015). RGC32 deficiency protects against high-fat diet-induced obesity and insulin resistance in mice. *J Endocrinol, 224*(2), 127-137. doi:10.1530/JOE-14-0548

Curado, S., Anderson, R. M., Jungblut, B., Mumm, J., Schroeter, E., & Stainier, D. Y. (2007). Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev Dyn, 236*(4), 1025-1035. doi:10.1002/dvdy.21100

D'Agati, G., Beltre, R., Sessa, A., Burger, A., Zhou, Y., Mosimann, C., & White, R. M. (2017). A defect in the mitochondrial protein Mpv17 underlies the transparent casper zebrafish. *Dev Biol, 430*(1), 11-17. doi:10.1016/j.ydbio.2017.07.017

Das, S. L., Kennedy, J. I., Murphy, R., Phillips, A. R., Windsor, J. A., & Petrov, M. S. (2014). Relationship between the exocrine and endocrine pancreas after acute pancreatitis. *World J Gastroenterol, 20*(45), 17196-17205. doi:10.3748/wjg.v20.i45.17196

Delaspre, F., Beer, R. L., Rovira, M., Huang, W., Wang, G., Gee, S., . . . Parsons, M. J. (2015). Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration. *Diabetes, 64*(10), 3499-3509. doi:10.2337/db15-0153

Delous, M., Yin, C., Shin, D., Ninov, N., Debrito Carten, J., Pan, L., . . . Stainier, D. Y. (2012). Sox9b is a key regulator of pancreaticobiliary ductal system development. *PLoS Genet, 8*(6), e1002754. doi:10.1371/journal.pgen.1002754

Demirci, N. S., Dogan, M., Erdem, G. U., Kacar, S., Turhan, T., Kilickap, S., . . . Zengin, N. (2017). Is plasma caveolin-1 level a prognostic biomarker in metastatic pancreatic cancer? *Saudi J Gastroenterol, 23*(3), 183-189. doi:10.4103/sjg.SJG_483_16

Deshpande, A. D., Harris-Hayes, M., & Schootman, M. (2008). Epidemiology of diabetes and diabetes-related complications. *Phys Ther, 88*(11), 1254-1264. doi:10.2522/ptj.20080020

di Magliano, M. P., & Logsdon, C. D. (2013). Roles for KRAS in pancreatic tumor development and progression. *Gastroenterology, 144*(6), 1220-1229. doi:10.1053/j.gastro.2013.01.071

Ding, L., Lu, Z., Foreman, O., Tatum, R., Lu, Q., Renegar, R., . . . Chen, Y. H. (2012). Inflammation and disruption of the mucosal architecture in claudin-7-deficient mice. *Gastroenterology, 142*(2), 305-315. doi:10.1053/j.gastro.2011.10.025

Dolle, L., Theise, N. D., Schmelzer, E., Boulter, L., Gires, O., & van Grunsven, L. A. (2015). EpCAM and the biology of hepatic stem/progenitor cells. *Am J Physiol Gastrointest Liver Physiol, 308*(4), G233-250. doi:10.1152/ajpgi.00069.2014

Dominguez-Gutierrez, G., Xin, Y., & Gromada, J. (2019). Heterogeneity of human pancreatic beta-cells. *Mol Metab, 27S*(Suppl), S7-S14. doi:10.1016/j.molmet.2019.06.015

Dong, P. D., Provost, E., Leach, S. D., & Stainier, D. Y. (2008). Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. *Genes Dev, 22*(11), 1445-1450. doi:10.1101/gad.1663208

Doyle, M. J., & Sussel, L. (2007). Nkx2.2 regulates beta-cell function in the mature islet. *Diabetes, 56*(8), 1999-2007. doi:10.2337/db06-1766

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., . . . Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development, 123*, 37-46. doi:10.1242/dev.123.1.37

Duque, M., Amorim, J. P., & Bessa, J. (2022). Ptf1a function and transcriptional cis-regulation, a cornerstone in vertebrate pancreas development. *FEBS J, 289*(17), 5121-5136. doi:10.1111/febs.16075

Durruthy-Durruthy, R., & Heller, S. (2015). Applications for single cell trajectory analysis in inner ear development and regeneration. *Cell Tissue Res, 361*(1), 49-57. doi:10.1007/s00441-014-2079-2

Dy, P., Wang, W., Bhattaram, P., Wang, Q., Wang, L., Ballock, R. T., & Lefebvre, V. (2012). Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. *Dev Cell, 22*(3), 597-609. doi:10.1016/j.devcel.2011.12.024

Eff, C., Faber, O., & Deckert, T. (1978). Persistent insulin secretion, assessed by plasma C-peptide estimation in long-term juvenile diabetics with a low insulin requirement. *Diabetologia, 15*(3), 169-172. doi:10.1007/BF00421234

England, A. R., Chaney, C. P., Das, A., Patel, M., Malewska, A., Armendariz, D., . . . Carroll, T. J. (2020). Identification and characterization of cellular heterogeneity within the developing renal interstitium. *Development, 147*(15). doi:10.1242/dev.190108

Espinet, E., Klein, L., Pure, E., & Singh, S. K. (2022). Mechanisms of PDAC subtype heterogeneity and therapy response. *Trends Cancer, 8*(12), 1060-1071. doi:10.1016/j.trecan.2022.08.005

Eze, U. C., Bhaduri, A., Haeussler, M., Nowakowski, T. J., & Kriegstein, A. R. (2021). Single-cell atlas of early human brain development highlights heterogeneity of human neuroepithelial cells and early radial glia. *Nat Neurosci, 24*(4), 584-594. doi:10.1038/s41593-020-00794-1

Fajans, S. S., & Bell, G. I. (2011). MODY: history, genetics, pathophysiology, and clinical decision making. *Diabetes Care, 34*(8), 1878-1884. doi:10.2337/dc11-0035

Faller, N., Gautschi, I., & Schild, L. (2014). Functional analysis of a missense mutation in the serine protease inhibitor SPINT2 associated with congenital sodium diarrhea. *PLoS One, 9*(4), e94267. doi:10.1371/journal.pone.0094267

Fang, J. Y., Lin, C. H., Huang, T. H., & Chuang, S. Y. (2019). In Vivo Rodent Models of Type 2 Diabetes and Their Usefulness for Evaluating Flavonoid Bioactivity. *Nutrients, 11*(3). doi:10.3390/nu11030530

Fang, Z., Weng, C., Li, H., Tao, R., Mai, W., Liu, X., . . . Li, Y. (2019). Single-Cell Heterogeneity Analysis and CRISPR Screen Identify Key beta-Cell-Specific Disease Genes. *Cell Rep, 26*(11), 3132-3144 e3137. doi:10.1016/j.celrep.2019.02.043

Fasolino, M., Schwartz, G. W., Patil, A. R., Mongia, A., Golson, M. L., Wang, Y. J., . . . Vahedi, G. (2022). Single-cell multi-omics analysis of human pancreatic islets reveals novel cellular states in type 1 diabetes. *Nat Metab, 4*(2), 284-299. doi:10.1038/s42255-022-00531-x

Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, C., Weller, P. A., Stevanovic, M., . . . et al. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature, 372*(6506), 525-530. doi:10.1038/372525a0

Froud, T., Baidal, D. A., Ponte, G., Ferreira, J. V., Ricordi, C., & Alejandro, R. (2006). Resolution of neurotoxicity and beta-cell toxicity in an islet transplant recipient following substitution of tacrolimus with MMF. *Cell Transplant, 15*(7), 613-620. doi:10.3727/000000006783981639

Furman, B. L. (2015). Streptozotocin-Induced Diabetic Models in Mice and Rats. *Curr Protoc Pharmacol, 70*, 5 47 41-45 47 20. doi:10.1002/0471141755.ph0547s70

Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., . . . Martin, C. (2020). Pathophysiology of Type 2 Diabetes Mellitus. *Int J Mol Sci, 21*(17). doi:10.3390/ijms21176275

Gangemi, A., Salehi, P., Hatipoglu, B., Martellotto, J., Barbaro, B., Kuechle, J. B., . . . Oberholzer, J. (2008). Islet transplantation for brittle type 1 diabetes: the UIC protocol. *Am J Transplant, 8*(6), 1250-1261. doi:10.1111/j.1600-6143.2008.02234.x

Gao, Y., Zhu, Y. Y., & Yuan, Z. (2015). Colloid (mucinous non-cystic) carcinoma of the pancreas: A case report. *Oncol Lett, 10*(5), 3195-3198. doi:10.3892/ol.2015.3733

Garside, V. C., Cullum, R., Alder, O., Lu, D. Y., Vander Werff, R., Bilenky, M., . . . Hoodless, P. A. (2015). SOX9 modulates the expression of key transcription factors required for heart valve development. *Development, 142*(24), 4340-4350. doi:10.1242/dev.125252

Gaulton, K. J., Willer, C. J., Li, Y., Scott, L. J., Conneely, K. N., Jackson, A. U., . . . Mohlke, K. L. (2008). Comprehensive association study of type 2 diabetes and related quantitative traits with 222 candidate genes. *Diabetes, 57*(11), 3136-3144. doi:10.2337/db07-1731

Gemberling, M., Bailey, T. J., Hyde, D. R., & Poss, K. D. (2013). The zebrafish as a model for complex tissue regeneration. *Trends Genet, 29*(11), 611-620. doi:10.1016/j.tig.2013.07.003

Geraldo, M. T., Valente, G. T., Nakajima, R. T., & Martins, C. (2016). Dimerization and Transactivation Domains as Candidates for Functional Modulation and Diversity of Sox9. *PLoS One, 11*(5), e0156199. doi:10.1371/journal.pone.0156199

Ghaye, A. P., Bergemann, D., Tarifeno-Saldivia, E., Flasse, L. C., Von Berg, V., Peers, B., . . . Manfroid, I. (2015). Progenitor potential of nkx6.1-expressing cells throughout zebrafish life and during beta cell regeneration. *BMC Biol, 13*, 70. doi:10.1186/s12915-015-0179-4

Giri, B., Sethi, V., Dudeja, V., Banerjee, S., Livingstone, A., & Saluja, A. (2017). Genetics of pancreatic cyst-cancer progression: standing on the shoulders of giants. *Curr Opin Gastroenterol, 33*(5), 404-410. doi:10.1097/MOG.0000000000000382

Goyal, S. N., Reddy, N. M., Patil, K. R., Nakhate, K. T., Ojha, S., Patil, C. R., & Agrawal, Y. O. (2016). Challenges and issues with streptozotocin-induced diabetes - A clinically relevant animal model to understand the diabetes pathogenesis and evaluate therapeutics. *Chem Biol Interact, 244*, 49-63. doi:10.1016/j.cbi.2015.11.032

Grussner, R., Nakhleh, R., Grussner, A., Tomadze, G., Diem, P., & Sutherland, D. (1993). Streptozotocin-induced diabetes mellitus in pigs. *Horm Metab Res, 25*(4), 199-203. doi:10.1055/s-2007-1002076

Guerrero-Juarez, C. F., Lee, G. H., Liu, Y., Wang, S., Karikomi, M., Sha, Y., . . . Atwood, S. X. (2022). Single-cell analysis of human basal cell carcinoma reveals novel regulators of tumor growth and the tumor microenvironment. *Sci Adv, 8*(23), eabm7981. doi:10.1126/sciadv.abm7981

Hald, J., Hjorth, J. P., German, M. S., Madsen, O. D., Serup, P., & Jensen, J. (2003). Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. *Dev Biol, 260*(2), 426-437. doi:10.1016/s0012-1606(03)00326-9

Hald, J., Sprinkel, A. E., Ray, M., Serup, P., Wright, C., & Madsen, O. D. (2008). Generation and characterization of Ptf1a antiserum and localization of Ptf1a in relation to Nkx6.1 and Pdx1 during the earliest stages of mouse pancreas development. *J Histochem Cytochem, 56*(6), 587-595. doi:10.1369/jhc.2008.950675

Hall, C. J., Astin, J. W., Mumm, J. S., & Ackerley, D. F. (2022). A New Transgenic Line for Rapid and Complete Neutrophil Ablation. *Zebrafish, 19*(3), 109-113. doi:10.1089/zeb.2022.0020

Han, J. H., Rall, L., & Rutter, W. J. (1986). Selective expression of rat pancreatic genes during embryonic development. *Proc Natl Acad Sci U S A, 83*(1), 110-114. doi:10.1073/pnas.83.1.110

Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W. M., 3rd, Zheng, S., Butler, A., . . . Satija, R. (2021). Integrated analysis of multimodal single-cell data. *Cell, 184*(13), 3573-3587 e3529. doi:10.1016/j.cell.2021.04.048

Hardt, P. D., Ewald, N., Brockling, K., Tanaka, S., Endo, T., Kloer, H. U., . . . Kobayashi, T. (2008). Distinct autoantibodies against exocrine pancreatic antigens in European patients with type 1 diabetes mellitus and non-alcoholic chronic pancreatitis. *JOP, 9*(6), 683-689.

Hart, P. A., Bellin, M. D., Andersen, D. K., Bradley, D., Cruz-Monserrate, Z., Forsmark, C. E., . . . Pancreatic, C. (2016). Type 3c (pancreatogenic) diabetes mellitus secondary to chronic pancreatitis and pancreatic cancer. *Lancet Gastroenterol Hepatol, 1*(3), 226-237. doi:10.1016/S2468-1253(16)30106-6

Harwood, H. J., Jr., Listrani, P., & Wagner, J. D. (2012). Nonhuman primates and other animal models in diabetes research. *J Diabetes Sci Technol, 6*(3), 503-514. doi:10.1177/193229681200600304

Hayashi, K., Takahashi, T., Kakita, A., & Yamashina, S. (1999). Regional differences in the cellular proliferation activity of the regenerating rat pancreas after partial pancreatectomy. *Arch Histol Cytol, 62*(4), 337-346. doi:10.1679/aohc.62.337

Hayashi, K. Y., Tamaki, H., Handa, K., Takahashi, T., Kakita, A., & Yamashina, S. (2003). Differentiation and proliferation of endocrine cells in the regenerating rat pancreas after 90% pancreatectomy. *Arch Histol Cytol, 66*(2), 163-174. doi:10.1679/aohc.66.163

He, X., Ohba, S., Hojo, H., & McMahon, A. P. (2016). AP-1 family members act with Sox9 to promote chondrocyte hypertrophy. *Development, 143*(16), 3012-3023. doi:10.1242/dev.134502

Heller, S., Li, Z., Lin, Q., Geusz, R., Breunig, M., Hohwieler, M., . . . Costa, I. G. (2021). Transcriptional changes and the role of ONECUT1 in hPSC pancreatic differentiation. *Commun Biol, 4*(1), 1298. doi:10.1038/s42003-021-02818-3

Hendley, A. M., Rao, A. A., Leonhardt, L., Ashe, S., Smith, J. A., Giacometti, S., . . . Hebrok, M. (2021). Single-cell transcriptome analysis defines heterogeneity of the murine pancreatic ductal tree. *Elife, 10*. doi:10.7554/eLife.67776

Hesselson, D., Anderson, R. M., & Stainier, D. Y. (2011). Suppression of Ptf1a activity induces acinar-to-endocrine conversion. *Curr Biol, 21*(8), 712-717. doi:10.1016/j.cub.2011.03.041

Hirshberg, B., Livi, A., Bartlett, D. L., Libutti, S. K., Alexander, H. R., Doppman, J. L., . . . Gorden, P. (2000). Forty-eight-hour fast: the diagnostic test for insulinoma. *J Clin Endocrinol Metab, 85*(9), 3222-3226. doi:10.1210/jcem.85.9.6807

Hoang, C. Q., Hale, M. A., Azevedo-Pouly, A. C., Elsasser, H. P., Deering, T. G., Willet, S. G., . . . MacDonald, R. J. (2016). Transcriptional Maintenance of Pancreatic Acinar Identity, Differentiation, and Homeostasis by PTF1A. *Mol Cell Biol, 36*(24), 3033-3047. doi:10.1128/MCB.00358-16

Horani, A., Ferkol, T. W., Shoseyov, D., Wasserman, M. G., Oren, Y. S., Kerem, B., . . . Kerem, E. (2013). LRRC6 mutation causes primary ciliary dyskinesia with dynein arm defects. *PLoS One, 8*(3), e59436. doi:10.1371/journal.pone.0059436

Houbracken, I., de Waele, E., Lardon, J., Ling, Z., Heimberg, H., Rooman, I., & Bouwens, L. (2011). Lineage tracing evidence for transdifferentiation of acinar to duct cells and plasticity of human pancreas. *Gastroenterology, 141*(2), 731-741, 741 e731-734. doi:10.1053/j.gastro.2011.04.050

Hruban, R. H., Goggins, M., Parsons, J., & Kern, S. E. (2000). Progression model for pancreatic cancer. *Clin Cancer Res, 6*(8), 2969-2972.

Hua, H., Zhang, Y. Q., Dabernat, S., Kritzik, M., Dietz, D., Sterling, L., & Sarvetnick, N. (2006). BMP4 regulates pancreatic progenitor cell expansion through Id2. *J Biol Chem, 281*(19), 13574-13580. doi:10.1074/jbc.M600526200

Huang, W., Beer, R. L., Delaspre, F., Wang, G., Edelman, H. E., Park, H., . . . Parsons, M. J. (2016). Sox9b is a mediator of retinoic acid signaling restricting endocrine progenitor differentiation. *Dev Biol, 418*(1), 28-39. doi:10.1016/j.ydbio.2016.08.019

Huang, W. Y., Aramburu, J., Douglas, P. S., & Izumo, S. (2000). Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat Med, 6*(5), 482-483. doi:10.1038/74914

Ibrahim, S., Harris-Kawano, A., Haider, I., Mirmira, R. G., Sims, E. K., & Anderson, R. M. (2020). A novel Cre-enabled tetracycline-inducible transgenic system for tissue-specific cytokine expression in the zebrafish: CETI-PIC3. *Dis Model Mech, 13*(6). doi:10.1242/dmm.042556

Inada, A., Nienaber, C., Katsuta, H., Fujitani, Y., Levine, J., Morita, R., . . . Bonner-Weir, S. (2008). Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proc Natl Acad Sci U S A, 105*(50), 19915-19919. doi:10.1073/pnas.0805803105

Ionut, V., Liu, H., Mooradian, V., Castro, A. V., Kabir, M., Stefanovski, D., . . . Bergman, R. N. (2010). Novel canine models of obese prediabetes and mild type 2 diabetes. *Am J Physiol Endocrinol Metab, 298*(1), E38-48. doi:10.1152/ajpendo.00466.2009

Ishii, H., Saitoh, M., Sakamoto, K., Kondo, T., Katoh, R., Tanaka, S., . . . Miyazawa, K. (2014). Epithelial splicing regulatory proteins 1 (ESRP1) and 2 (ESRP2) suppress cancer cell motility via different mechanisms. *J Biol Chem, 289*(40), 27386-27399. doi:10.1074/jbc.M114.589432

Jacquemin, P., Lemaigre, F. P., & Rousseau, G. G. (2003). The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. *Dev Biol, 258*(1), 105-116. doi:10.1016/s0012-1606(03)00115-5

Jones, S., Zhang, X., Parsons, D. W., Lin, J. C., Leary, R. J., Angenendt, P., . . . Kinzler, K. W. (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science, 321*(5897), 1801-1806. doi:10.1126/science.1164368

Juiz, N., Elkaoutari, A., Bigonnet, M., Gayet, O., Roques, J., Nicolle, R., . . . Dusetti, N. (2020). Basal-like and classical cells coexist in pancreatic cancer revealed by single-cell analysis on biopsy-derived pancreatic cancer organoids from the classical subtype. *FASEB J, 34*(9), 12214-12228. doi:10.1096/fj.202000363RR

Kadaja, M., Keyes, B. E., Lin, M., Pasolli, H. A., Genander, M., Polak, L., . . . Fuchs, E. (2014). SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. *Genes Dev, 28*(4), 328-341. doi:10.1101/gad.233247.113

Kamachi, Y., & Kondoh, H. (2013). Sox proteins: regulators of cell fate specification and differentiation. *Development, 140*(20), 4129-4144. doi:10.1242/dev.091793

Kanda, M., Matthaei, H., Wu, J., Hong, S. M., Yu, J., Borges, M., . . . Goggins, M. (2012). Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology, 142*(4), 730-733 e739. doi:10.1053/j.gastro.2011.12.042

Kanno, S., Hirano, S., Sakamoto, T., Furuyama, A., Takase, H., Kato, H., . . . Aoki, Y. (2020). Scavenger receptor MARCO contributes to cellular internalization of exosomes by dynamin-dependent endocytosis and macropinocytosis. *Sci Rep, 10*(1), 21795. doi:10.1038/s41598-020-78464-2

Kawai, T., Yasuchika, K., Ishii, T., Miyauchi, Y., Kojima, H., Yamaoka, R., . . . Uemoto, S. (2016). SOX9 is a novel cancer stem cell marker surrogated by osteopontin in human hepatocellular carcinoma. *Sci Rep, 6*, 30489. doi:10.1038/srep30489

Kawakami, K. (2004). Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. *Methods Cell Biol, 77*, 201-222. doi:10.1016/s0091-679x(04)77011-9

Keck, F. S., & Duntas, L. H. (2007). Brunner's missing 'Aha experience' delayed progress in diabetes research by 200 years. *Hormones (Athens), 6*(3), 251-254.

Keewan, E., & Naser, S. A. (2020). The Role of Notch Signaling in Macrophages during Inflammation and Infection: Implication in Rheumatoid Arthritis? *Cells, 9*(1). doi:10.3390/cells9010111

Kikkas, I., Mallone, R., Larger, E., Volland, H., & Morel, N. (2014). A rapid lateral flow immunoassay for the detection of tyrosine phosphatase-like protein IA-2 autoantibodies in human serum. *PLoS One, 9*(7), e103088. doi:10.1371/journal.pone.0103088

Kim, S. K., & MacDonald, R. J. (2002). Signaling and transcriptional control of pancreatic organogenesis. *Curr Opin Genet Dev, 12*(5), 540-547. doi:10.1016/s0959-437x(02)00338-6

Kimmel, R. A., Dobler, S., Schmitner, N., Walsen, T., Freudenblum, J., & Meyer, D. (2015). Diabetic pdx1-mutant zebrafish show conserved responses to nutrient overload and anti-glycemic treatment. *Sci Rep, 5*, 14241. doi:10.1038/srep14241

Kimmel, R. A., & Meyer, D. (2016). Zebrafish pancreas as a model for development and disease. *Methods Cell Biol, 134*, 431-461. doi:10.1016/bs.mcb.2016.02.009

Kinkel, M. D., & Prince, V. E. (2009). On the diabetic menu: zebrafish as a model for pancreas development and function. *Bioessays, 31*(2), 139-152. doi:10.1002/bies.200800123

Kopinke, D., Brailsford, M., Pan, F. C., Magnuson, M. A., Wright, C. V., & Murtaugh, L. C. (2012). Ongoing Notch signaling maintains phenotypic fidelity in the adult exocrine pancreas. *Dev Biol, 362*(1), 57-64. doi:10.1016/j.ydbio.2011.11.010

Kopinke, D., Brailsford, M., Shea, J. E., Leavitt, R., Scaife, C. L., & Murtaugh, L. C. (2011). Lineage tracing reveals the dynamic contribution of Hes1+ cells to the developing and adult pancreas. *Development, 138*(3), 431-441. doi:10.1242/dev.053843

Kopinke, D., & Murtaugh, L. C. (2010). Exocrine-to-endocrine differentiation is detectable only prior to birth in the uninjured mouse pancreas. *BMC Dev Biol, 10*, 38. doi:10.1186/1471-213X-10-38

Kopp, J. L., Dubois, C. L., Schaffer, A. E., Hao, E., Shih, H. P., Seymour, P. A., . . . Sander, M. (2011). Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. *Development, 138*(4), 653-665. doi:10.1242/dev.056499

Kopp, J. L., von Figura, G., Mayes, E., Liu, F. F., Dubois, C. L., Morris, J. P. t., . . . Sander, M. (2012). Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. *Cancer Cell, 22*(6), 737-750. doi:10.1016/j.ccr.2012.10.025

Kott, E., Duquesnoy, P., Copin, B., Legendre, M., Dastot-Le Moal, F., Montantin, G., . . . Amselem, S. (2012). Loss-of-function mutations in LRRC6, a gene essential for proper axonemal assembly of inner and outer dynein arms, cause primary ciliary dyskinesia. *Am J Hum Genet, 91*(5), 958-964. doi:10.1016/j.ajhg.2012.10.003

Kottaisamy, C. P. D., Raj, D. S., Prasanth Kumar, V., & Sankaran, U. (2021). Experimental animal models for diabetes and its related complications-a review. *Lab Anim Res, 37*(1), 23. doi:10.1186/s42826-021-00101-4

Kramer-Zucker, A. G., Wiessner, S., Jensen, A. M., & Drummond, I. A. (2005). Organization of the pronephric filtration apparatus in zebrafish requires Nephrin, Podocin and the FERM domain protein Mosaic eyes. *Dev Biol, 285*(2), 316-329. doi:10.1016/j.ydbio.2005.06.038

Krieger, T. G., Le Blanc, S., Jabs, J., Ten, F. W., Ishaque, N., Jechow, K., . . . Conrad, C. (2021). Single-cell analysis of patient-derived PDAC organoids reveals cell state heterogeneity and a conserved developmental hierarchy. *Nat Commun, 12*(1), 5826. doi:10.1038/s41467-021-26059-4

Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazer, S., . . . Baetge, E. E. (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol, 26*(4), 443-452. doi:10.1038/nbt1393

Kuri, P., Ellwanger, K., Kufer, T. A., Leptin, M., & Bajoghli, B. (2017). A high-sensitivity bi-directional reporter to monitor NF-kappaB activity in cell culture and zebrafish in real time. *J Cell Sci, 130*(3), 648-657. doi:10.1242/jcs.196485

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., . . . Kharchenko, P. V. (2018). RNA velocity of single cells. *Nature, 560*(7719), 494-498. doi:10.1038/s41586-018-0414-6

Lancman, J. J., Zvenigorodsky, N., Gates, K. P., Zhang, D., Solomon, K., Humphrey, R. K., . . . Dong, P. D. (2013). Specification of hepatopancreas progenitors in zebrafish by hnf1ba and wnt2bb. *Development, 140*(13), 2669-2679. doi:10.1242/dev.090993

Lawson, N. D., Li, R., Shin, M., Grosse, A., Yukselen, O., Stone, O. A., . . . Zhu, L. (2020). An improved zebrafish transcriptome annotation for sensitive and comprehensive detection of cell type-specific genes. *Elife, 9*. doi:10.7554/eLife.55792

Lee, A. Y. L., Dubois, C. L., Sarai, K., Zarei, S., Schaeffer, D. F., Sander, M., & Kopp, J. L. (2019). Cell of origin affects tumour development and phenotype in pancreatic ductal adenocarcinoma. *Gut, 68*(3), 487-498. doi:10.1136/gutjnl-2017-314426

Lee, R. D., Munro, S. A., Knutson, T. P., LaRue, R. S., Heltemes-Harris, L. M., & Farrar, M. A. (2021). Single-cell analysis identifies dynamic gene expression networks that govern B cell development and transformation. *Nat Commun, 12*(1), 6843. doi:10.1038/s41467-021-27232-5

Lefebvre, V., & Dvir-Ginzberg, M. (2017). SOX9 and the many facets of its regulation in the chondrocyte lineage. *Connect Tissue Res, 58*(1), 2-14. doi:10.1080/03008207.2016.1183667

Leung, V. Y., Gao, B., Leung, K. K., Melhado, I. G., Wynn, S. L., Au, T. Y., . . . Cheah, K. S. (2011). SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct

concomitant transactivation and repression. *PLoS Genet, 7*(11), e1002356. doi:10.1371/journal.pgen.1002356
Lewis, K. L., Del Cid, N., & Traver, D. (2014). Perspectives on antigen presenting cells in zebrafish. *Dev Comp Immunol, 46*(1), 63-73. doi:10.1016/j.dci.2014.03.010
Li, D., Mukai, K., Suzuki, T., Suzuki, R., Yamashita, S., Mitani, F., & Suematsu, M. (2007). Adrenocortical zonation factor 1 is a novel matricellular protein promoting integrin-mediated adhesion of adrenocortical and vascular smooth muscle cells. *FEBS J, 274*(10), 2506-2522. doi:10.1111/j.1742-4658.2007.05786.x
Li, Z., Korzh, V., & Gong, Z. (2009). DTA-mediated targeted ablation revealed differential interdependence of endocrine cell lineages in early development of zebrafish pancreas. *Differentiation, 78*(4), 241-252. doi:10.1016/j.diff.2009.05.009
Lieber, M., Mazzetta, J., Nelson-Rees, W., Kaplan, M., & Todaro, G. (1975). Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. *Int J Cancer, 15*(5), 741-747. doi:10.1002/ijc.2910150505
Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L., & Raible, D. W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development, 126*(17), 3757-3767. doi:10.1242/dev.126.17.3757
Liu, H., Kiseleva, A. A., & Golemis, E. A. (2018). Ciliary signalling in cancer. *Nat Rev Cancer, 18*(8), 511-524. doi:10.1038/s41568-018-0023-6
Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H., & Ke, N. J. (1999). Is green fluorescent protein toxic to the living cells? *Biochem Biophys Res Commun, 260*(3), 712-717. doi:10.1006/bbrc.1999.0954
Lohr, M., & Kloppel, G. (1987). Residual insulin positivity and pancreatic atrophy in relation to duration of chronic type 1 (insulin-dependent) diabetes mellitus and microangiopathy. *Diabetologia, 30*(10), 757-762. doi:10.1007/BF00275740
Lowenfels, A. B., Maisonneuve, P., Cavallini, G., Ammann, R. W., Lankisch, P. G., Andersen, J. R., . . . Domellof, L. (1993). Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med, 328*(20), 1433-1437. doi:10.1056/NEJM199305203282001
Lynn, F. C., Smith, S. B., Wilson, M. E., Yang, K. Y., Nekrep, N., & German, M. S. (2007). Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A, 104*(25), 10500-10505. doi:10.1073/pnas.0704054104
Maclean, N., & Ogilvie, R. F. (1959). Observations on the pancreatic islet tissue of young diabetic subjects. *Diabetes, 8*(2), 83-91. doi:10.2337/diab.8.2.83
Maetzel, D., Denzel, S., Mack, B., Canis, M., Went, P., Benk, M., . . . Gires, O. (2009). Nuclear signalling by tumour-associated antigen EpCAM. *Nat Cell Biol, 11*(2), 162-171. doi:10.1038/ncb1824
Maghzal, N., Kayali, H. A., Rohani, N., Kajava, A. V., & Fagotto, F. (2013). EpCAM controls actomyosin contractility and cell adhesion by direct inhibition of PKC. *Dev Cell, 27*(3), 263-277. doi:10.1016/j.devcel.2013.10.003
Maghzal, N., Vogt, E., Reintsch, W., Fraser, J. S., & Fagotto, F. (2010). The tumor-associated EpCAM regulates morphogenetic movements through intracellular signaling. *J Cell Biol, 191*(3), 645-659. doi:10.1083/jcb.201004074
Manfroid, I., Ghaye, A., Naye, F., Detry, N., Palm, S., Pan, L., . . . Peers, B. (2012). Zebrafish sox9b is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration. *Dev Biol, 366*(2), 268-278. doi:10.1016/j.ydbio.2012.04.002
Marfil-Garza, B. A., Imes, S., Verhoeff, K., Hefler, J., Lam, A., Dajani, K., . . . Shapiro, A. M. J. (2022). Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada. *Lancet Diabetes Endocrinol, 10*(7), 519-532. doi:10.1016/S2213-8587(22)00114-0

Marino, C. R., Matovcik, L. M., Gorelick, F. S., & Cohn, J. A. (1991). Localization of the cystic fibrosis transmembrane conductance regulator in pancreas. *J Clin Invest, 88*(2), 712-716. doi:10.1172/JCI115358

Matsuda, H., Parsons, M. J., & Leach, S. D. (2013). Aldh1-expressing endocrine progenitor cells regulate secondary islet formation in larval zebrafish pancreas. *PLoS One, 8*(9), e74350. doi:10.1371/journal.pone.0074350

McCall, M., & Shapiro, A. M. (2012). Update on islet transplantation. *Cold Spring Harb Perspect Med, 2*(7), a007823. doi:10.1101/cshperspect.a007823

Meeker, N. D., Hutchinson, S. A., Ho, L., & Trede, N. S. (2007). Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *Biotechniques, 43*(5), 610, 612, 614. doi:10.2144/000112619

Meier, J. J., Bhushan, A., Butler, A. E., Rizza, R. A., & Butler, P. C. (2005). Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia, 48*(11), 2221-2228. doi:10.1007/s00125-005-1949-2

Meier, J. J., Butler, A. E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., . . . Butler, P. C. (2008). Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes, 57*(6), 1584-1594. doi:10.2337/db07-1369

Melancon, E., Gomez De La Torre Canny, S., Sichel, S., Kelly, M., Wiles, T. J., Rawls, J. F., . . . Guillemin, K. (2017). Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. *Methods Cell Biol, 138*, 61-100. doi:10.1016/bs.mcb.2016.11.005

Mi, J., & Andersson, O. (2023). Efficient knock-in method enabling lineage tracing in zebrafish. *Life Sci Alliance, 6*(5). doi:10.26508/lsa.202301944

Mitrowska, K., Pekala, A., & Posyniak, A. (2015). Tissue distribution and residue depletion of metronidazole in rainbow trout (Oncorhynchus mykiss). *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32*(6), 841-848. doi:10.1080/19440049.2015.1036320

Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., . . . Leach, S. D. (2003). Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell, 3*(6), 565-576. doi:10.1016/s1535-6108(03)00140-5

Miyatsuka, T., Matsuoka, T. A., Sasaki, S., Kubo, F., Shimomura, I., Watada, H., . . . Hara, M. (2014). Chronological analysis with fluorescent timer reveals unique features of newly generated beta-cells. *Diabetes, 63*(10), 3388-3393. doi:10.2337/db13-1312

Monberg, M. E., Geiger, H., Lee, J. J., Sharma, R., Semaan, A., Bernard, V., . . . Maitra, A. (2022). Occult polyclonality of preclinical pancreatic cancer models drives in vitro evolution. *Nat Commun, 13*(1), 3652. doi:10.1038/s41467-022-31376-3

Moro, E., Gnugge, L., Braghetta, P., Bortolussi, M., & Argenton, F. (2009). Analysis of beta cell proliferation dynamics in zebrafish. *Dev Biol, 332*(2), 299-308. doi:10.1016/j.ydbio.2009.05.576

Moss, J. B., Koustubhan, P., Greenman, M., Parsons, M. J., Walter, I., & Moss, L. G. (2009). Regeneration of the pancreas in adult zebrafish. *Diabetes, 58*(8), 1844-1851. doi:10.2337/db08-0628

Muller-Pillasch, F., Wallrapp, C., Bartels, K., Varga, G., Friess, H., Buchler, M., . . . Gress, T. M. (1998). Cloning of a new Kunitz-type protease inhibitor with a putative transmembrane domain overexpressed in pancreatic cancer. *Biochim Biophys Acta, 1395*(1), 88-95. doi:10.1016/s0167-4781(97)00129-2

Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L., & Baranski, T. J. (2011). A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. *Dis Model Mech, 4*(6), 842-849. doi:10.1242/dmm.007948

Navis, A., & Bagnat, M. (2015). Loss of cftr function leads to pancreatic destruction in larval zebrafish. *Dev Biol, 399*(2), 237-248. doi:10.1016/j.ydbio.2014.12.034

Nicolle, R., Blum, Y., Marisa, L., Loncle, C., Gayet, O., Moutardier, V., . . . Iovanna, J. (2017). Pancreatic Adenocarcinoma Therapeutic Targets Revealed by Tumor-Stroma Cross-Talk Analyses in Patient-Derived Xenografts. *Cell Rep, 21*(9), 2458-2470. doi:10.1016/j.celrep.2017.11.003

Niederhuber, J. E., Brennan, M. F., & Menck, H. R. (1995). The National Cancer Data Base report on pancreatic cancer. *Cancer, 76*(9), 1671-1677. doi:10.1002/1097-0142(19951101)76:9<1671::aid-cncr2820760926>3.0.co;2-r

Ninov, N., Borius, M., & Stainier, D. Y. (2012). Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. *Development, 139*(9), 1557-1567. doi:10.1242/dev.076000

Nir, T., Melton, D. A., & Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest, 117*(9), 2553-2561. doi:10.1172/JCI32959

Nizzi, F., Rees, M., Salzberg, D., & Ngwube, A. (2020). Successful management of sirolimus toxicity in a hematopoietic stem cell transplant patient using automated red blood cell exchange. *Transfusion, 60*(12), 3060-3063. doi:10.1111/trf.16064

Ntimbane, T., Comte, B., Mailhot, G., Berthiaume, Y., Poitout, V., Prentki, M., . . . Levy, E. (2009). Cystic fibrosis-related diabetes: from CFTR dysfunction to oxidative stress. *Clin Biochem Rev, 30*(4), 153-177.

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., . . . Tanaka, T. (2010). Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiol, 10*, 21. doi:10.1186/1472-6793-10-21

Olsen, A. S., Sarras, M. P., Jr., & Intine, R. V. (2010). Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. *Wound Repair Regen, 18*(5), 532-542. doi:10.1111/j.1524-475X.2010.00613.x

Olsen, A. S., Sarras, M. P., Jr., Leontovich, A., & Intine, R. V. (2012). Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. *Diabetes, 61*(2), 485-491. doi:10.2337/db11-0588

Orth, M., Metzger, P., Gerum, S., Mayerle, J., Schneider, G., Belka, C., . . . Lauber, K. (2019). Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. *Radiat Oncol, 14*(1), 141. doi:10.1186/s13014-019-1345-6

Pagliuca, F. W., Millman, J. R., Gurtler, M., Segel, M., Van Dervort, A., Ryu, J. H., . . . Melton, D. A. (2014). Generation of functional human pancreatic beta cells in vitro. *Cell, 159*(2), 428-439. doi:10.1016/j.cell.2014.09.040

Panicot, L., Mas, E., Thivolet, C., & Lombardo, D. (1999). Circulating antibodies against an exocrine pancreatic enzyme in type 1 diabetes. *Diabetes, 48*(12), 2316-2323. doi:10.2337/diabetes.48.12.2316

Papizan, J. B., Singer, R. A., Tschen, S. I., Dhawan, S., Friel, J. M., Hipkens, S. B., . . . Sussel, L. (2011). Nkx2.2 repressor complex regulates islet beta-cell specification and prevents beta-to-alpha-cell reprogramming. *Genes Dev, 25*(21), 2291-2305. doi:10.1101/gad.173039.111

Parekh, D., & Natarajan, S. (2015). Surgical Management of Chronic Pancreatitis. *Indian J Surg, 77*(5), 453-469. doi:10.1007/s12262-015-1362-0

Parsons, M. J., Pisharath, H., Yusuff, S., Moore, J. C., Siekmann, A. F., Lawson, N., & Leach, S. D. (2009). Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. *Mech Dev, 126*(10), 898-912. doi:10.1016/j.mod.2009.07.002

Path, G., Perakakis, N., Mantzoros, C. S., & Seufert, J. (2019). Stem cells in the treatment of diabetes mellitus - Focus on mesenchymal stem cells. *Metabolism, 90*, 1-15. doi:10.1016/j.metabol.2018.10.005

Pathak, V., Pathak, N. M., O'Neill, C. L., Guduric-Fuchs, J., & Medina, R. J. (2019). Therapies for Type 1 Diabetes: Current Scenario and Future Perspectives. *Clin Med Insights Endocrinol Diabetes, 12*, 1179551419844521. doi:10.1177/1179551419844521

Patriarca, C., Macchi, R. M., Marschner, A. K., & Mellstedt, H. (2012). Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. *Cancer Treat Rev, 38*(1), 68-75. doi:10.1016/j.ctrv.2011.04.002

Peng, J., Sun, B. F., Chen, C. Y., Zhou, J. Y., Chen, Y. S., Chen, H., . . . Wu, W. (2019). Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell Res, 29*(9), 725-738. doi:10.1038/s41422-019-0195-y

Permuth-Wey, J., & Egan, K. M. (2009). Family history is a significant risk factor for pancreatic cancer: results from a systematic review and meta-analysis. *Fam Cancer, 8*(2), 109-117. doi:10.1007/s10689-008-9214-8

Piper, K., Ball, S. G., Keeling, J. W., Mansoor, S., Wilson, D. I., & Hanley, N. A. (2002). Novel SOX9 expression during human pancreas development correlates to abnormalities in Campomelic dysplasia. *Mech Dev, 116*(1-2), 223-226. doi:10.1016/s0925-4773(02)00145-4

Pisharath, H., & Parsons, M. J. (2009). Nitroreductase-mediated cell ablation in transgenic zebrafish embryos. *Methods Mol Biol, 546*, 133-143. doi:10.1007/978-1-60327-977-2_9

Pisharath, H., Rhee, J. M., Swanson, M. A., Leach, S. D., & Parsons, M. J. (2007). Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. *Mech Dev, 124*(3), 218-229. doi:10.1016/j.mod.2006.11.005

Plerhoples, T. A., Ahdoot, M., DiMaio, M. A., Pai, R. K., Park, W. G., & Poultsides, G. A. (2011). Colloid carcinoma of the pancreas. *Dig Dis Sci, 56*(5), 1295-1298. doi:10.1007/s10620-011-1573-6

Pourghasem, M., Shafi, H., & Babazadeh, Z. (2015). Histological changes of kidney in diabetic nephropathy. *Caspian J Intern Med, 6*(3), 120-127.

Prince, V. E., Anderson, R. M., & Dalgin, G. (2017). Zebrafish Pancreas Development and Regeneration: Fishing for Diabetes Therapies. *Curr Top Dev Biol, 124*, 235-276. doi:10.1016/bs.ctdb.2016.10.005

Puri, S., Folias, A. E., & Hebrok, M. (2015). Plasticity and dedifferentiation within the pancreas: development, homeostasis, and disease. *Cell Stem Cell, 16*(1), 18-31. doi:10.1016/j.stem.2014.11.001

Qadir, M. M. F., Alvarez-Cubela, S., Klein, D., van Dijk, J., Muniz-Anquela, R., Moreno-Hernandez, Y. B., . . . Dominguez-Bendala, J. (2020). Single-cell resolution analysis of the human pancreatic ductal progenitor cell niche. *Proc Natl Acad Sci U S A, 117*(20), 10876-10887. doi:10.1073/pnas.1918314117

Qian, P., Kang, J., Liu, D., & Xie, G. (2022). Single Cell Transcriptome Sequencing of Zebrafish Testis Revealed Novel Spermatogenesis Marker Genes and Stronger Leydig-Germ Cell Paracrine Interactions. *Front Genet, 13*, 851719. doi:10.3389/fgene.2022.851719

Quintanilla Rodriguez, B. S., & Mahdy, H. (2023). Gestational Diabetes. In *StatPearls*. Treasure Island (FL).

Reiten, I., Uslu, F. E., Fore, S., Pelgrims, R., Ringers, C., Diaz Verdugo, C., . . . Jurisch-Yaksi, N. (2017). Motile-Cilia-Mediated Flow Improves Sensitivity and Temporal Resolution of Olfactory Computations. *Curr Biol, 27*(2), 166-174. doi:10.1016/j.cub.2016.11.036

Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D. C., Kessler, B., . . . Wolf, E. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes, 59*(5), 1228-1238. doi:10.2337/db09-0519

Rezania, A., Bruin, J. E., Riedel, M. J., Mojibian, M., Asadi, A., Xu, J., . . . Kieffer, T. J. (2012). Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes, 61*(8), 2016-2029. doi:10.2337/db11-1711

Rhim, A. D., Mirek, E. T., Aiello, N. M., Maitra, A., Bailey, J. M., McAllister, F., . . . Stanger, B. Z. (2012). EMT and dissemination precede pancreatic tumor formation. *Cell, 148*(1-2), 349-361. doi:10.1016/j.cell.2011.11.025

Rhim, A. D., & Stanger, B. Z. (2010). Molecular biology of pancreatic ductal adenocarcinoma progression: aberrant activation of developmental pathways. *Prog Mol Biol Transl Sci, 97*, 41-78. doi:10.1016/B978-0-12-385233-5.00002-7

Ribeiro, F. F., & Xapelli, S. (2021). An Overview of Adult Neurogenesis. *Adv Exp Med Biol, 1331*, 77-94. doi:10.1007/978-3-030-74046-7_7

Ro, C., Chai, W., Yu, V. E., & Yu, R. (2013). Pancreatic neuroendocrine tumors: biology, diagnosis,and treatment. *Chin J Cancer, 32*(6), 312-324. doi:10.5732/cjc.012.10295

Robert, T., De Mesmaeker, I., Stange, G. M., Suenens, K. G., Ling, Z., Kroon, E. J., & Pipeleers, D. G. (2018). Functional Beta Cell Mass from Device-Encapsulated hESC-Derived Pancreatic Endoderm Achieving Metabolic Control. *Stem Cell Reports, 10*(3), 739-750. doi:10.1016/j.stemcr.2018.01.040

Roche, E. F., Menon, A., Gill, D., & Hoey, H. (2005). Clinical presentation of type 1 diabetes. *Pediatr Diabetes, 6*(2), 75-78. doi:10.1111/j.1399-543X.2005.00110.x

Roversi, F. M., Olalla Saad, S. T., & Machado-Neto, J. A. (2018). Serine peptidase inhibitor Kunitz type 2 (SPINT2) in cancer development and progression. *Biomed Pharmacother, 101*, 278-286. doi:10.1016/j.biopha.2018.02.100

Rovira, M., Scott, S. G., Liss, A. S., Jensen, J., Thayer, S. P., & Leach, S. D. (2010). Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. *Proc Natl Acad Sci U S A, 107*(1), 75-80. doi:10.1073/pnas.0912589107

Rutter, W. J., Kemp, J. D., Bradshaw, W. S., Clark, W. R., Ronzio, R. A., & Sanders, T. G. (1968). Regulation of specific protein synthesis in cytodifferentiation. *J Cell Physiol, 72*(2), Suppl 1:1-18. doi:10.1002/jcp.1040720403

Sabaliauskas, N. A., Foutz, C. A., Mest, J. R., Budgeon, L. R., Sidor, A. T., Gershenson, J. A., . . . Cheng, K. C. (2006). High-throughput zebrafish histology. *Methods, 39*(3), 246-254. doi:10.1016/j.ymeth.2006.03.001

Sancho, R., Gruber, R., Gu, G., & Behrens, A. (2014). Loss of Fbw7 reprograms adult pancreatic ductal cells into alpha, delta, and beta cells. *Cell Stem Cell, 15*(2), 139-153. doi:10.1016/j.stem.2014.06.019

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., . . . German, M. (2000). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development, 127*(24), 5533-5540. doi:10.1242/dev.127.24.5533

Sarras, M. P., Jr., Leontovich, A. A., & Intine, R. V. (2015). Use of zebrafish as a model to investigate the role of epigenetics in propagating the secondary complications observed in diabetes mellitus. *Comp Biochem Physiol C Toxicol Pharmacol, 178*, 3-7. doi:10.1016/j.cbpc.2015.07.001

Schaffer, A. E., Freude, K. K., Nelson, S. B., & Sander, M. (2010). Nkx6 transcription factors and Ptf1a function as antagonistic lineage determinants in multipotent pancreatic progenitors. *Dev Cell, 18*(6), 1022-1029. doi:10.1016/j.devcel.2010.05.015

Schaffer, A. E., Taylor, B. L., Benthuysen, J. R., Liu, J., Thorel, F., Yuan, W., . . . Sander, M. (2013). Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. *PLoS Genet, 9*(1), e1003274. doi:10.1371/journal.pgen.1003274

Schnell, U., Cirulli, V., & Giepmans, B. N. (2013). EpCAM: structure and function in health and disease. *Biochim Biophys Acta, 1828*(8), 1989-2001. doi:10.1016/j.bbamem.2013.04.018

Schroder, B. (2016). The multifaceted roles of the invariant chain CD74--More than just a chaperone. *Biochim Biophys Acta, 1863*(6 Pt A), 1269-1281. doi:10.1016/j.bbamcr.2016.03.026

Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., & Ristow, M. (2007). Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab, 6*(4), 280-293. doi:10.1016/j.cmet.2007.08.011

Scott, C. E., Wynn, S. L., Sesay, A., Cruz, C., Cheung, M., Gomez Gaviro, M. V., . . . Briscoe, J. (2010). SOX9 induces and maintains neural stem cells. *Nat Neurosci, 13*(10), 1181-1189. doi:10.1038/nn.2646

Seeley, E. S., Carriere, C., Goetze, T., Longnecker, D. S., & Korc, M. (2009). Pancreatic cancer and precursor pancreatic intraepithelial neoplasia lesions are devoid of primary cilia. *Cancer Res, 69*(2), 422-430. doi:10.1158/0008-5472.CAN-08-1290

Seita, M., Noguchi, H., Kubota, Y., Kawamoto, H., Nakaji, S., Kobayashi, N., & Fujiwara, T. (2013). Development of Canine Models of Type 1 Diabetes With Partial Pancreatectomy and the Administration of Streptozotocin. *Cell Med, 6*(1-2), 25-31. doi:10.3727/215517913X674289

Seymour, P. A. (2014). Sox9: a master regulator of the pancreatic program. *Rev Diabet Stud, 11*(1), 51-83. doi:10.1900/RDS.2014.11.51

Seymour, P. A., Freude, K. K., Dubois, C. L., Shih, H. P., Patel, N. A., & Sander, M. (2008). A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation. *Dev Biol, 323*(1), 19-30. doi:10.1016/j.ydbio.2008.07.034

Seymour, P. A., Freude, K. K., Tran, M. N., Mayes, E. E., Jensen, J., Kist, R., . . . Sander, M. (2007). SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A, 104*(6), 1865-1870. doi:10.1073/pnas.0609217104

Shapiro, A. M., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., . . . Lakey, J. R. (2006). International trial of the Edmonton protocol for islet transplantation. *N Engl J Med, 355*(13), 1318-1330. doi:10.1056/NEJMoa061267

Sharrock, A. V., Mulligan, T. S., Hall, K. R., Williams, E. M., White, D. T., Zhang, L., . . . Mumm, J. S. (2022). NTR 2.0: a rationally engineered prodrug-converting enzyme with substantially enhanced efficacy for targeted cell ablation. *Nat Methods, 19*(2), 205-215. doi:10.1038/s41592-021-01364-4

Shetty, A. K., & Hattiangady, B. (2016). Grafted Subventricular Zone Neural Stem Cells Display Robust Engraftment and Similar Differentiation Properties and Form New Neurogenic Niches in the Young and Aged Hippocampus. *Stem Cells Transl Med, 5*(9), 1204-1215. doi:10.5966/sctm.2015-0270

Shi, C., Pan, F. C., Kim, J. N., Washington, M. K., Padmanabhan, C., Meyer, C. T., . . . Means, A. L. (2019). Differential Cell Susceptibilities to Kras(G12D) in the Setting of Obstructive Chronic Pancreatitis. *Cell Mol Gastroenterol Hepatol, 8*(4), 579-594. doi:10.1016/j.jcmgh.2019.07.001

Shi, Z., Chiang, C. I., Labhart, P., Zhao, Y., Yang, J., Mistretta, T. A., . . . Mori-Akiyama, Y. (2015). Context-specific role of SOX9 in NF-Y mediated gene regulation in colorectal cancer cells. *Nucleic Acids Res, 43*(13), 6257-6269. doi:10.1093/nar/gkv568

Shih, H. P., Kopp, J. L., Sandhu, M., Dubois, C. L., Seymour, P. A., Grapin-Botton, A., & Sander, M. (2012). A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. *Development, 139*(14), 2488-2499. doi:10.1242/dev.078634

Shih, H. P., Seymour, P. A., Patel, N. A., Xie, R., Wang, A., Liu, P. P., . . . Sander, M. (2015). A Gene Regulatory Network Cooperatively Controlled by Pdx1 and Sox9 Governs Lineage Allocation of Foregut Progenitor Cells. *Cell Rep, 13*(2), 326-336. doi:10.1016/j.celrep.2015.08.082

Shih, H. P., Wang, A., & Sander, M. (2013). Pancreas organogenesis: from lineage determination to morphogenesis. *Annu Rev Cell Dev Biol, 29*, 81-105. doi:10.1146/annurev-cellbio-101512-122405

Shin, C. H., Chung, W. S., Hong, S. K., Ober, E. A., Verkade, H., Field, H. A., . . . Stainier, D. Y. (2008). Multiple roles for Med12 in vertebrate endoderm development. *Dev Biol, 317*(2), 467-479. doi:10.1016/j.ydbio.2008.02.031

Shiota, M., & Printz, R. L. (2012). Diabetes in Zucker diabetic fatty rat. *Methods Mol Biol, 933*, 103-123. doi:10.1007/978-1-62703-068-7_8

Shukla, U. V., & Tripathy, K. (2022). Diabetic Retinopathy. In *StatPearls*. Treasure Island (FL).

Siegel, R. L., Miller, K. D., & Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J Clin, 68*(1), 7-30. doi:10.3322/caac.21442

Singh, R., Kishore, L., & Kaur, N. (2014). Diabetic peripheral neuropathy: current perspective and future directions. *Pharmacol Res, 80*, 21-35. doi:10.1016/j.phrs.2013.12.005

Singh, S. P., Chawla, P., Hnatiuk, A., Kamel, M., Silva, L. D., Spanjaard, B., . . . Ninov, N. (2022). A single-cell atlas of de novo beta-cell regeneration reveals the contribution of hybrid beta/delta-cells to diabetes recovery in zebrafish. *Development, 149*(2). doi:10.1242/dev.199853

Sivagnanam, M., Mueller, J. L., Lee, H., Chen, Z., Nelson, S. F., Turner, D., . . . Hoffman, H. M. (2008). Identification of EpCAM as the gene for congenital tufting enteropathy. *Gastroenterology, 135*(2), 429-437. doi:10.1053/j.gastro.2008.05.036

Slanchev, K., Carney, T. J., Stemmler, M. P., Koschorz, B., Amsterdam, A., Schwarz, H., & Hammerschmidt, M. (2009). The epithelial cell adhesion molecule EpCAM is required for epithelial morphogenesis and integrity during zebrafish epiboly and skin development. *PLoS Genet, 5*(7), e1000563. doi:10.1371/journal.pgen.1000563

Solar, M., Cardalda, C., Houbracken, I., Martin, M., Maestro, M. A., De Medts, N., . . . Ferrer, J. (2009). Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. *Dev Cell, 17*(6), 849-860. doi:10.1016/j.devcel.2009.11.003

Spilsbury, K., O'Mara, M. A., Wu, W. M., Rowe, P. B., Symonds, G., & Takayama, Y. (1995). Isolation of a novel macrophage-specific gene by differential cDNA analysis. *Blood, 85*(6), 1620-1629.

Stanger, B. Z., Stiles, B., Lauwers, G. Y., Bardeesy, N., Mendoza, M., Wang, Y., . . . Dor, Y. (2005). Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell, 8*(3), 185-195. doi:10.1016/j.ccr.2005.07.015

Staples, C. J., Myers, K. N., Beveridge, R. D., Patil, A. A., Howard, A. E., Barone, G., . . . Collis, S. J. (2014). Ccdc13 is a novel human centriolar satellite protein required for ciliogenesis and genome stability. *J Cell Sci, 127*(Pt 13), 2910-2919. doi:10.1242/jcs.147785

Stevanovic, M., Lovell-Badge, R., Collignon, J., & Goodfellow, P. N. (1993). SOX3 is an X-linked gene related to SRY. *Hum Mol Genet, 2*(12), 2013-2018. doi:10.1093/hmg/2.12.2013

Stooke-Vaughan, G. A., Huang, P., Hammond, K. L., Schier, A. F., & Whitfield, T. T. (2012). The role of hair cells, cilia and ciliary motility in otolith formation in the zebrafish otic vesicle. *Development, 139*(10), 1777-1787. doi:10.1242/dev.079947

Storz, P. (2017). Acinar cell plasticity and development of pancreatic ductal adenocarcinoma. *Nat Rev Gastroenterol Hepatol, 14*(5), 296-304. doi:10.1038/nrgastro.2017.12

Suri, A., Calderon, B., Esparza, T. J., Frederick, K., Bittner, P., & Unanue, E. R. (2006). Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. *Science, 311*(5768), 1778-1780. doi:10.1126/science.1123500

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L., & German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development, 125*(12), 2213-2221. doi:10.1242/dev.125.12.2213

Szabo, R., Hobson, J. P., Christoph, K., Kosa, P., List, K., & Bugge, T. H. (2009). Regulation of cell surface protease matriptase by HAI2 is essential for placental development, neural tube closure and embryonic survival in mice. *Development, 136*(15), 2653-2663. doi:10.1242/dev.038430

Tajiri, Y., Igarashi, T., Li, D., Mukai, K., Suematsu, M., Fukui, E., . . . Matsumoto, H. (2010). Tubulointerstitial nephritis antigen-like 1 is expressed in the uterus and binds with integrins in decidualized endometrium during postimplantation in mice. *Biol Reprod, 82*(2), 263-270. doi:10.1095/biolreprod.109.080028

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*(4), 663-676. doi:10.1016/j.cell.2006.07.024

Tanaka, H., Takechi, M., Kiyonari, H., Shioi, G., Tamura, A., & Tsukita, S. (2015). Intestinal deletion of Claudin-7 enhances paracellular organic solute flux and initiates colonic inflammation in mice. *Gut, 64*(10), 1529-1538. doi:10.1136/gutjnl-2014-308419

Taniguchi, T., Okazaki, K., Okamoto, M., Seko, S., Tanaka, J., Uchida, K., . . . Seino, Y. (2003). High prevalence of autoantibodies against carbonic anhydrase II and lactoferrin in type 1 diabetes: concept of autoimmune exocrinopathy and endocrinopathy of the pancreas. *Pancreas, 27*(1), 26-30. doi:10.1097/00006676-200307000-00004

Taniguchi, T., Tanaka, J., Seko, S., Okazaki, K., & Okamoto, M. (2001). Association of rapid-onset type 1 diabetes and clinical acute pancreatitis positive for autoantibodies to the exocrine pancreas. *Diabetes Care, 24*(12), 2156-2157. doi:10.2337/diacare.24.12.2156

Tarifeno-Saldivia, E., Lavergne, A., Bernard, A., Padamata, K., Bergemann, D., Voz, M. L., . . . Peers, B. (2017). Transcriptome analysis of pancreatic cells across distant species highlights novel important regulator genes. *BMC Biol, 15*(1), 21. doi:10.1186/s12915-017-0362-x

Tateishi, K., He, J., Taranova, O., Liang, G., D'Alessio, A. C., & Zhang, Y. (2008). Generation of insulin-secreting islet-like clusters from human skin fibroblasts. *J Biol Chem, 283*(46), 31601-31607. doi:10.1074/jbc.M806597200

Taylor, B. L., Liu, F. F., & Sander, M. (2013). Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. *Cell Rep, 4*(6), 1262-1275. doi:10.1016/j.celrep.2013.08.010

Thorel, F., Nepote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S., & Herrera, P. L. (2010). Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature, 464*(7292), 1149-1154. doi:10.1038/nature08894

Tiso, N., Moro, E., & Argenton, F. (2009). Zebrafish pancreas development. *Mol Cell Endocrinol, 312*(1-2), 24-30. doi:10.1016/j.mce.2009.04.018

Towns, R., & Pietropaolo, M. (2011). GAD65 autoantibodies and its role as biomarker of Type 1 diabetes and Latent Autoimmune Diabetes in Adults (LADA). *Drugs Future, 36*(11), 847. doi:10.1358/dof.2011.036.11.1710754

Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., . . . Rinn, J. L. (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol, 32*(4), 381-386. doi:10.1038/nbt.2859

Tsuji-Tamura, K., Morino-Koga, S., Suzuki, S., & Ogawa, M. (2021). The canonical smooth muscle cell marker TAGLN is present in endothelial cells and is involved in angiogenesis. *J Cell Sci, 134*(15). doi:10.1242/jcs.254920

Tucker, T. R., Knitter, C. A., Khoury, D. M., Eshghi, S., Tran, S., Sharrock, A. V., . . . Parsons, M. J. (2023). A new model of inducible chronic hyperglycemia. *Dis Model Mech*. doi:10.1242/dmm.050215

Umeyama, K., Watanabe, M., Saito, H., Kurome, M., Tohi, S., Matsunari, H., . . . Nagashima, H. (2009). Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. *Transgenic Res, 18*(5), 697-706. doi:10.1007/s11248-009-9262-3

Uribe, R. A., Kwon, T., Marcotte, E. M., & Gross, J. M. (2012). Id2a functions to limit Notch pathway activity and thereby influence the transition from proliferation to differentiation of retinoblasts during zebrafish retinogenesis. *Dev Biol, 371*(2), 280-292. doi:10.1016/j.ydbio.2012.08.032

Van de Casteele, M., Leuckx, G., Cai, Y., Yuchi, Y., Coppens, V., De Groef, S., . . . Heimberg, H. (2014). Partial duct ligation: beta-cell proliferation and beyond. *Diabetes, 63*(8), 2567-2577. doi:10.2337/db13-0831

van der Gun, B. T., Melchers, L. J., Ruiters, M. H., de Leij, L. F., McLaughlin, P. M., & Rots, M. G. (2010). EpCAM in carcinogenesis: the good, the bad or the ugly. *Carcinogenesis, 31*(11), 1913-1921. doi:10.1093/carcin/bgq187

Villasenor, A., Chong, D. C., Henkemeyer, M., & Cleaver, O. (2010). Epithelial dynamics of pancreatic branching morphogenesis. *Development, 137*(24), 4295-4305. doi:10.1242/dev.052993

Volarevic, V., Markovic, B. S., Gazdic, M., Volarevic, A., Jovicic, N., Arsenijevic, N., . . . Stojkovic, M. (2018). Ethical and Safety Issues of Stem Cell-Based Therapy. *Int J Med Sci, 15*(1), 36-45. doi:10.7150/ijms.21666

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., . . . Scherer, G. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell, 79*(6), 1111-1120. doi:10.1016/0092-8674(94)90041-8

Walker, S. L., Ariga, J., Mathias, J. R., Coothankandaswamy, V., Xie, X., Distel, M., . . . Mumm, J. S. (2012). Automated reporter quantification in vivo: high-throughput screening method for reporter-based assays in zebrafish. *PLoS One, 7*(1), e29916. doi:10.1371/journal.pone.0029916

Wang, G., Rajpurohit, S. K., Delaspre, F., Walker, S. L., White, D. T., Ceasrine, A., . . . Mumm, J. S. (2015). First quantitative high-throughput screen in zebrafish identifies novel pathways for increasing pancreatic beta-cell mass. *Elife, 4*. doi:10.7554/eLife.08261

Wang, L., Wang, L., Wang, S., Zhou, Z., Liu, Z., Xu, P., . . . Yan, J. (2021). N2E4, a Monoclonal Antibody Targeting Neuropilin-2, Inhibits Tumor Growth and Metastasis in Pancreatic Ductal Adenocarcinoma via Suppressing FAK/Erk/HIF-1alpha Signaling. *Front Oncol, 11*, 657008. doi:10.3389/fonc.2021.657008

Wang, R., Li, Q., & Tang, D. D. (2006). Role of vimentin in smooth muscle force development. *Am J Physiol Cell Physiol, 291*(3), C483-489. doi:10.1152/ajpcell.00097.2006

Wang, Y., Rovira, M., Yusuff, S., & Parsons, M. J. (2011). Genetic inducible fate mapping in larval zebrafish reveals origins of adult insulin-producing beta-cells. *Development, 138*(4), 609-617. doi:10.1242/dev.059097

Wang, Y., & Zhang, S. (2011). Identification and expression of liver-specific genes after LPS challenge in amphioxus: the hepatic cecum as liver-like organ and "pre-hepatic" acute phase response. *Funct Integr Genomics, 11*(1), 111-118. doi:10.1007/s10142-010-0199-7

Wang, Y. J., Park, J. T., Parsons, M. J., & Leach, S. D. (2015). Fate mapping of ptf1a-expressing cells during pancreatic organogenesis and regeneration in zebrafish. *Dev Dyn, 244*(6), 724-735. doi:10.1002/dvdy.24271

Wang, Y. J., Schug, J., Won, K. J., Liu, C., Naji, A., Avrahami, D., . . . Kaestner, K. H. (2016). Single-Cell Transcriptomics of the Human Endocrine Pancreas. *Diabetes, 65*(10), 3028-3038. doi:10.2337/db16-0405

Went, P., Vasei, M., Bubendorf, L., Terracciano, L., Tornillo, L., Riede, U., . . . Baeuerle, P. A. (2006). Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. *Br J Cancer, 94*(1), 128-135. doi:10.1038/sj.bjc.6602924

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., . . . Zon, L. I. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell, 2*(2), 183-189. doi:10.1016/j.stem.2007.11.002

Wiggenhauser, L. M., Qi, H., Stoll, S. J., Metzger, L., Bennewitz, K., Poschet, G., . . . Kroll, J. (2020). Activation of Retinal Angiogenesis in Hyperglycemic pdx1 (-/-) Zebrafish Mutants. *Diabetes, 69*(5), 1020-1031. doi:10.2337/db19-0873

Wingett, D., Forcier, K., & Nielson, C. P. (1999). A role for CD99 in T cell activation. *Cell Immunol, 193*(1), 17-23. doi:10.1006/cimm.1999.1470

Wolf, E., Braun-Reichhart, C., Streckel, E., & Renner, S. (2014). Genetically engineered pig models for diabetes research. *Transgenic Res, 23*(1), 27-38. doi:10.1007/s11248-013-9755-y

Wolfien, M., David, R., & Galow, A. M. (2021). Single-Cell RNA Sequencing Procedures and Data Analysis. In I. N. Helder (Ed.), *Bioinformatics*. Brisbane (AU).

Wu, C. J., Feng, X., Lu, M., Morimura, S., & Udey, M. C. (2017). Matriptase-mediated cleavage of EpCAM destabilizes claudins and dysregulates intestinal epithelial homeostasis. *J Clin Invest, 127*(2), 623-634. doi:10.1172/JCI88428

Xi, S., Yin, W., Wang, Z., Kusunoki, M., Lian, X., Koike, T., . . . Zhang, Q. (2004). A minipig model of high-fat/high-sucrose diet-induced diabetes and atherosclerosis. *Int J Exp Pathol, 85*(4), 223-231. doi:10.1111/j.0959-9673.2004.00394.x

Xiao, X., Chen, Z., Shiota, C., Prasadan, K., Guo, P., El-Gohary, Y., . . . Gittes, G. K. (2013). No evidence for beta cell neogenesis in murine adult pancreas. *J Clin Invest, 123*(5), 2207-2217. doi:10.1172/JCI66323

Xiao, X., Guo, P., Shiota, C., Prasadan, K., El-Gohary, Y., Wiersch, J., . . . Gittes, G. K. (2013). Neurogenin3 activation is not sufficient to direct duct-to-beta cell transdifferentiation in the adult pancreas. *J Biol Chem, 288*(35), 25297-25308. doi:10.1074/jbc.M113.484022

Xin, Y., Dominguez Gutierrez, G., Okamoto, H., Kim, J., Lee, A. H., Adler, C., . . . Gromada, J. (2018). Pseudotime Ordering of Single Human beta-Cells Reveals States of Insulin Production and Unfolded Protein Response. *Diabetes, 67*(9), 1783-1794. doi:10.2337/db18-0365

Xing, T., Benderman, L. J., Sabu, S., Parker, J., Yang, J., Lu, Q., . . . Chen, Y. H. (2020). Tight Junction Protein Claudin-7 Is Essential for Intestinal Epithelial Stem Cell Self-Renewal and Differentiation. *Cell Mol Gastroenterol Hepatol, 9*(4), 641-659. doi:10.1016/j.jcmgh.2019.12.005

Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., . . . Heimberg, H. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell, 132*(2), 197-207. doi:10.1016/j.cell.2007.12.015

Yau, B., & Kebede, M. A. (2021). Islet Biology and Metabolism. *Metabolites, 11*(11). doi:10.3390/metabo11110786

Ye, D., Liu, T., Li, Y., Wang, Y., Hu, W., Zhu, Z., & Sun, Y. (2023). Identification of fish spermatogenic cells through high-throughput immunofluorescence against testis with an antibody set. *Front Endocrinol (Lausanne), 14*, 1044318. doi:10.3389/fendo.2023.1044318

Ye, L., Robertson, M. A., Hesselson, D., Stainier, D. Y., & Anderson, R. M. (2015). Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. *Development, 142*(8), 1407-1417. doi:10.1242/dev.117911

Yee, N. S., Lorent, K., & Pack, M. (2005). Exocrine pancreas development in zebrafish. *Dev Biol, 284*(1), 84-101. doi:10.1016/j.ydbio.2005.04.035

Yu, J., Ma, J., Li, Y., Zhou, Y., Luo, L., & Yang, Y. (2023). Pax4-Ghrelin mediates the conversion of pancreatic epsilon-cells to beta-cells after extreme beta-cell loss in zebrafish. *Development, 150*(6). doi:10.1242/dev.201306

Yu, K. H., Barry, C. G., Austin, D., Busch, C. M., Sangar, V., Rustgi, A. K., & Blair, I. A. (2009). Stable isotope dilution multidimensional liquid chromatography-tandem mass spectrometry for pancreatic cancer serum biomarker discovery. *J Proteome Res, 8*(3), 1565-1576. doi:10.1021/pr800904z

Zang, L., Shimada, Y., & Nishimura, N. (2017). Development of a Novel Zebrafish Model for Type 2 Diabetes Mellitus. *Sci Rep, 7*(1), 1461. doi:10.1038/s41598-017-01432-w

Zappia, L., & Oshlack, A. (2018). Clustering trees: a visualization for evaluating clusterings at multiple resolutions. *Gigascience, 7*(7). doi:10.1093/gigascience/giy083

Zeng, C., Mulas, F., Sui, Y., Guan, T., Miller, N., Tan, Y., . . . Sander, M. (2017). Pseudotemporal Ordering of Single Cells Reveals Metabolic Control of Postnatal beta Cell Proliferation. *Cell Metab, 25*(5), 1160-1175 e1111. doi:10.1016/j.cmet.2017.04.014

Zettler, S., Renner, S., Kemter, E., Hinrichs, A., Klymiuk, N., Backman, M., . . . Wolf, E. (2020). A decade of experience with genetically tailored pig models for diabetes and metabolic research. *Anim Reprod, 17*(3), e20200064. doi:10.1590/1984-3143-AR2020-0064

Zhang, Z. Y., Miao, L. F., Qian, L. L., Wang, N., Qi, M. M., Zhang, Y. M., . . . Wang, R. X. (2019). Molecular Mechanisms of Glucose Fluctuations on Diabetic Complications. *Front Endocrinol (Lausanne), 10*, 640. doi:10.3389/fendo.2019.00640

Zhong, F., & Jiang, Y. (2019). Endogenous Pancreatic beta Cell Regeneration: A Potential Strategy for the Recovery of beta Cell Deficiency in Diabetes. *Front Endocrinol (Lausanne), 10*, 101. doi:10.3389/fendo.2019.00101

Zhou, H., Qin, Y., Ji, S., Ling, J., Fu, J., Zhuang, Z., . . . Chiao, P. J. (2018). SOX9 activity is induced by oncogenic Kras to affect MDC1 and MCMs expression in pancreatic cancer. *Oncogene, 37*(7), 912-923. doi:10.1038/onc.2017.393

Zhou, Q., Law, A. C., Rajagopal, J., Anderson, W. J., Gray, P. A., & Melton, D. A. (2007). A multipotent progenitor domain guides pancreatic organogenesis. *Dev Cell, 13*(1), 103-114. doi:10.1016/j.devcel.2007.06.001

Ziegler, B. L., Valtieri, M., Porada, G. A., De Maria, R., Muller, R., Masella, B., . . . Peschle, C. (1999). KDR receptor: a key marker defining hematopoietic stem cells. *Science, 285*(5433), 1553-1558. doi:10.1126/science.285.5433.1553

Ziv, O., Glaser, B., & Dor, Y. (2013). The plastic pancreas. *Dev Cell, 26*(1), 3-7. doi:10.1016/j.devcel.2013.06.013