Variables that influence transcription factor-mediated acinar to beta cell reprogramming

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3TF	Pdx1, Neurog3 and MafA
A→β	Acinar to beta cell
ADM	Acinar-to-ductal metaplasia
AdV-CMV-3TF	3TF-expressing adenovirus
AdV-CMV-GFP	GFP-expressing adenovirus
β	beta
$[Ca^{2+}]_i$	intracellular calcium concentration
DAVID	Database for annotation, visualization and integrated discovery
DM	Diabetes mellitus
Dox	Doxycycline
E	Embryonic day
CFP	Cerulean
GDM	Gestational diabetes mellitus
GFP	Green fluorescent protein
FACS	Fluorescence-activated cell sorting
$GdCl_3$	Gadolinium chloride
iPSC	Induced pluripotent stem cell
mESC	Mouse embryonic stem cells
MPC	Multipotent progenitor cell
OSKM	Oct4, Sox2, Klf4, and Myc

PanINs	Pancreatic intraepithelial neoplasia
PBS-T	PBS with 0.2% Tween-20
PDAC	Pancreatic ductal adenocarcinomas
Pfu	Plaque-forming units
PKD1	Protein Kinase D1
PORT	Pipeline Of RNA-Seq Transformations
PP	Pancreatic polypeptide
tTA	Tet-TransActivator
RFP	Red fluorescent protein
RNA-seq	RNA-sequencing
RPKM	Reads per kilobase of gene model per million mapped reads
rtTA	Reverse-tetracycline transactivator
RUM	RNA-Seq Unified
SCNT	Somatic cell nuclear transfer
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TPSR	Vanderbilt Tissue Pathology Shared Resource
YFP	Yellow fluorescent protein

CHAPTER 1

INTRODUCTION

Pancreas physiology

The pancreas is a dual functioning organ with both endocrine and exocrine functions (**Figure 1.1**) that resides in the abdominal cavity adjacent to the stomach. The exocrine compartment, comprised of acinar and ductal cells, constitutes the bulk of the pancreatic mass and aids in the digestion of food. The acinar cell is the functional unit of the exocrine pancreas whose major role is the synthesis, storage, and production of digestive enzymes, which are secreted into the small intestine through a system of ducts. Conversely, the endocrine pancreas consists of the Islets of Langerhans that function to regulate blood glucose levels. The Islets of Langerhans are comprised of five hormone-producing cells: beta, alpha, delta, pancreatic polypeptide, and epsilon cells that secrete insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin, respectively. While the murine islet has a well-defined cellular architecture with beta cells positioned in the core and the other endocrine cells positioned at the periphery, this spatial distribution is not observed in humans (Bosco et al., 2010).

A critical role of the endocrine pancreas is the regulation of plasma glucose levels. Blood glucose is tightly regulated by the endocrine hormones insulin and glucagon secreted by the beta and alpha cells. In response to elevated glucose, beta cells release insulin to stimulate glucose uptake by the adipose and muscle tissue, induce glycogen synthesis, and inhibit gluconeogenesis in the liver (Barthel & Schmoll, 2003). Conversely, glucagon is the major counterpart to insulin and is released during hypoglycemia to promote hepatic glucose production (Gromada, Franklin, & Wollheim, 2007; Shepherd & Kahn, 1999).

The exocrine pancreas is responsible for the secretion of digestive enzymes, ions, and water into the duodenum. The digestive enzymes are essential for the proper digestion and absorption of food across the gastrointestinal surface of the epithelium with loss of exocrine function resulting in compromised absorption of nutrients and malnutrition. The secretion of water and ions is necessary for the transportation of the digestive enzymes from the pancreatic acinar cells to the intestine. Tight junctions between acinar cells prevent the passage of large molecules, such as the digestive enzymes, while allowing for the paracellular passage of water and ions (Logsdon & Ji, 2013).

Overview of pancreas development

Pancreas development begins midway through gestation with the evagination of the endoderm to form the dorsal and ventral buds, which eventually fuse together (Gittes, 2009; Oliver-Krasinski & Stoffers, 2008; Pan & Wright, 2011). These buds are comprised of multipotent pancreatic progenitor cells (MPCs) that express many genes, such as *Ptf1a*, *Pdx1*, *Sox9*, *Hnf6*, and *Nkx6.*1. MPCs give rise to all three lineages of the pancreas (acinar, endocrine, and duct) and become committed to the pancreatic fate with the expression of *Ptf1a* (Kawaguchi et al., 2002). *Ptf1a* expression is maintained in MPCs that give rise to immature acinar cells, where it interacts with *Rbpj.* As acinar cells mature, *Rbpjl* replaces *Rbpj* in the trimeric PTF1 complex, a complex important for the expression of acinar-specific genes (Masui et al., 2010) (Holmstrom et al., 2011). MPCs also give rise to $Sox9^{+}/Nkx6.1^{+}$ bipotent cells (Arda, Benitez, & Kim, 2013), which further differentiate into exocrine ductal cells expressing *Sox9* (McDonald et al., 2012) or into a transient population of *Neurog3* expressing endocrine precursor cells. High *Neurog3* expressing progenitors give rise to all hormone-secreting endocrine cells of the

pancreas. A number of transcription factors are required for beta cell differentiation and maturation including *Pax4, Nkx2.2,* and *Pdx1.* Beta cell-specific deletion of these factors results in a reduction of beta cells. *MafA* is important for beta cell maturation and activates many genes important for its function (Kaneto et al., 2005; Kataoka et al., 2002; H. Wang, Brun, Kataoka, Sharma, & Wollheim, 2007; C. Zhang et al., 2005). During development, beta cells proceed from a *MafB* immature state to a *MafB* intermediate state and finally to a *MafA* mature state (Artner et al., 2007; Artner et al., 2010).

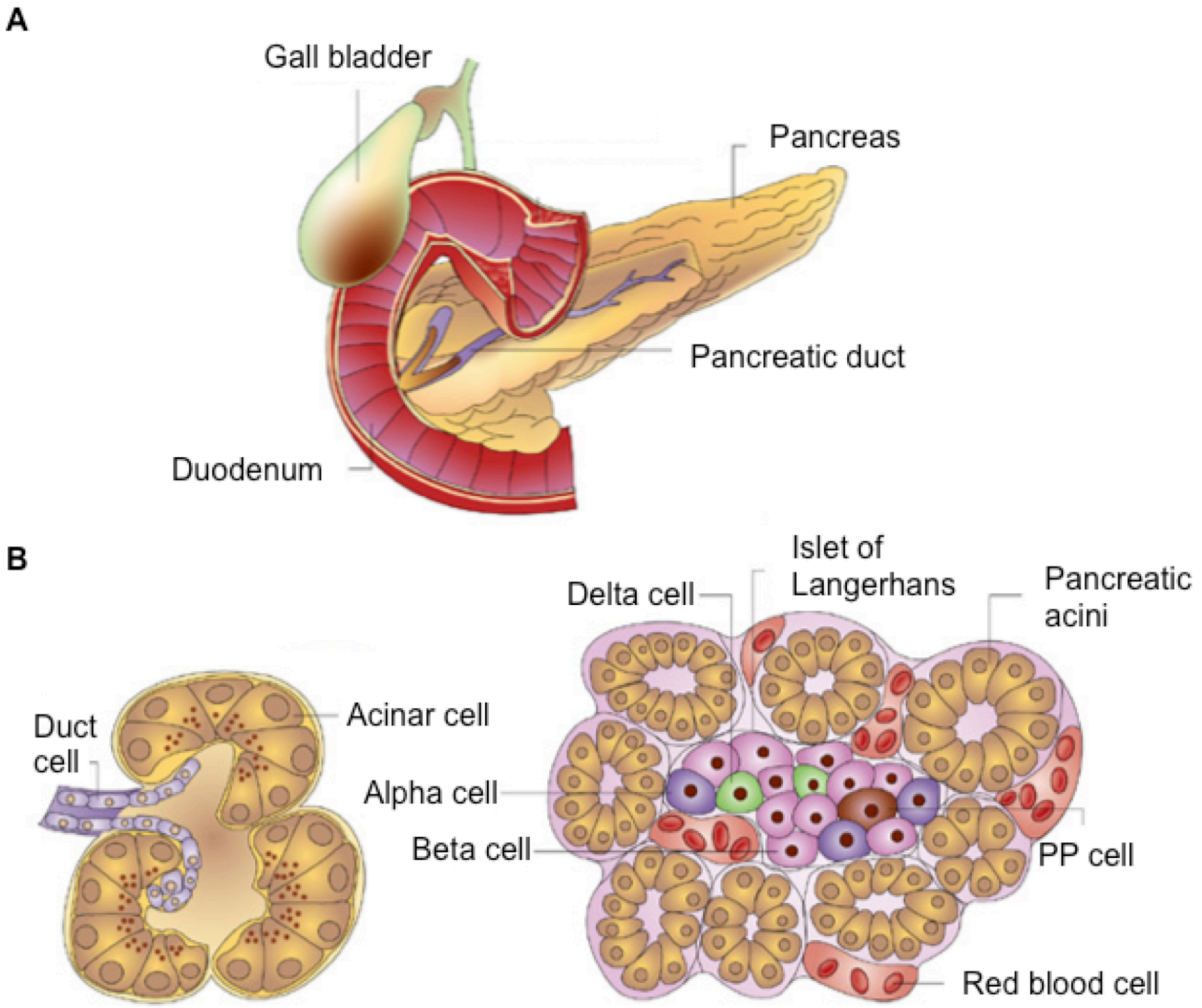


Figure 1.1 Human pancreas anatomy. (A) Gross anatomy of the pancreas. (B) Exocrine (left) and endocrine (right) pancreas. The exocrine pancreas consists of acinar and ductal cells and aids in the digestion of food while the exocrine pancreas is composed of four specialized cell types, beta-, alpha-, delta-, and PP-cells, which comprise the Islets of Langerhans and secrete hormones into the blood stream to regulate blood glucose levels. Image adapted (Bardeesy & DePinho, 2002).

Diabetes mellitus

Diabetes mellitus (DM) is an endocrine disorder associated with hyperglycemia and results in severe damage to the blood vessels, eyes, kidneys, and heart. According to the latest survey, 417 million people worldwide suffer from DM and the number affected is expected to grow exponentially in the next few decades with 642 million people worldwide predicted to suffer from diabetes in 2040. Diabetes places a huge financial burden on individuals and their families, and it also imposes a huge economic burden on countries, with the majority of countries spending between 5% and 15% of their total health expenditure on diabetes (P. Zhang et al., 2010). This chronic disease is categorized into three major types: Type 1 (T1D), Type 2 (T2D), and gestational diabetes.

T1D occurs predominantly in young people and is due to the autoimmune destruction of pancreatic beta cells, leading to insulin deficiency. T1D arises in genetically susceptible individuals, most likely as a result of an environmental trigger. Genetic data identifies the following genes as susceptibility genes: HLA, insulin, PTPN22, IL2Ra, and CTLA4 (van Belle, Coppieters, & von Herrath, 2011). There is currently no cure for T1D. Alternatively, T2D is much more common, comprising 90-95% of those diagnosed, and results from both insulin resistance and impaired beta cell function (Cavaghan, Ehrmann, & Polonsky, 2000). Risk factors for T2D include excess body weight, physical inactivity, poor nutrition, genetics, family history of diabetes, and older age. T2D can go undiagnosed for years and can often be managed with dietary changes, increased physical activities, and medications. T1D and T2D are polygenic disorders, and multiple genes and environmental factors contribute to the development of the disease. Gestational diabetes, characterized by slightly elevated blood glucose levels during

pregnancy, imposes serious health risk to both the mother and child and is associated with an increased risk to both the mother and child in developing T2D later in life.

Current and future treatments for T1D

T1D is due to the autoimmune-mediated destruction of pancreatic beta cells leading to a profound impairment of insulin secretion. A curative therapy for T1D requires both the restoration of the lost beta cell mass and amelioration of the autoimmune attack. For nearly the past one hundred years, the only effective treatment for T1D has been lifelong insulin therapy. However, for many people blood glucose control is inadequate, thereby leading to serious complications, including hypoglycemia, nephropathy, retinopathy, and other vasculopathies. Over the last few decades, transplantation of whole pancreas and isolated beta cells indicate that diabetes can be cured by replenishment of deficient beta cells (Shapiro et al., 2006). These promising results, paired with the shortage of cadaver pancreases, have lent strong motivation to the search for new sources of beta cells. Strategies to produce beta cells suitable for transplantation include expansion of existing beta cells, directed differentiation of embryonic stem cells to beta cells, and reprogramming of other terminally differentiated cells into beta cells (**Figure 1.2**) (Bonner-Weir & Weir, 2005; Pagliuca & Melton, 2013).

A potential source of new beta cells is through expansion of existing beta cells, which, if achieved, offer an autologous source of new beta cells capable of regulating blood glucose. While young beta cells have relatively high replication rates, adult beta cells have very low replication rates (Teta, Long, Wartschow, Rankin, & Kushner, 2005). However, the expansion of adult beta cells mass increases during pregnancy and obesity (Nichols, New, & Annes, 2014), and a landmark study demonstrated that pre-existing beta cells, rather than stem cells, are the

major source of new beta cells during adult life and after pancreatectomy in mice (Dor, Brown, Martinez, & Melton, 2004). This observation has led to a profound interest in defining pharmacologic approaches to therapeutically control beta cell growth and mass. While the therapeutic potential of harnessing the latent growth potential of mature beta cells for the treatment of diabetes exists, at present, a clinically viable strategy does not exist.

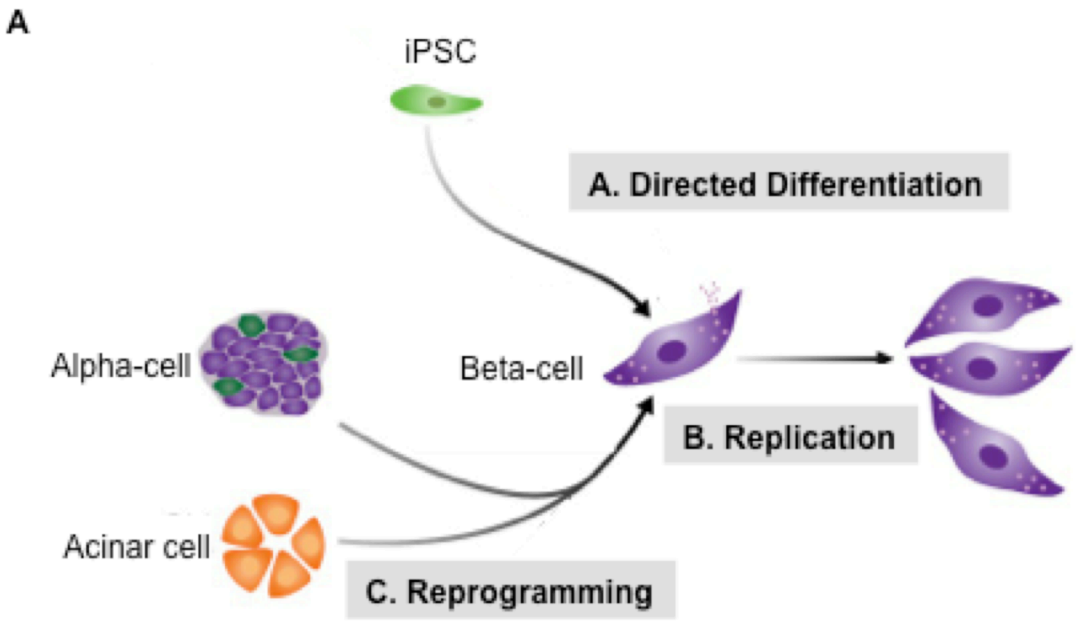


Figure 1.2 Strategies for creating new beta cells. Three strategies to generate new beta cells include (A) directed differentiation of induced pluripotent stem cells (iPSCs), (B) replication of existing beta cells, and (C) reprogramming or mature cells types into new beta cells. Image adapted (Pagliuca & Melton, 2013).

Cellular reprogramming overview

Cellular reprogramming, defined as the process in which a differentiated cell is changed into that of another state (Halley-Stott, Pasque, & Gurdon, 2013), holds great promise for regenerative medicine. The ultimate goal of regenerative medicine is to replace damaged cells. This can potentially be accomplished through the different avenues of cellular reprogramming: dedifferentiation followed by directed differentiation or transdifferentiation. Indeed, the forced overexpression of key transcription factors has been shown to convert fully differentiated cells into pluripotent stem cells (dedifferentiation), convert pluripotent cells into fully differentiated cells (directed differentiation) and can also induce fully differentiated cells to convert into another mature cell type (transdifferentiation).

The first instance of experimentally induced cellular reprogramming was in 1962 when Sir John Gurdon reported cellular reprogramming through somatic cell nuclear transfer (SCNT) (Gurdon, 1962). SCNT involves transferring the nucleus of a mature cell into an enucleated egg resulting in the production of an embryo that is a clone of the donor somatic cell. This work demonstrated the feasibility of experimentally manipulating a cell's identity. However, the field of cellular reprogramming experienced limited progress following Gurdon's work. It wasn't until the late 20th century that several groups expanded the field of cellular reprogramming, reporting the direct cell fate conversion (transdifferentiation) through the introduction of a single factor. Indeed, it was shown that the forced overexpression of MYOD converts fibroblasts into myoblast followed by the finding that the forced expression of the GATA 1 reprograms myeloblasts into eosinophils (J. Choi et al., 1990; Kulessa, Frampton, & Graf, 1995). However, it was the seminal discovery of induced pluripotent stem cells (iPSCs) that has paved the way for cell fate conversion studies in which the overexpression of a set of defined transcription factors can

induce the conversion of cell fates. In a groundbreaking study, Yamanaka demonstrated that viral transfection of four transcription factors, Oct4, Sox2, Klf4, and Myc (OSKM), reprograms somatic cells into iPSCs. These cells are then capable of being reprogrammed into any cell type (Takahashi & Yamanaka, 2006).

Although OSKM converts somatic cells into iPSCs, the reprogramming efficiency is low and the reprogramming is a stochastic event often leading to only partially reprogrammed iPSCs (Takahashi & Yamanaka, 2006, 2016). Thus, researchers have attempted to refine the reprogramming protocol and identify reprogramming enhancers to overcome these limitations. Such studies have reported that the addition of other pluripotency-associated genes, such as TBX3, can enhance the reprogramming efficiency. Furthermore, the addition of certain microRNAs, including miR-291-3p, miR-294, and MIR-295, has been shown to enhance OSK reprogramming (Han et al., 2010; Judson, Babiarz, Venere, & Blelloch, 2009). Furthermore, studies have also identified the transcriptional and epigenetic changes involved in the reprogramming of a somatic cell into an iPSC. Gene expression profiling at defined points during reprogramming has revealed three stages of reprogramming: initiation, maturation, and stabilization (Samavarchi-Tehrani et al., 2010). The initiation phase is marked by an increase in proliferation, histone modifications, mesenchymal-to-epithelial transition, and activation of DNA repair and RNA processing. In the intermediate stage, reprogrammed cells undergo activation of pluripotency markers, developmental regulators, and glycolysis. In the final stage, the cells establish a pluripotent signature independent of transgenes expression (Buganim, Faddah, & Jaenisch, 2013; David & Polo, 2014). Studies have also revealed that Oct4, Sox2, and Klf4 function as “pioneer factors” binding to inaccessible chromatin regions, while MYC enhances

the binding of pluripotency markers to the chromatin leading to chromatin remodeling and activation of repressed genes (Soufi, Donahue, & Zaret, 2012).

The ability to modulate cell identity and the knowledge gained from dedifferentiation studies have spurred investigators to define transcription factors that allow for cell fate conversions without reverting back to the pluripotent state (transdifferentiation). Indeed, the generation of iPSCs suggest that, rather than a single factor, a specific combination of transcription factors could alter a cell's identity, allowing for greater cellular plasticity than once believed. Furthermore, the ability to obtain a desired, differentiated cell-type would allow for studies on disease modeling, regenerative medicine, and drug interactions. Thus, researchers have identified transcription factors that allows for the conversion of pancreatic acinar cells into insulin-secreting beta cells, fibroblast into neurons, and fibroblast into cardiomyocytes (Ieda et al., 2010; Vierbuchen et al., 2010; Zhou, Brown, Kanarek, Rajagopal, & Melton, 2008). In addition, databases, such as CellNet, have been developed allowing researchers to assess the fidelity of cellular reprogramming in order to determine the degree to which the reprogrammed cell resembles the corresponding target cell in molecular and functional terms (Cahan et al., 2014).

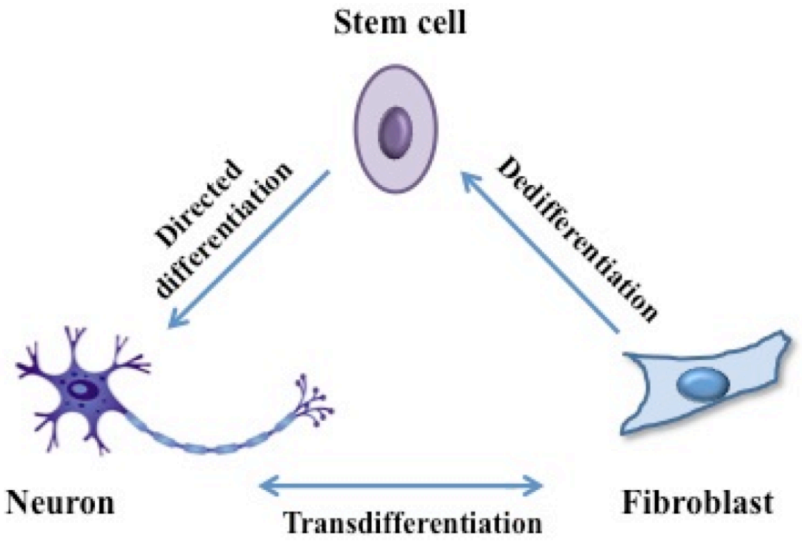


Figure 1.3 Routes of cellular reprogramming. A cell's identity is defined by its epigenetic and transcriptional landscape. Once believed to be immutable, a number of experimental manipulations can alter a cell's identity resulting in cellular reprogramming. Processes include dedifferentiation, directed differentiation, and transdifferentiation. Image adapted (Cherry & Daley, 2012).

Cellular reprogramming as a therapy for Type 1 diabetes

Until recently, cellular identities were thought to be largely immutable. However, in 2006 Yamanaka converted fibroblasts into iPSCs by the introduction of four different transcription factors (Takahashi & Yamanaka, 2006). The ability to perform cellular reprogramming has opened a new paradigm for regenerative medicine in which specific cell types can be obtained by reprogramming other cell types. These advances may enable a truly curative therapy for T1D.

iPSCs have the potential to differentiate into any somatic cell, making them ideal candidates for cell replacement therapies. Towards this goal, much progress has been made in developing protocols for the directed differentiation of iPSCs to insulin-producing beta cells. These protocols follow a stepwise conversion that closely mimics the development of beta cells, differentiating iPSCs cells towards the definitive endoderm, pancreatic endoderm, endocrine lineages, and finally to the beta cell. Many of these differentiation protocols derive pancreatic progenitors *in vitro* and then subsequently transplant these progenitors into immunodeficient mice to allow them to further develop and mature into functional beta cells (D'Amour et al., 2006; Kroon et al., 2008; Rezania et al., 2012). Indeed, the majority of protocols that derived insulin-expressing cells wholly from *in vitro* cells did not produce fully mature or functional beta cells (Cheng et al., 2012; Hrvatin et al., 2014; Narayanan et al., 2014). However, improved protocols were able to generate glucose-responsive insulin-producing cells *in vitro* that share significant similarities with endogenous beta cells (Pagliuca et al., 2014; Rezania et al., 2014). While the molecular mechanisms underlying the generation of functional beta cells remain to be elucidated, cell-based therapeutics to treat T1D have advanced to the point where the first Phase I/II trials in humans have begun.

An alternative strategy to differentiating iPSCs in a step-wise fashion into beta cells is the reprogramming of terminally differentiated cell-types into new, functional beta cells. Indeed,

hepatocytes, fibroblasts, intestinal and gastric crypt cells, gallbladder cells, pancreatic alpha cells, and pancreatic acinar cells have all been reprogrammed into beta-like cells (Akinci et al., 2013; Ariyachet et al., 2016; Banga, Akinci, Greder, Dutton, & Slack, 2012; S. X. Chen et al., 2011; Y. J. Chen et al., 2014; Hickey et al., 2013; Luo et al., 2014; Thorel et al., 2010; Zhou & Melton, 2008). The pancreatic acinar cells represent an appealing reprogramming target. They are located in the pancreas, are highly abundant, are derived from a common progenitor cell during pancreatic organogenesis (Gu, Dubauskaite, & Melton, 2002), and exhibit transcriptional plasticity (W. Li, M. Nakanishi, et al., 2014; Puri, Folias, & Hebrok, 2015; Ziv, Glaser, & Dor, 2013). Indeed, pancreatic acinar cells have been converted into new beta-like cells by cellular reprogramming in response to both physiological processes and experimental interventions (Baeyens et al., 2013; Zhou et al., 2008). Acinar cells have been successfully converted into insulin-expressing beta-like cells by the transient administration of epidermal growth factor and ciliary neurotrophic factor (Baeyens et al., 2013). In addition, the adenoviral delivery of *Pdx1*, *Neurog3,* and *MafA* (3TF) to the pancreas of immunocompromised *Rag1*$^{-/-}$ mice has also been shown to reprogram pancreatic acinar cells into insulin-expressing beta-like cells (Zhou et al., 2008). Subsequent reports have shown that these three transcription factors can also reprogram hepatocytes, fibroblasts, intestinal crypt cells, and gallbladder cells into new insulin-expressing beta-like cells (Akinci et al., 2013; Banga et al., 2012; Y. J. Chen et al., 2014; Hickey et al., 2013; Luo et al., 2014).

Biological differences between acinar and beta cells

The effects of acinar to beta cell (A→β) reprogramming on the microscopic anatomy, cellular function, and physiological function of the pancreas has not been studied but would be

expected to be substantial. Acinar cells, which constitute the bulk of the pancreas, synthesize copious amounts of digestive enzymes that they secrete directly into the pancreatic ductal tree (Logsdon & Ji, 2013). In contrast, pancreatic beta cells, like other pancreatic endocrine cells, are co-located in the Islets of Langerhans where they secrete the hormones they produce into the bloodstream (Kulkarni, 2004). These very marked physiological and histological differences raise important questions about the consequences of A→β conversion, especially when it involves a cell whose function is to produce enzymes for the digestion of proteins, complex carbohydrates, lipids, and nucleic acids.

Digestive enzymes

Pancreatic acinar cells synthesize and secrete more proteins than any other cell-type (Logsdon & Ji, 2013) with the majority being digestive enzymes. The synthesis, storage, and secretion of digestive enzymes is influenced by external inputs from nerves and hormones and tightly regulated to ensure that the production and delivery of the digestive enzymes matches dietary need. The trimeric PTF1-L complex binds to the pancreas consensus element and activates the expression of the digestive enzymes such as amylase, chymotrypsin B, carboxypeptidase A, and elastase 1 (Boulet, Erwin, & Rutter, 1986; Masui et al., 2008). The relative synthesis rate of the specific digestive enzymes changes as a function of the dietary intake. A carbohydrate-rich diet increases expression of amylase and decreases chymotrypsinogen while a lipid-rich diet enhances lipase expression (Pandol, 2010).

Digestive enzymes are stored in zymogen granules at the apical surface of the acinar cell. The basolateral membrane of acinar cells contains receptors for neurohumoral agents such as gastrin-releasing peptide, secretin, cholecystokinin, acetylcholine, and vasoactive intestinal

polypeptide. Activation of these receptors causes changes in intracellular calcium ($[Ca^{2+}]_i$) or cAMP, which in turn mediates exocytosis of the digestive enzymes.

The enzymes of the pancreas (**Table 1.1**) are divided into two main groups: the endopeptidases and the exopeptidases. The exopeptides hydrolyze the terminal bonds of proteins or peptide molecules, whereas the endopeptidases attack the internal peptide bonds (Beck, 1973). These digestive enzymes normally become activated when they enter the duodenum. Activation occurs at the surface of the duodenal lumen where trypsinogen is activated by enterokinase, a glycoprotein peptidase, which through hydrolysis removes an N-terminal hexapeptide fragment (Rinderknecht, 1986). The active form of trypsin then catalyzes the activation of the other proenzymes. These digestive enzymes, if prematurely activated, are capable of digesting the acinar cell and can cause significant damage to the pancreas.

Table 1.1 Digestive enzymes of the pancreas

Proteolytic enzymes
Endopeptidases:
Trypsin
Chymotrypsin
Elastase
Exopeptidases:
Carboxypeptidase A
Carboxypeptidase B
Lypolytic enzymes
Lipase
Cholesterol esterase
Phospholipase A
Amylolytic enzymes
α-Amylase
Other enzymes
Ribonuclease
Desoxyreibonuclease

Pancreatic inflammation and acinar-to-ductal metaplasia

The exocrine pancreas protects itself from autodigestion by the potent proteases, lipases, and ribonucleases it produces through several mechanisms. First, many of the enzymes are secreted as inactive pro-enzymes, or zymogens, which only become active within the duodenum (Neurath & Walsh, 1976). Second, the proteolytic enzymes are co-secreted with a trypsin inhibitor that prevents the premature activation of typsinogen, which normally becomes activated in the small intestine and is responsible for the activation of the other precursor digestive enzymes (Logsdon & Ji, 2013). Third, acinar-to-ductal metaplasia (ADM) occurs (Bockman, Muller, Buchler, Friess, & Beger, 1997; Liou et al., 2013; Pan et al., 2013) and has been suggested to limit autodigestion in the face of inflammation or injury (Puri et al., 2015).

ADM is characterized by the formation of duct-like complexes and fibrosis (R. N. Wang, Kloppel, & Bouwens, 1995) and is accompanied by transcriptional changes in acinar cells, most notably by expression of cytokeratins (Strobel et al., 2007), *Pdx1* (Rooman, Heremans, Heimberg, & Bouwens, 2000; Song et al., 1999), and ductal transcription factors *Sox9* and *Onecut1* (Rooman & Real, 2012). However, the precise mechanisms that initiate both pancreatic inflammation and ADM remain in dispute. Some have argued that pancreatic inflammation is due to intracellular activation of trypsinogen (Halangk et al., 2000; Szilagyi et al., 2001; Van Acker et al., 2002; Whitcomb et al., 1996) whereas others have suggested that calcium overload (Li, Zhou, Zhang, & Li, 2014) and endoplasmic reticulum (ER) stress (Ji et al., 2003; Logsdon & Ji, 2013) are principally responsible.

***Pdx1, Neurog3,* and *MafA* mediated acinar to beta cell reprogramming**

A complex series of events must occur in order for an acinar cell to convert into a beta cell. Acinar cells must cease zymogen production, delaminate and migrate to the surrounding

mesenchyme, then cluster into new vascularized islets. It is vital to gain a clear understanding of the cellular dynamics of A→β conversion for an *in vivo* beta cell restorative therapy to ever be clinically feasible. Indeed, much care must be taken to prevent acinar cell damage and pancreatic inflammation during A→β reprogramming. Due to the physiological role of acinar cells, whose function is to produce digestive enzymes, it is not unfathomable that, if not properly regulated, A→β reprogramming could cause pancreatic damage. For one, the complex cellular changes that occur during A→β conversion may require substantial time to allow acinar cells to 1) cease the production and secretion of tissue-digesting enzymes and 2) establish a new endocrine cell-like state of internal homeostasis. If the pace of such events occurs at a rate that exceeds the ability of the cell to undergo an orderly transition from one cell state to another, this could lead to cellular damage. Indeed, a recent study that used adenoviral delivery of the three transcription factors demonstrated that it took two months for the reprogrammed acinar cells to adopt a DNA methylation and transcriptional profile similar to that of endogenous beta cells and seven months for the reprogramed cells to functionally mimic beta cells (W. Li, C. Cavelti-Weder, et al., 2014). Second, pancreatic acinar cells are especially vulnerable to ER dysfunction owing to their high level of protein synthetic and secretory activity (Ji et al., 2003; Logsdon & Ji, 2013). It is possible that high levels of factor expression could induce ER stress and subesquently pancreatic inflammation. In fact, multiple studies have reported acinar cell inflammation or ADM in response to ectopic factor overexpression. For instance, the overexpression of either *Pdx1, Isl1* or *Sox9* have all been shown to induce metaplastic pancreatic changes (Kopp et al., 2011; Miyatsuka et al., 2006; Miyazaki, Tashiro, Fujikura, Yamato, & Miyazaki, 2012).

While it is vital to understand the cellular changes associated with A→β reprogramming in order to prevent adverse consequences such as pancreatic damage and metaplasia, this does

not diminish the important implications that 3TF-mediated A→β reprogramming has for advancing the field of regentative medicine and cellular reprogramming. For starters, researchers can begin to invesitigate why and how these particular factors (3TF) are able to induce A→β conversion when other pancreatic/beta cell specific factors were unable to induce such a conversion. To identify transcriptional factors capable of reprogramming acinar cells into beta cells, Zhou et al. screened a pool of nine beta cell-associated candidate genes. The combination of all nine, when co-expressed from adenoviral vectors, induced a modest conversion of acinar cells into beta-like cells. Successive rounds of elimination of individual factors led to the identification of the minimally required set of factors comprised of *Pdx1, Neurog3*, and *MafA* required to achieve the highest level of A→β reprogramming. These reprogrammed cells expressed insulin along with beta cell markers including Glut2, PC1/3, NeuroD, Nkx2.2, and Nkx6.1 (Zhou et al., 2008).

Pdx1, Neurog3, and *MafA* all play critical roles in beta cell development, maturation, and function. *Pdx1*-expressing epithelium gives rise to all three lineages of the pancreas (acinar, endocrine and duct) (Gu, Brown, & Melton, 2003) and is required for the embryonic development of the pancreas and beta cell function. Homozygous deletion of *Pdx1* results in pancreatic agenesis (Offield et al., 1996); while *Pdx1* haploinsufficiency causes compromised beta cell function resulting in impaired glucose tolerance and glucose-stimulated insulin secretion (Brissova et al., 2002). Indeed, *Pdx1* activates insulin as well as many genes involved in insulin biosynthesis and secretion (Khoo et al., 2012).

Neurog3 is a master regulator required for the MPCs to enter the endocrine lineage. Indeed, *Neurog3*$^{+}$ cells contribute to all endocrine cells of the mature pancreas (Gradwohl, Dierich, LeMeur, & Guillemot, 2000; Gu et al., 2002). *Neurog3* is transiently expressed in MPCs

and is progressively down regulated as the endocrine program is initiated. Mice deficient for *Neurog3* fail to generate pancreatic endocrine cells and die postnatally (Gradwohl et al., 2000) while Neurog3 haploinsufficiency results in decreased endocrine cell mass (S. Wang et al., 2010). Direct targets of *Neurog3* include genes critical for endocrine differentiation such as *NeuroD1, Pax4, Insm1*, and *Nkx2.2* (H. P. Huang et al., 2000; Smith et al., 2003; Watada, Scheel, Leung, & German, 2003).

MafA is vital for beta cell maturation and function with *MafA*-deficient mice developing diabetes as they age (C. Zhang et al., 2005). Furthermore, *MafA* is crucial for the functional maturation process as nascent beta cells acquire glucose-responsive insulin secretion (Aguayo-Mazzucato et al., 2011). Direct targets of *MafA* include genes critical for beta cell function, including glucose sensing, vesicle maturation, and Ca^{2+} signaling (H. Wang et al., 2007). Indeed, *Pdx1, Neurog3,* and *MafA* play important roles in beta cell development, function, and maturation. Thus, it is not surprising that in combination these factors are able to induce A→β reprogramming. However, much work remains elucidating the cellular and molecular changes associated with A→β reprogramming and the role of each factor in the reprogramming process.

Aims of Dissertation

Over the past decade, efforts have intensified to develop new therapies that will replace or regenerate beta cells that have been destroyed by autoimmune attack in people with T1D. Among the therapeutic avenues being explored, reprogramming other non-beta pancreatic cell types into new beta cells is particularly appealing. If achievable, this approach has the advantage that newly generated beta cells would be autologous and would be produced in their natural anatomical location, the pancreas. There has been marked progress in achieving this goal with

both pancreatic acinar- and alpha-cells being converted into insulin-secreting beta-like cells (Baeyens et al., 2014; Thorel et al., 2010; Zhou et al., 2008). Indeed, it has been shown that adenoviral delivery of 3TF to the pancreas of immunocompromised mice converts pancreatic acinar cells into insulin-secreting beta-like cells (W. Li, C. Cavelti-Weder, et al., 2014; Zhou et al., 2008). The primary goal of the research included in this dissertation is to understand the factors that promote or inhibit 3TF-mediated A→β reprogramming as well as the physiological effects that inducing such a conversion might cause. A better understanding of the cellular dynamics of A→β conversion is vital for an *in vivo* beta cell restorative therapy to ever be clinically feasible.

In order to understand the dynamics of A→β, the Magnuson lab developed and validated a novel diallelic transgene-based mouse model that co-expresses 3TF and a fluorescent reporter, mCherry, in both an acinar cell- and doxycycline (dox)-dependent manner. The derivation and validation of this mouse model is described in Chapter 3.

Using this mouse model, I discovered that the outcome of transcription factor-mediated A→β reprogramming is highly dependent on both the magnitude of 3TF expression and reprogramming-induced inflammation. Overly robust 3TF expression causes acinar cell necrosis resulting in marked inflammation and ADM. New beta-like cells are observed only when reprogramming-induced inflammation is attenuated by either reducing 3TF expression or eliminating macrophages. In Chapter 4, the inflammatory response associated with high levels of 3TF overexpression and the corresponding pancreatic histological changes are characterized.

In the following chapters, experimental variables that may influence the outcome of A→β reprogramming are investigated. Since the reprogramming outcome using transgene-mediated 3TF expression in *Rag1*$^{+/+}$ mice differed so markedly from the use of adenoviral-

mediated expression in *Rag1*$^{-/-}$ mice, I examined the role of variables that differ between these two experimental designs. Two obvious differences are the use of a transgene to express 3TF, opposed to a viral vector, and the presence of *Rag1*. The role of these variables is investigated in Chapter 5. In Chapter 6, I investigate the role of inflammation on influencing the outcome of 3TF-mediated A→β reprogramming.

Furthermore, to determine the specific role of the individual factors in A→β reprogramming, the Magnuson lab developed mice that express either *Pdx1* or *Neurog3* in both an acinar cell- and dox-dependent manner. In chapter 7, I briefly discuss the derivation and characterization of these mice models.

The materials and methods used to conduct these studies are described in Chapter 2, and the overall conclusion and future directions are presented in Chapter 8.

Some material in this dissertation has been published in Worchel & Magnuson, 2014 (Worchel & Magnuson, 2014) and Clayton et al., 2016 (Clayton et al., 2016)

CHAPTER 2

MATERIALS AND METHODS

Mouse lines and husbandry

All animals were housed at the Vanderbilt Institutional Animal Care Facility and experimental protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee. Mice were treated with dox (Sigma) dissolved in a 5% sucrose solution beginning at 6 to 8 weeks of age. Dox was provided *ad libitum* in lieu of the normal water supply. *Rosa26*$^{rtTA/+}$ (Hochedlinger, Yamada, Beard, & Jaenisch, 2005) and *Rag1*$^{-/-}$ mice (Mombaerts et al., 1992) were purchased from Jackson Laboratory. *Ptf1a*$^{YFP/+}$ (Burlison, Long, Fujitani, Wright, & Magnuson, 2008) and *MIP.GFP* mice (Hara et al., 2003) were genotyped as previously described.

Mutant alleles and genotyping

The *Rosa26*$^{3TF.\text{mCherry}}$, *Rosa26*$^{Neurog3.CFP}$, *Rosa26*$^{Pdx1.YFP}$ and *Ptf1a*^{rtTA} alleles were derived by recombinase-mediated cassette exchange using mouse ES cells (mESCs) that had been previously engineered to contain loxed cassette acceptor alleles with methods that have been previously described (S. X. Chen et al., 2011). The exchange vector for *Rosa26*$^{3TF.mCherry}$ was made by cloning both the mCherry DNA sequences and a 2A peptide-based expression cassette containing sequences for *Neurog3*, *Pdx1,* and *MafA* into separate sites of a pTRE-Tight-BI vector (Clontech). The bi-Tet operator assemblage was then inserted between the Lox71 and Lox2272 sites in pMCS.71/2272.Hygro. The exchange vector for *Rosa26*$^{Neurog3.CFP}$ was made by cloning both the CFP (Cerulean) *Neurog3* and DNA sequences into pTRE-Tight-BI vector

(Clontech). The bi-Tet operator assemblage was then inserted between the Lox71 and Lox2272 sites in pMCS.71/2272.Hygro. The exchange vector for *Rosa26*^{Pdx1,YFP} was made by cloning both the YFP (Yellow fluorescent protein) and *Pdx1* DNA sequences into pTRE-Tight-BI vector (Clontech). The bi-Tet operator assemblage was then inserted between the Lox71 and Lox2272 sites in pMCS.71/2272.Hygro. The bi-Tet operator assemblage was then inserted between the Lox71 and Lox2272 sites in pMCS.71/2272.Hygro. For the *Ptf1a*^{rtTA} allele, pPtf1a.Ex, a plasmid containing two inverted LoxP sites flanking the *Ptf1a* gene sequences and a FRT-flanked Hygromycin-resistance cassette, was modified using standard methods to insert the 5' UTR sequences from *X. laevis,* rtTA sequences from pTET-ON Advanced vector (Clontech), and an intron-containing rabbit *β-globin* poly A site. After electroporation of the exchange vectors into *Rosa26*^{LCA} (S. X. Chen et al., 2011) and *Ptf1a*^{LCA} containing mESCs (Burlison et al., 2008) respectively, clones surviving dual selection with hygromycin (Invitrogen) and gancyclovir (Sigma) were screened by PCR (**Table 2.1**). Blastocyst microinjections, chimeric matings and excision of the FRT-flanked hygromycin selection cassette were performed as previously described (S. X. Chen et al., 2011).

Microscopy

mCherry fluorescence intensity measurements in unfixed tissues were performed using a Leica MZ16 FA stereoscope at an exposure time of 39.5 milliseconds. For paraffin sections, pancreatic tissue was fixed with 20% formaldehyde and processed by the Vanderbilt Tissue Pathology Shared Resource (TPSR). H&E, Masson's Trichrome Blue, CD3, F4/80, and Cytokeratin staining was performed by TPSR according to manufacturer's directions. For routine immunodetection using frozen sections, whole pancreata were fixed for 4 hours at 4°C in 4%

paraformaldehyde in PBS, washed three times in PBS, and incubated overnight at 4°C in 30% sucrose in PBS. Fixed tissues were then embedded in O.C.T. (Tissue Tek) and frozen on dry ice. 8 μm sections were permeabilized for 30 minutes with 0.1% Triton X-100 in PBS and then preincubated with blocking solution (0.5% bovine serum albumin in PBS with 0.2% Tween-20 (PBS-T)) for 1 hour before applying primary antibody (**Table 2.2**). Primary antibodies were diluted in blocking solution, incubated overnight at 4°C, then washed 3 times with PBS-T. The slides were incubated with secondary antibodies (**Table 2.3**) diluted in blocking solution for 2 hours, washed three times in PBS-T and once in PBS, and then mounted with Prolong Gold with DAPI (Invitrogen).

Images were acquired using a Zeiss Axioplan-II upright microscope or a LSM 710 META inverted confocal microscope and then pseudo-colored using either ImageJ (NIH) or Zeiss LSM browser software. All images are representative of phenotypes observed in at least three different animals.

Adenovirus construction and injection

The AdV-CMV-3TF virus was made using pAd/CMV/V5-DEST (Invitrogen). High titer virus (6.5 $x10^{10}$ plaque-forming units (pfu)) was obtained by purification (Vector BioLabs). Mice were subjected to laparotomy under general anesthesia (Ketamine/Xylazine). The splenic lobe of the dorsal pancreas of 8 week old *Rag1*$^{-/-}$ mice was injected with 100 μl of purified AdV-CMV-3TF ($2x10^{10}$ pfu) and AdV-CMV-GFP ($1x10^{9}$ pfu) (Vector BioLabs) and animals were euthanized 7 days later.

Macrophage depletion

To study the effect of macrophages on the outcome of 3TF-mediated acinar cell reprogramming, 6-8 week old mice were intravenously injected with either saline (control) or gadolinium chloride ($GdCl_3$) (10 mg/kg, every 2 days for 1 week prior to dox treatment and then every 3rd day during dox treatment) and administered dox (2.0 or 0.2 mg/ml). Pancreata were harvested 7 days after dox. Only animals in which macrophages were reduced to less than 15% of the total DAPI-stained cells were used in the study.

Physiological studies

Adult mice were rendered diabetic with a single intraperitoneal injection of streptozotocin (180 mg per kg body weight dissolved in citrate buffer (pH 4.5)) after 4 hours fast. Mice with blood glucose levels >300 mg/dL were used for experiments. Glucose tolerance test was performed by fasting animals overnight (16-hours) followed by an intraperitoneal injection of D-glucose (2 g per kg body weight). Blood glucose concentrations were measured using a BD Logic glucometer.

FACS and RNA extraction

Pancreata were removed, perfused with 2.0 mg/mL collagenase P (Roche) in HBSS, minced and incubated at 37°C for 5 minutes. Cells were further dispersed by manual pipetting, washed with FACS staining buffer (R&D Systems), filtered through a 100 μm mesh cell strainer, then centrifuged. Cell pellets were resuspended in AccuMax (Sigma) and DNase1 (Ambion) and incubated at 37°C for 4 minutes. Afterwards, cells were washed with FACS staining buffer, centrifuged, and resuspended in FACS buffer containing DNAse1 (1 U/ml, Ambion) and 1 mM

EDTA. DAPI was used to stain for cell viability at a dilution of 1:1,000. Viable mCherry^{+} cells were sorted into TRIzol LS (Invitrogen) and total RNA was isolated using TRIzol LS (Invitrogen), DNase-treated, and column-purified (Zymo Research) as previously described (Osipovich et al., 2014).

Immunoblot analysis

Pancreatic tissues were lysed in 20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM EDTA, 20 mM beta-glycerophosphate, 5 mM EGTA, 1 mM PMSF, 1 μg/ml DTT, and 1x protease inhibitor (Sigma, P8340). Proteins were size fractionated in 4-20% SDS-PAGE gels (BioRad), transferred to polyvinylidene difluoride membranes (Millipore), blocked in 5% milk (BioRad) in PBS-T for 3 hours, then incubated with goat anti-Pdx1 (1:100; BCBC) or mouse anti-β actin (1:1000, Sigma) overnight at 4°C. The membranes were then washed, incubated with anti-goat and anti-mouse horseradish peroxidase-conjugated antibodies (Sigma) for 1 hour, washed again, then imaged using Western Lightning Plus ECL chemiluminescence kit (Perkin Elmer).

Luciferase Reporter Assays

The NeuroD1- and Insulin II-luciferase fusion gene constructs were previously described (Anderson et al., 2009; Zhao et al., 2005) as well as the design of the cytomegalovirus (CMV) enhancer-driven p300 (Lee et al., 2012), Beta2 (Qiu, Sharma, & Stein, 1998), and rtTA (Clontech 631069) expression vectors. Coding sequences for Neurog3, MafA, and Pdx1 were cloned into TRE-Tight (Clontech) in order to compare the function of the wild-type to the 2A peptide-modified variants. HeLa and Panc1 cell lines were grown in Dulbecco's modified Eagle's medium using 10% heat-inactivated fetal bovine serum, 25 mM glucose, penicillin (100

units/ml), and streptomycin (100 units/ml). To test the functionality of 2A peptide-modified *MafA* and *Pdx1*, HeLa cells were co-transfected in 6-well plates with CMV-driven rtTA (0.375 μg), p300 (0.375 μg), and Beta2 (0.375 μg) expression vectors and dox-inducible *MafA* (0.375 μg), Pdx1 (0.375 μg), or 3TF (0.375 μg) expression vectors in the presence of either -238 wild-type Insulin (0.375 μg), -238 Ins C1 mutant (0.375 μg), or -238 Ins A1/A3 mutant (0.375 μg) luciferase vectors. To test the functionality of 2A peptide modified *Neurog3*, Panc1 cells were co-transfected in 6-well plates with rtTA (0.5 μg) and either *Neurog3* (0.5 μg) or 3TF (0.5 μg) in the presence of either the NDFull (0.5 μg) or NDΔ1 (0.5 μg) expression vectors. All transfections were performed using Polyfect Transfection reagent (Quiagen 301105) in the presence of dox (50 μM). A CMV-driven *Renilla* luciferase expression vector (7.5 ng) was used to correct for differences in transfection efficiency. Fusion gene expression was measured 40–48 hours after transfection using a Dual Luciferase reporter assay system (Promega) and an automated luminometer (BioTek). Each transfection condition was tested in triplicate (n=3). Firefly luciferase readings were normalized to *Renilla* luciferase values.

RNA-seq pre-processing and differential expression analyses

Three independent RNA isolates from each genotype were used for sequencing. RNA-sequencing methods were described previously (E. Choi et al., 2012). Single-end sequencing (110 bp) was performed on Illumina HiSeq2000 genome analyzer. Read alignment to the mouse genome (mm10) was performed using RNA-Seq Unified Mapper (RUM) (Grant et al., 2011). Genome alignment of sequencing data yielded 32-84 million uniquely mapped reads. Data was pre-processed with the PORT pipeline (https://github.com/itmat/Normalization). PORT extends the idea of resampling to resample for more than just read depth, but also for

exon/intron/intergenic balance and ribosomal depletion balance (Li & Tibshirani, 2013). Moreover, PORT enables for separate treatment of genes that are predominantly expressed in a subset of the samples. This is particularly important in my case due to the peculiar composition of the acinar cell mRNA population. 90-95% of the mRNA molecules in a pancreatic acinar cell code for fewer than 30 secretory enzymes (Harding et al., 1977) and the Amylase-2a and Trypsinogen gene families have nearly identical members that account for nearly half of the mRNA molecules. Differential expression of non-protease genes was analyzed with edgeR (FDR 0.01) (McCarthy, Chen, & Smyth, 2012) and PADE (FDR 0.1) (https://github.com/itmat/pade; an extension of PaGE,(Grant, Liu, & Stoeckert, 2005)) in my 1 and 7 day induced acinar cells compared to uninduced acinar cells. Genes that were found to be differential expressed by both approaches were considered differential expressed. Functional Annotation Clustering was performed using DAVID Bioinformatics Resources v.6.7 (Huang da, Sherman, & Lempicki, 2009).

cDNA was prepared from RNA using a high-capacity cDNA Archive Kit (Life Technologies) and amplified using real-time PCR with Power SYBR Green PCR master mix (*Life Technologies*) using gene specific primers (**Table 2.1**). Three experimental RNA replicates for each genotype were assayed. PCR was performed with an ABI 7900HT real-time PCR system (Life Technologies) and amplification data were analyzed using Sequence Detection System version 2.1 (Life Technologies) and Excel software (Microsoft). *Hprt* was used as endogenous housekeeping control for normalization and comparative C_t method was used to calculate relative fold expression by $2^{-\Delta\Delta Ct}$.

Quantification of necrosis

Necrosis in pancreatic acini was quantified as previously described (Liu et al., 2014; Yuan et al., 2012). Quantification of necrosis was performed on pancreatic tissue (collected after 2 day of 2.0 mg/ml dox) sections stained with H&E. Cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into interstitium were considered necrotic. Over 1000 acinar cells were counted from each mouse and three mice per condition were counted.

Cell quantification and statistics

Cells co-expressing specific genes were determined by manual counting. For each animal, over 500 cells were counted using ImageJ from five sections per animal that were separated by approximately 50 μM. All key experimental findings were observed in 3 or more animals. The total number of cells in the adult mouse pancreas was previously calculated (Dore, Grogan, Madge, & Webb, 1981) and used to determine the number of new beta cells produced. Statistical difference between two groups was assessed using Student's t-tests. All data represent mean $\pm$ SEM.

Accession number

The RNA-seq data and annotation have been submitted to ArrayExpress with accession ID: E-MTAB-3921.

Table 2.1 Primers used

Primer	Sequence (5' to 3')	Product (bps)	Application
Rosa26.S1 Hygro-3'	AGACTTATCTACCTCATAGGTG GTGAGAACAGAGTACCTACAT	761	Screening of RMCE-derived mESCs
Rosa26.R1 Rosa26.S2	GAGGATCATAATCAGCCATACC TCACAAGCAATAATAACCTGTAGT	512	Screening of mESCs and genotyping of *ROSA*$^{3TF.mCherry}$ allele
F-p48 R-5'p48	CCTTCTGACTTCTCCAAGAAGGCA CCCTTTATGCCTGGCATTTCACTG	Targeted: 670 Wild-type: 636	Genotyping *of* *Ptf1a*^{rtTA} allele
Ins1-Fwd Ins1-Rv	CCAGCCCTTAGTGACCAGCTAT CCCAGGCTTTTGTCAAACAG	143 (exons 1-2)	RT-qPCR of *Ins1*
Ins2-Fwd Ins2-Rv	CCACCCAGGCTTTTGTCAAA CCCAGCTCCAGTTGTTCCAC	149 (exons 2-3)	RT-qPCR of *Ins2*
Hprt-Fwd Hprt-Rv	TACGAGGAGTCCTGTTGATGTTGC GGGACGCAGCAACTGACATTTCTA	138 (exon 9)	RT-qPCR of *Hprt* (Endogenous control)

Table 2.2 Primary antibodies for immunohistochemistry

Antigen	Species	Dilution	Source	Catalog #
Amylase	Goat	1:100	Santa Cruz Biotechnology	SC-212821
CD45	Rat	1:50	BD Biosciences	550539
Chromogranin A	Rabbit	1:1000	Abcam	ab15160
Cytokeratin	Rabbit	1:500	Dako	Z0226
F4/80	Rat	1:50	Invitrogen	MF48000
GFP	Chicken	1:1000	Invitrogen	A10262
Ghrelin	Rabbit	1:1000	Phoenix Pharmaceuticals	G-031-30
Glucagon	Rabbit	1:1000	Linco	4030-01F
Insulin	Guinea pig	1:1000	Invitrogen	18-0067
MafA	Rabbit	1:500	Bethyl laboratories	IHC-00352
Neurog3	Goat	1:1000	BCBC	AB5684
Pancreatic polypeptide	Guinea pig	1:1000	Linco	4041-01
Pdx1	Guinea pig	1:1000	Gift from Chris Wright	
RFP	Rabbit	1:1000	Rockland	600-401-379
RFP	Chicken	1:1000	Rockland	600-901-379
Somatostatin	Sheep	1:1000	American Research Products	13-2366

Table 2.3 Secondary antibodies for immunohistochemistry

Target Species	Host Species	Fluorophore	Dilution	Source
Chicken	Donkey	Cy3	1:1000	Jackson ImmunoResearch
Goat		Alexa Flour 488	1:1000	Invitrogen
Rabbit		Alexa Flour 488	1:1000	Invitrogen
Rabbit		Alexa Flour 555	1:1000	Invitrogen
Rat		Alexa Flour 488	1:1000	Invitrogen
Sheep		Alexa Flour 488	1:1000	Invitrogen
Chicken	Goat	Alexa Flour 555	1:1000	Invitrogen
Guinea pig		Alexa Fluor 488	1:1000	Invitrogen

CHAPTER 3

DESIGN AND VALIDATION OF A DIALLELIC, TRANSGENE-BASED MODEL FOR THE OVEREXPRESSION OF NUEROG3, PDX1, AND MAFA IN PANCREATIC ACINAR CELLS

Introduction

Cellular reprogramming is a potential therapy for replacing beta cells destroyed by autoimmune attack in T1D (Bramswig et al., 2013; W. Li, M. Nakanishi, et al., 2014; Thorel et al., 2010; Zhou et al., 2008). Towards this end, in 2008 it was reported that adenoviral-mediated expression of three pancreas-specific transcription factors *MafA, Pdx1,* and *Neurog3* (3TF) in immunocompromised mice resulted in the conversion of pancreatic acinar cells into new insulin-secreting beta-like cells (Zhou et al., 2008). In order to gain a better understanding of A→β conversion, the Magnuson lab derived a novel mouse model that enables simultaneous expression 3TF and a fluorescent reporter in a pancreatic acinar cell- and doxycycline-dependent manner. In this chapter, I discuss the derivation and validation of the transgenic mouse model.

Tet-regulated alleles

An effective system for regulating gene expression in mice is the diallelic, tet-regulated gene expression system (Freundlieb, Schirra-Muller, & Bujard, 1999; Lamartina et al., 2003), which has been used successfully for many *in vitro* and *in vivo* experiments, including adult pancreas studies (Hale et al., 2005; Holland, Hale, Kagami, Hammer, & MacDonald, 2002). The original Tet-Off strategy consists of a tetracycline-inhibited tet-TransActivator (tTA) driver and a tTA-dependent tetO-promoter responder gene. The Tet-On strategy consists of two parts: a

reverse-tetracycline transactivator (rtTA) driver gene and a tetracycline dependent responder gene. When coupled with the use of a cell-restricted promoter, both the Tet-Off and Tet-On strategies enable exquisite control of transgene expression.

Ptfa1a

Ptf1a (p48) is crucial for pancreas development and function and also serves as a specific marker for adult pancreatic acinar cells. *Ptf1a* is the defining central component of the trimeric PTF1 complex that plays important roles in both pancreas development and the maintenance of pancreatic acinar cell identity (Jonsson, Carlsson, Edlund, & Edlund, 1994; Kawaguchi et al., 2002; Krapp et al., 1998; Offield et al., 1996; Rose, Swift, Peyton, Hammer, & MacDonald, 2001; Roux, Strubin, Hagenbuchle, & Wellauer, 1989). During early organogenesis, *Ptf1a* (in the trimeric $PTF1^{RBPJ}$ isoform comprising Ptf1a, E47 and RBPJ) commits cells to the pancreatic MPC state in the undifferentiated, nascent pancreatic rudiment (Kawaguchi et al., 2002; Masui et al., 2008). Midway through development, $PTF1^{RBPJ}$ increases the level *RBPJL* expression, resulting in change in the composition of the PTF complex to $PTF1^{RBPJL}$ (Hale et al., 2005; Masui, Long, Beres, Magnuson, & MacDonald, 2007; Masui et al., 2010). $PTF1^{RBPJL}$ activates many acinar-specific target genes, including many for digestive enzymes (Holmstrom et al., 2011; Masui et al., 2010), which are crucial for the terminal differentiation of acinar cells and for maintaining the acinar phenotype in adult pancreas. $PTF1^{RBPJL}$, which does not bind the Notch intracellular domain (Beres et al., 2006), auto-regulates itself through the *Ptf1a* promoter-enhancer to reinforce and maintain its expression (Masui et al., 2008).

Results

Design and validation of mouse alleles

To better understand the cellular dynamics of pancreatic A→β reprogramming, the Magnuson lab developed a diallelic transgene-based mouse model that co-expresses both 3TF and mCherry in a pancreatic acinar cell- and doxycycline-dependent manner (**Figure 3.1A**). The first allele was made by replacing the coding sequences for *Ptf1a* with those for rtTA. In the second allele, a bi-directional Tet-operator cassette that in one direction expresses a 2A peptide-linked fusion gene of *MafA*, *Pdx1,* and *Neurog3* and in the other direction the red fluorescent protein *mCherry,* was inserted into a functionally disabled *Rosa26/SetD5* gene locus (S. X. Chen et al., 2011). When adult mice containing both the $Ptf1a^{rtTA}$ and $Rosa26^{3TF.mCherry}$ alleles were given 2.0 mg/ml dox in their drinking water for 1 day, red fluorescence was observed in the pancreas but no other visceral organs (**Figure 3.1B,C**). Immunofluorescent staining for mCherry and for the acinar cell marker amylase, showed that 78% (± 2.5%; n=3) of acinar cells expressed mCherry, and staining for mCherry and for each of the reprogramming factors showed that virtually all $mCherry^{+}$ cells expressed the reprogramming factors (**Figure 3.2A**). Furthermore, after 1 day of dox, mCherry expression was restricted to the pancreatic acinar cells and was not observed in pancreatic ducts or endocrine cells, as expected due to the well-established acinar cell-restricted expression of *Ptf1a* (**Figure 3.1C** and **Figure 3.2B**).

To further validate this experimental model, the function of the 2A peptide-cleaved transcription factors generated by the $Rosa26^{3TF.mCherry}$ allele was analyzed. First, immunoblot analysis for PDX1, which is flanked by MAFA and NEUROG3 protein sequences in the 2A peptide containing cassette, showed it to be properly cleaved from the two surrounding proteins (**Figure 3.2C**). Second, analysis of protein function using reporter genes showed that each of the

2A peptide-modified proteins functioned in a normal manner, indistinguishable from that of their wild-type counterparts (**Figure 3.3**). Third, when Rosa26^{rtTA} was used to drive expression of 3TF, insulin^{+} cells are induced within 3 days in intestinal crypts (**Figure 3.4A**), a previously reported finding (Y. J. Chen et al., 2014). Fourth, a recombinant adenovirus containing the 3TF fusion gene, when injected together with a GFP-expressing virus into the pancreas of *Rag1*$^{-/-}$ mice, resulted in scattered insulin^{+}/GFP^{+} co-expressing cells within the exocrine compartment of the pancreas, similar to those observed by Zhou et al. (Zhou et al., 2008) (**Figure 3.4B,C**). Together, these findings confirmed that the 2A peptide-modified MAFA, PDX1 and NEUROG3, made in response to dox-induction, functioned normally.

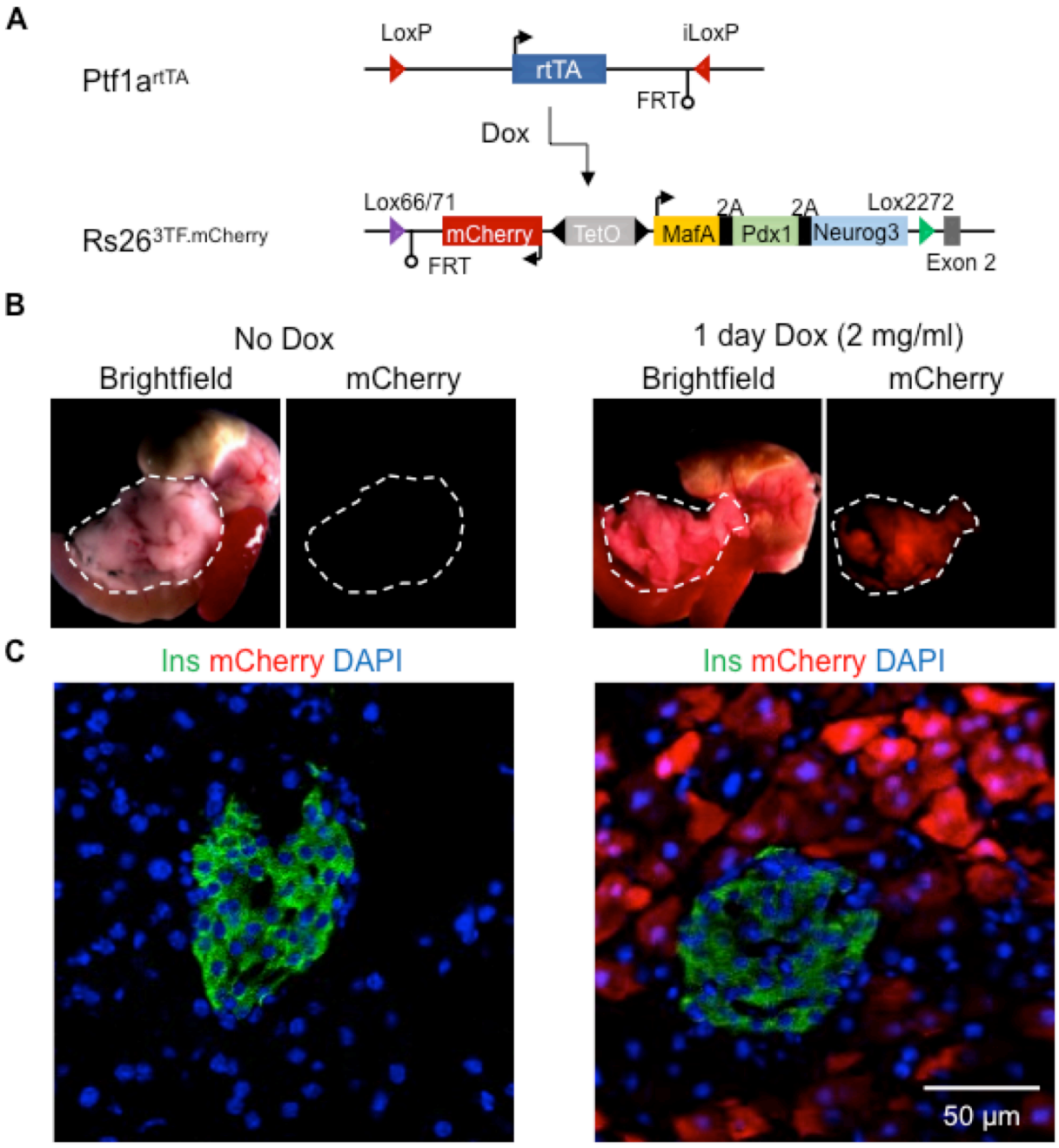


Figure 3.1 Mouse model for the acinar cell- and doxycycline-dependent overexpression of 3TF. (A) *Ptf1a*^{rtTA} and *Rosa26*$^{3TF.mCherry}$ alleles were generated by recombinase-mediated cassette exchange. When interbred, the two alleles resulted in dox-inducible expression of *MafA, Pdx1*, *Neurog3,* and mCherry in a pancreatic acinar cell-specific manner. (B) mCherry expression was visible after administering 2.0 mg/ml of dox for 1 day (n=5). mCherry fluorescence was restricted to the pancreas (outlined) of dox treated mice and was not observed in other tissues. (C) Dox-inducible, acinar cell-specific expression of mCherry was confirmed with immunofluorescence analysis. Pancreas sections stained with insulin and mCherry showed that the two proteins were not co-localized.

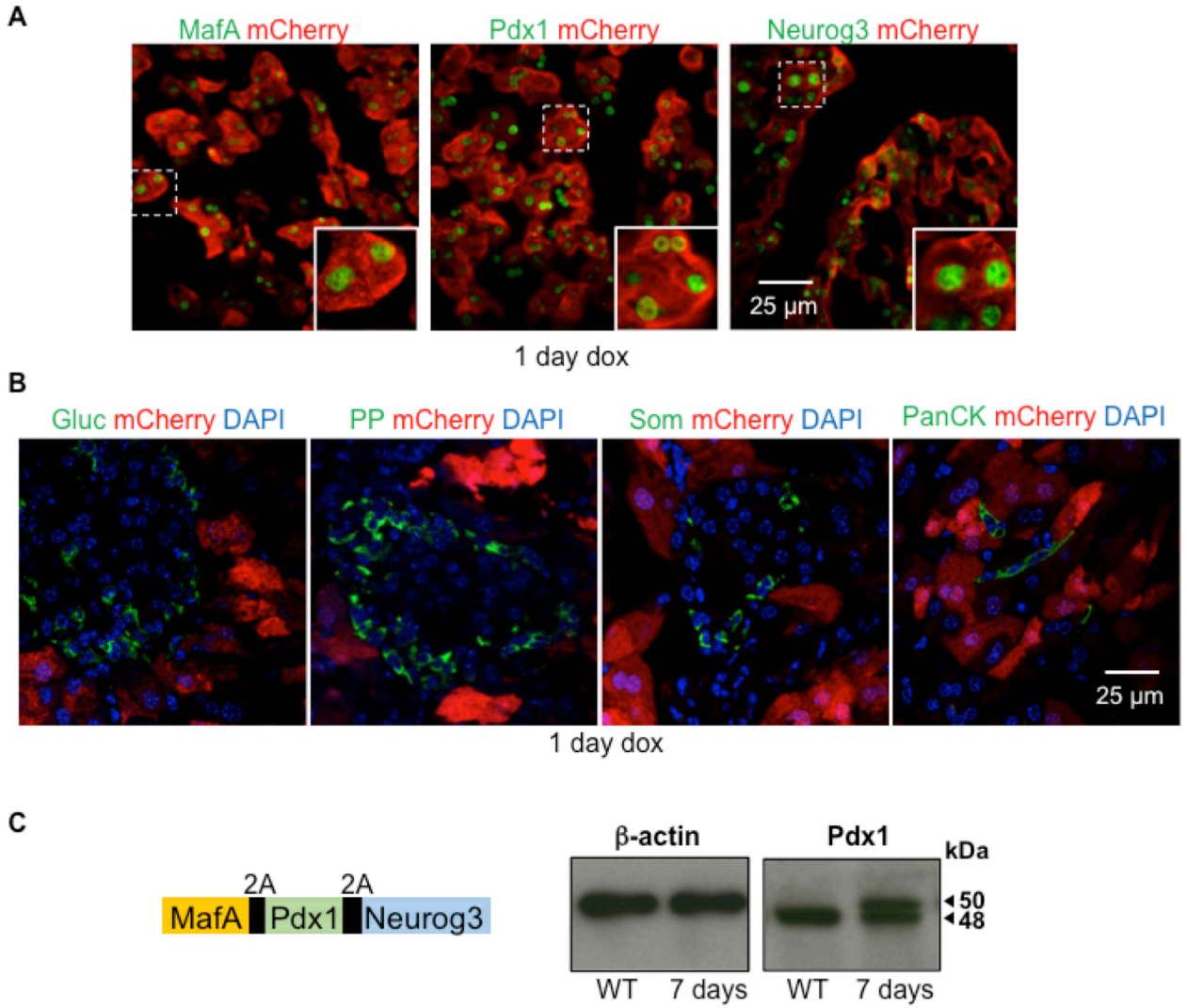


Figure 3.2 Reprogramming factors are expressed specifically in pancreatic acinar cells. (A) Pancreas sections stained with antibodies against mCherry and either *MafA, Pdx1*, or *Neurog3* showed co-expression of mCherry and 3TF after 1 day of dox. (B) After 1 day of dox, mCherry was not expressed in glucagon-, pancreatic polypeptide-, or somatostatin-expressing endocrine cells, or in ductal cells, marked by PanCK staining. (C) Schematic of the transgene showing that the protein sequences for MAFA and NEUROG3 flank PDX1. To determine whether proper 2A mediated cleavage of 3TF was achieved, western blot against PDX1 was performed on pancreatic lysate from wild-type (WT) and 7 day induced, *Ptf1a*$^{rtTA/+}$*; Rosa26*$^{3TF.mCherry/+}$ (3TF) mice. Arrows indicate both the 50 kDa PDX1 protein with the addition of the 2A peptide sequences and the endogenous PDX1 at 48 kDa

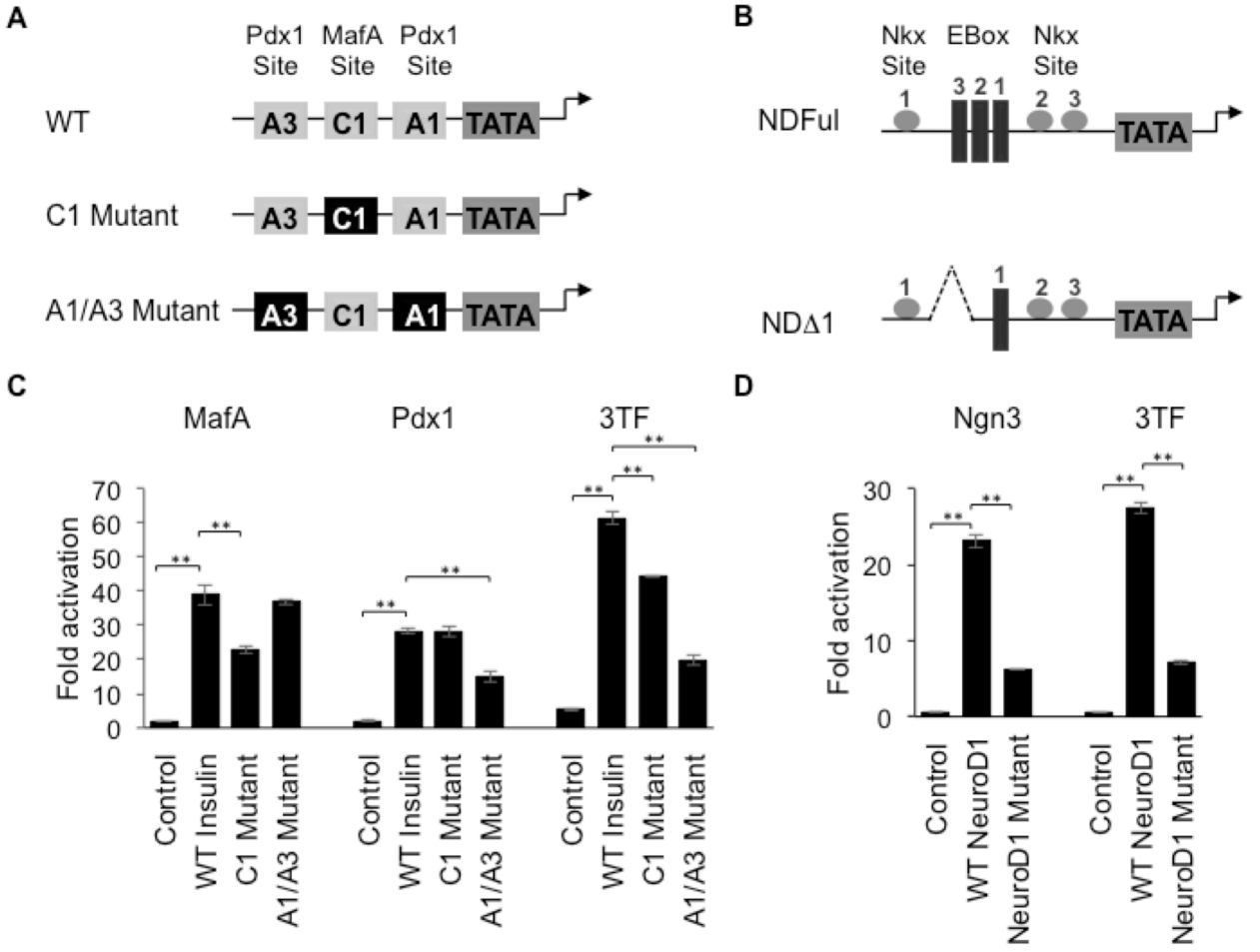


Figure 3.3 2A peptide-modified proteins function in a normal manner. (A) Promoter constructs used for Dual Luciferase Assay to test the functionality of the 2A peptide modified MAFA and PDX1 proteins. Constructs used were a wild-type (WT), MafA-binding mutant (mutant C1), or Pdx1-binding mutant (double mutant A1/A3) of the insulin promoter fused to luciferase. (B) Promoter constructs used for Dual Luciferase Assay to test the functionality of the 2A peptide modified NEUROG3 protein. Constructs used were a WT *NeuroD1* promoter fused to luciferase (NDFul) or a *NeuroD1* promoter containing a deletion of the Neurog3 binding site fused to luciferase (NDΔ1). (C) Dual Luciferase Assay was performed in transfected HeLa cells using either the WT or mutated insulin promoter constructs. Control was no transcription factors. Data are represented as mean ± SEM. **p < 0.001, student's t-test, n=3. (D) Dual Luciferase Assay was performed in transfected Panc1 cells using either the WT or mutated *NeuroD1* promoter fused to luciferase. Control was no transcription factors. Data are represented as mean ± SEM. **p < 0.001, student's t-test, n=3.

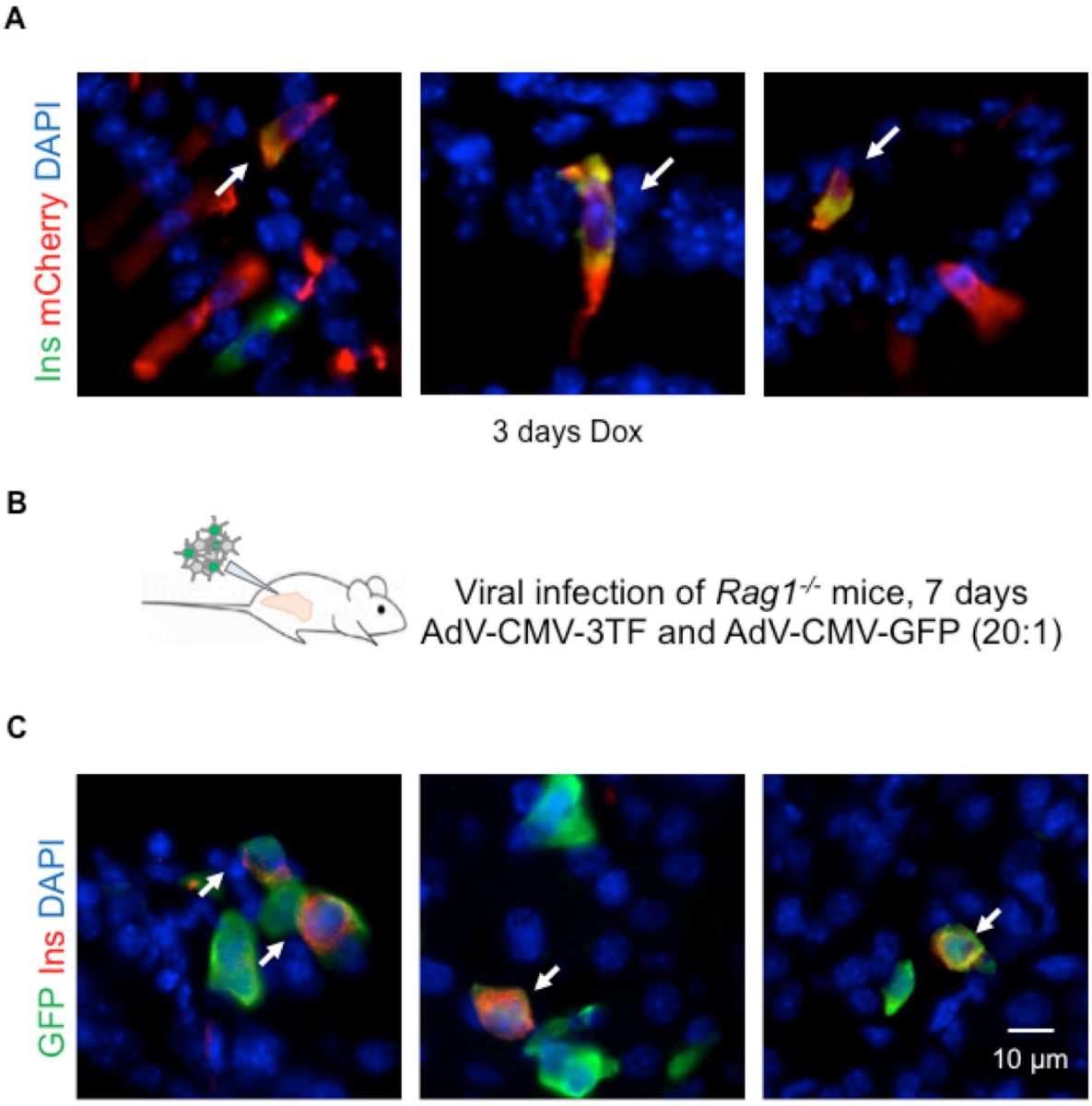


Figure 3.4 Validation of mouse model (B) The splenic lobe of the dorsal pancreas of 8 week old *Rag1*$^{-/-}$ mice was injected with 100 µl of a purified 3TF-expressing adenovirus (AdV-CMV-3TF; $2x10^{10}$ pfu) and a GFP-expressing adenovirus (AdV-CMV-GFP, $1x10^{9}$ pfu). Animals were euthanized 7 days later. (C) Pancreatic immunofluorescence staining of insulin and GFP. Arrows indicate infected acinar cells that have been reprogrammed into insulin-expressing cells.

Discussion

In order to investigate the cellular dynamics of 3TF-mediated A→β reprogramming, the Magnuson lab developed a biallelic transgenic mouse. This mouse model utilizes two different alleles, *Ptf1a*^{rtTA} and *Rosa26*$^{3TF.mCherry}$, that allow for the dox-inducible expression of 3TF and mCherry specifically in pancreatic acinar cells. 2A peptide technology was used to simultaneously express 3TF and mCherry from a single transcript. To validate the biallelic mouse model and ensure that the 2A peptide-modified MAFA, PDX1, and NEUROG3, made in response to dox-induction, functioned normally, multiple validation studies were performed.

First, I showed that 3TF/mCherry is expressed specifically in the pancreatic acinar cells in a dox-dependent manner. When biallelic mice are given 2.0 mg/ml dox in their drinking water for one day, red fluorescence is observed in the pancreas but no other visceral organs. Immunohistochemical staining for mCherry and for each of the reprogramming factors has shown that all four proteins are simultaneously expressed in at least 70% of acinar cells. Furthermore, immunohistochemical staining shows that mCherry expression is restricted to acinar cells and is not observed in pancreatic ducts or endocrine cells, as expected, due to the well-established acinar cell-restricted expression of *Ptf1a,* the driver gene for *rtTA*.

Second, multiple validation studies to ensure that the three reprogramming factors functioned properly were performed. **1.** Immunoblotting has shown proper peptide-2A-mediated cleavage of the nascent protein transcript. **2.** Reporter studies in cell lines have shown that the 2A peptide-modified factors each function in a manner indisguishable from the wild-type proteins. **3.** When *Rosa26*^{rtTA} was used to drive expression of 3TF, insulin^{+} cells were observed within 3 days in intestinal crypts, similar to a report by Chen et al. (Y. J. Chen et al., 2014). **5.** Finally, when the 3TF cassette in *Rosa26*$^{3TF.mCherry}$ allele was inserted into a recombinant adenovirus and

injected into the pancreas of *Rag1*$^{-/-}$ mice, I observed scattered insulin^{+} cells, precisely mimicking the results from Zhou et al. (Zhou et al., 2008).

This mouse model allows me to express 3TF in the majority of pancreatic acinar cells in a regulated manner. With viral delivery of the factors, only a small portion of the acinar cells were infected, and only a few of the infected cells produced insulin. Use of the Tet-O system allows for the controlled expression of transgenes in the majority of pancreatic acinar cells. This make it possible to acquire enough tissue in sufficient purity for biochemical, cell-biological, and molecular analyses of synchronized cellular changes. Indeed, this mouse model enables 3TF expression to be modulated in a manner that is unachievable using a virus-based expression system, thereby allowing the effects of both 3TF concentration and duration on generating new beta-like cells to be examined.

CHAPTER 4

TRANSGENE-BASED EXPRESSION OF *NEUROG3, PDX1,* AND *MAFA* CAUSES PANCREATIC INFLAMMATION AND ACINAR-TO-DUCTAL METAPLASIA

Introduction

The diallelic, transgenic mouse model that the Magnuson lab developed and validated, which expresses 3TF in both a pancreatic acinar- and dox-dependent manner, allows the dynamics of A→β reprogramming to be investigated. Such a model allows for both 3TF expression and duration to be modulated in a manner that is unachievable using a virus-based expression system. However, using this transgene-based mouse model, I was unable to achieve A→β reprogramming and found that robust 3TF expression causes acinar cell necrosis resulting in marked inflammation and ADM. In this chapter, I discuss the effects of transgene-mediated 3TF overexpression in pancreatic acinar cells on pancreatic histology and architecture.

Acinar cell plasticity

Acinar cells exhibit plasticity converting into endocrine cells (Baeyens et al., 2014; W. Li, C. Cavelti-Weder, et al., 2014; W. Li, M. Nakanishi, et al., 2014; Pan et al., 2013; Zhou et al., 2008), duct cells (Kopp et al., 2011), and adipocytes (Bonal et al., 2009). The ability of acinar cells to undergo cellular conversions has significant implications for both health and disease. First, acinar cell reprogramming into beta cells represents a potential therapy for T1D. Second, the conversion of acinar cells into duct-like cells, also referred to as ADM, is a major contributor to the generation ductal of pancreatic intraepithelial neoplasia (PanINs) (Habbe et al., 2008), the

predecessors to pancreatic ductal adenocarcinomas (PDAC) (Habbe et al., 2008), one of the most deadly forms of cancer accounting for the fourth leading cause of cancer-related deaths in the United States (Siegel, Naishadham, & Jemal, 2013).

In response to injury, the pancreas activates a regeneration process to maintain tissue homeostasis. In acinar cells, inflammation activates cytokines, inflammatory proteins, signaling pathways involved in stress, such as the unfolded protein response (UPR), and developmental and regeneration proteins (Pin, Ryan, & Mehmood, 2015). Recent studies have shown that after injury, acinar cells undergo ADM where they dedifferentiate into a ductal epithelium that expresses early developmental factors. These duct-like cells are suggested to be "facultative progenitor cells" that, once inflammation is resolved, can re-differentiate into mature acinar cells (Husain & Thrower, 2009). However, when combined with Kras mutations, these cells have an increased chance of forming PanIN lesions (De La et al., 2008; Habbe et al., 2008). A deep understanding of the molecular mechanism governing acinar cell plasticity is vital and may be exploited to drive acinar cells towards and endocrine cell fate and away from a potentially pre-cancerous, duct-like cell.

Results

Lack of A→β reprogramming in transgenic mice after 3TF induction

I was first interested to see if transgene-mediated 3TF expression resulted in the reprogramming of acinar cells into beta cells. Since viral mediated expression of 3TF in the pancreas has previously been reported to cause A→β conversion in 10 days or less (Zhou et al., 2008), I treated my diallelic mice with dox for 1, 7, and 28 days and performed immunostaining for acinar and endocrine cell markers, including insulin. At 1 day, the 3TF-induced cells

resembled acinar cells with all mCherry$^+$ cells seen to express amylase, an acinar cell-specific marker. However, at 7 and 28 days, the expression of amylase in mCherry$^+$ cells was greatly diminished or absent (**Figure 4.1**). In addition, the mCherry$^+$ cells were smaller in size and located in tubular-like cell clusters (**Figure 4.1A**). Chromogranin A, an endocrine cell marker that was absent in the 1 day sample, was expressed in nearly 100% of mCherry$^+$ cells at 7 and 28 days (**Figure 4.2**). Interestingly, despite the widespread expression of this endocrine marker in mCherry$^+$ cells, I failed to observe any insulin, glucagon, somatostatin, or pancreatic polypeptide expression by immunofluorescence staining. However, after 7 days of dox treatment, I did observe that approximately 40% of mCherry$^+$ cells expressed ghrelin, a hormone that is normally expressed in 1% or fewer adult pancreatic endocrine cells (Arnes, Hill, Gross, Magnuson, & Sussel, 2012). The portion of cells expressing ghrelin rose to nearly 60% in the 28 day sample (**Figure 4.2)**.

RNA profiling of the 7 day 3TF-treated cells

To better understand why new beta-like cells were not observed and to corroborate the immunostaining results, I performed RNA-Seq on FACS-purified mCherry$^+$ cells after 1 and 7 days of dox administration and compared their transcriptional profiles to FACS-purified uninduced acinar cells (**ArrayExpress:E-MTAB-3921**). These datasets revealed that *MafA, Pdx1,* and *Neurog3* mRNAs were all highly up-regulated after 1 day of dox treatment compared to uninduced acinar cells. Indeed, *MafA* increased from 0.67 $\pm$ 0.67 to 4069 $\pm$ 169, *Pdx1* from 20 $\pm$ 4 to 7037 $\pm$ 471, and *Neurog3* from 0 to 10931 $\pm$ 629 normalized counts, strongly suggesting that the lack of insulin gene expression was not due to insufficient 3TF expression. Furthermore, inspection of the 7 day, 3TF-induced RNA-seq dataset showed that while some genes that

characterize either immature or mature beta cells, such as *ChgA, Ghrl, Neurod1,* and *Insm1,* were highly up-regulated compared to uninduced acinar cells, other genes that are normally present in beta cells such as *Ins1*, *Ins2*, *Nkx6.1*, *Isl1*, and *Pax6* were not identified as significantly up-regulated in the 3TF-induced acinar cells (**Figure 4.3A** and **ArrayExpress:E-MTAB-3921**). In addition, many of the endocrine-specific genes that were up-regulated have been previously shown to be direct DNA binding targets for either *Neurog3, Pdx1,* or *MafA*. For instance, *Neurod1*, *Nkx2.2,* and *Insm1* are targets of *Neurog3* (H. P. Huang et al., 2000; Smith et al., 2003; Watada et al., 2003), and *Pdx1* binds to the promoters of both *Gck* and *Slc2a2* (Khoo et al., 2012; Watada et al., 1996). Importantly, I also noticed that many of the upregulated genes from the RNA-seq data set of 7 day, dox-induced acinar cells were associated with inflammation (**Figure 4.3B**)**.** Taken together, these findings suggest that while 3TF expression increased expression of several endocrine-specific genes, it did not cause acinar cells to thoroughly adopt a beta cell-like gene expression profile.

Transgene-based expression of 3TF results in acinar-to-ductal metaplasia

To confirm that inflammation-associated genes were expressed in response to 3TF-induction, I began by staining pancreata for inflammatory cells using CD45, a pan-leukocyte marker, and observed that nearly a third of the cells present in the pancreas were leukocytes (**Figure 4.4A, B**). Further staining for F4/80, a macrophage marker, and CD3, a T-cell marker, revealed that both types of inflammatory cells were present with the majority being macrophages (**Figure 4.5A, B**). Finally, Masson's trichrome staining revealed extensive fibrosis, further indicating a potent inflammatory response (**Figure 4.5C**).

Since inflammation in the pancreas has been linked to metaplastic changes, I examined the histological appearance of 7 day-induced pancreatic tissue. After 7 days of 3TF expression, pancreata of the diallelic mice were smaller than control mice (**Figure 4.6A**), were characterized by the presence of many tubular complexes (**Figure 4.6B**), and had marked acinar cell necrosis (**Figure 4.6C**), all of which are hallmarks of ADM (Jura, Archer, & Bar-Sagi, 2005; Parsa et al., 1985). Immunostaining for cytokeratin, a marker for pancreatic duct cells, further revealed that the 3TF-expressing $mCherry^{+}$ cells had adopted duct-like characteristics (**Figure 4.7A, B**). Since marked inflammation and metaplasia were not observed in my viral control experiments (**Figure 4.7C, D**) or previously reported by others when an adenovirus was used to introduce 3TF to the pancreas of normoglycemic mice (Cavelti-Weder et al., 2016; Zhou et al., 2008), I hypothesized that the overly robust 3TF expression that occurred using 2.0 mg/ml of dox was responsible for both the immune response and histologic changes consistent with ADM.

To determine why 3TF expression in my diallelic transgenic model was causing pancreatic inflammation, I considered the fact that pancreatic acinar cells are especially vulnerable to ER dysfunction owing to their high level of protein synthetic and secretory activity (Ji et al., 2003; Logsdon & Ji, 2013). In support of this notion, I noticed that 44 of 83 genes involved in the activation of the unfolded protein response were upregulated after 7 days of 3TF expression (**Figure 4.8A**). In addition, the expression of genes encoding voltage-gated and other Ca^{2+} channels (**Figure 4.8B**) were also markedly increased. In acinar cells, a rise in $[Ca^{2+}]_i$ has been associated with ADM and is known to cause activation of inflammatory genes and the ER stress response (Sah et al., 2014). These findings are consistent with activation of the ER stress response in 3TF-expressing acinar cells, most likely through a disruption in calcium homeostasis.

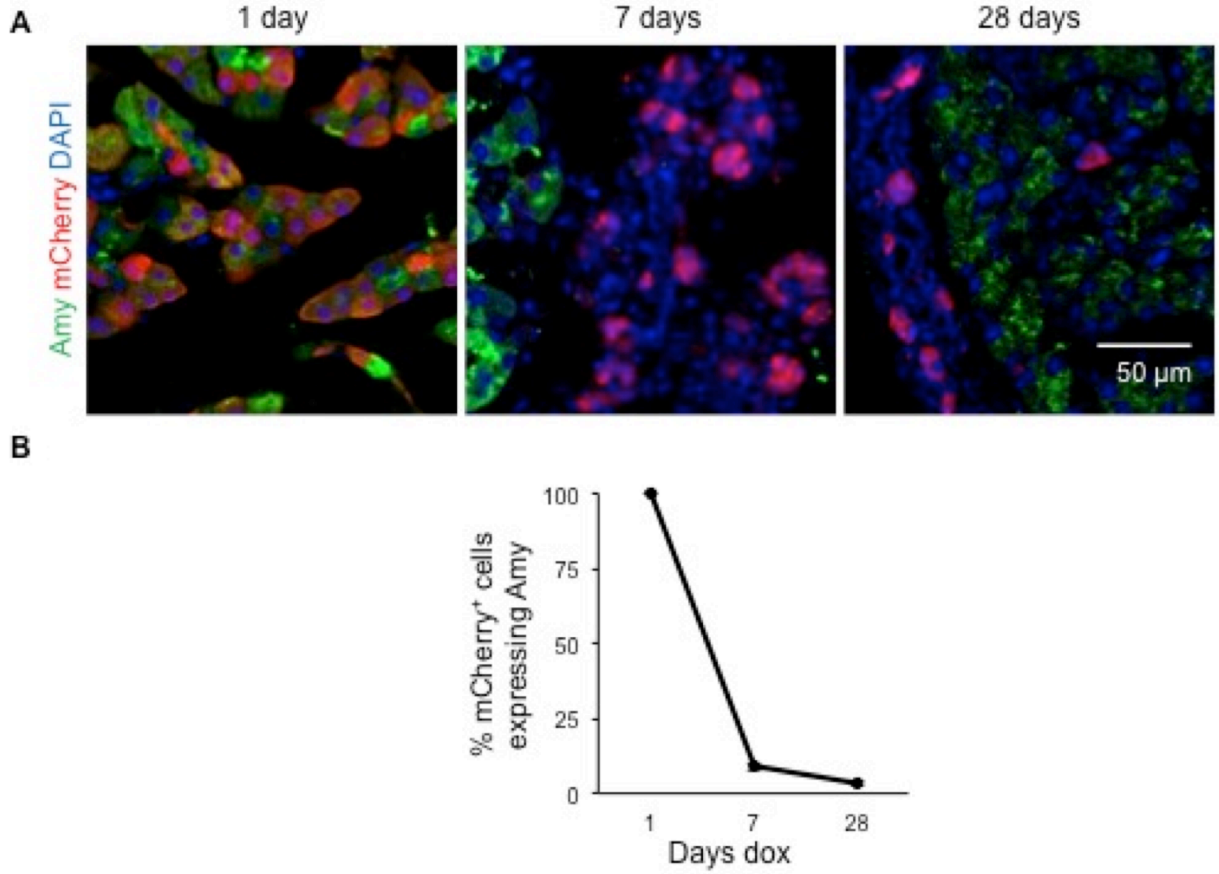


Figure 4.1 Amylase expression decreases during reprogramming. (A) Animals were induced for 1, 7, or 28 days with dox (2.0 mg/ml). Immunofluorescence analysis revealed that amylase expression in mCherry^{+} cells was present after 1 day of dox but decreased by 7 days and continued to decreased throughout the time course. (B) Percentage of cells expressing amylase among mCherry^{+} cells. Three mice per time point. Over 500 mCherry^{+} cells counted for each mouse. Data are represented as mean $\pm$ SEM.

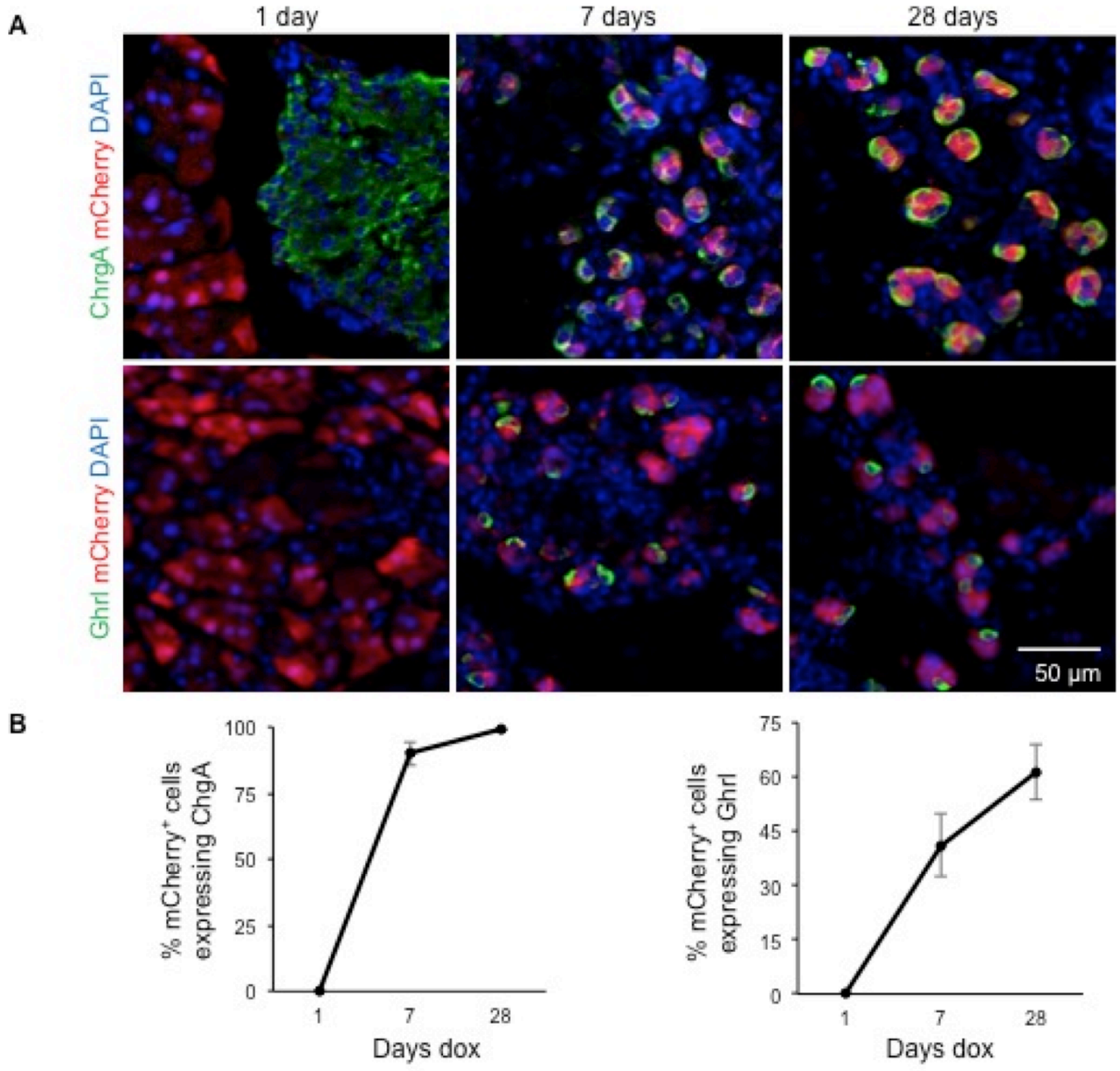


Figure 4.2 Expression of endocrine markers increase during reprogramming. (A) Animals were induced for 1, 7, or 28 days with dox (2.0 mg/ml). Immunofluorescence analysis revealed that the expression of the endocrine markers chromogranin A and ghrelin began at 7 days and increased during the reprogramming time-course. (B) Percentage of cells expressing chromogranin A and ghrelin among mCherry^{+} cells. Three mice per time point. Over 500 mCherry^{+} cells counted for each mouse. Data are represented as mean $\pm$ SEM.

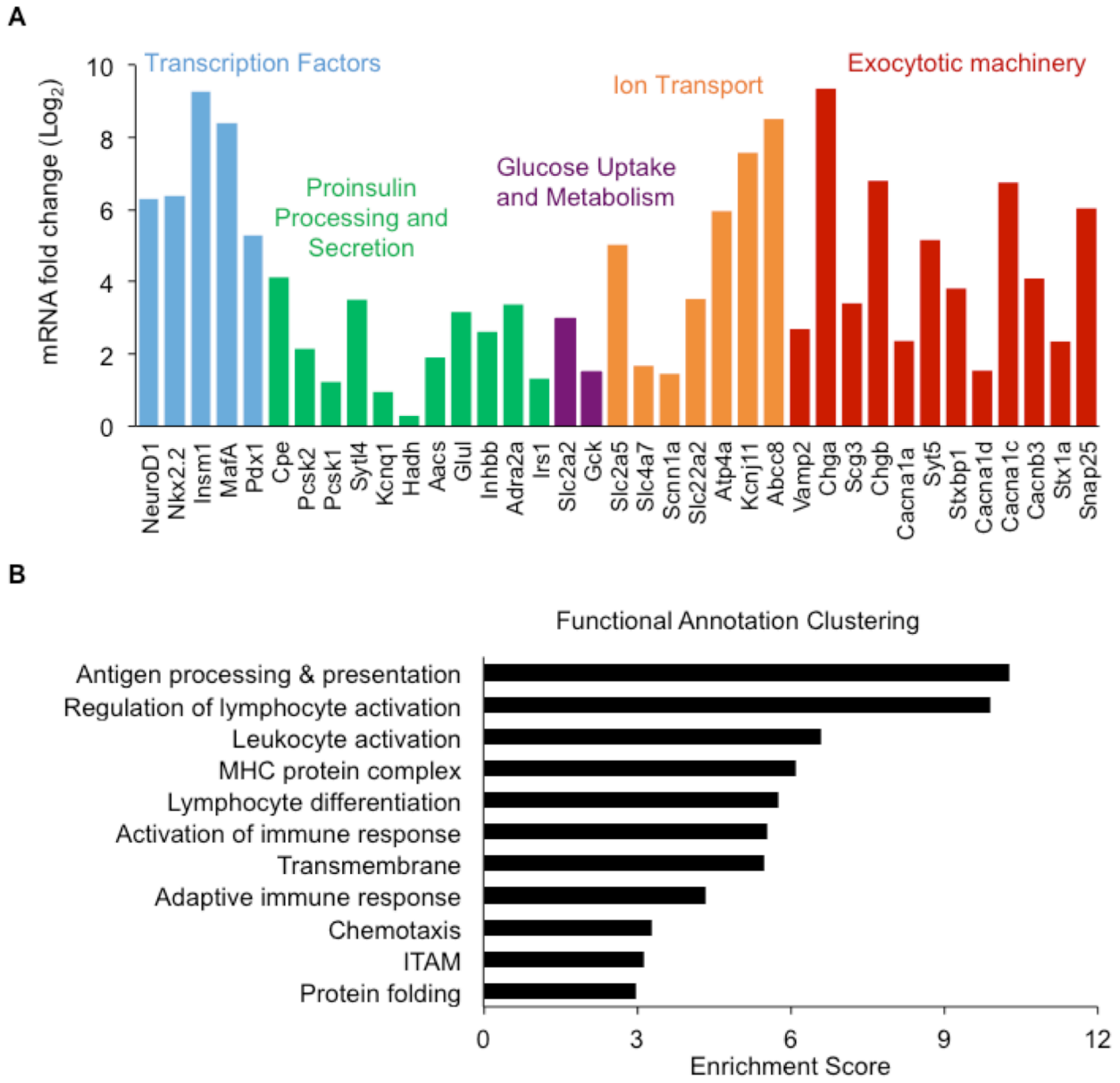


Figure 4.3 Expression analysis of reprogrammed cells (A) Fold changes (log2 scale; pseudocounts added) in the expression of beta cell specific genes calculated from my RNA-seq data set of 7 day 3TF-induced acinar cells compared to uninduced acinar cells. These mRNAs were identified as significantly differentially expressed by both edgeR and PADE. (B) The top eleven functional annotation clusters compiled from the top 200 up-regulated genes in my RNA-seq data set of 7 day 3TF-induced acinar cells compared to uninduced acinar cells using DAVID analysis.

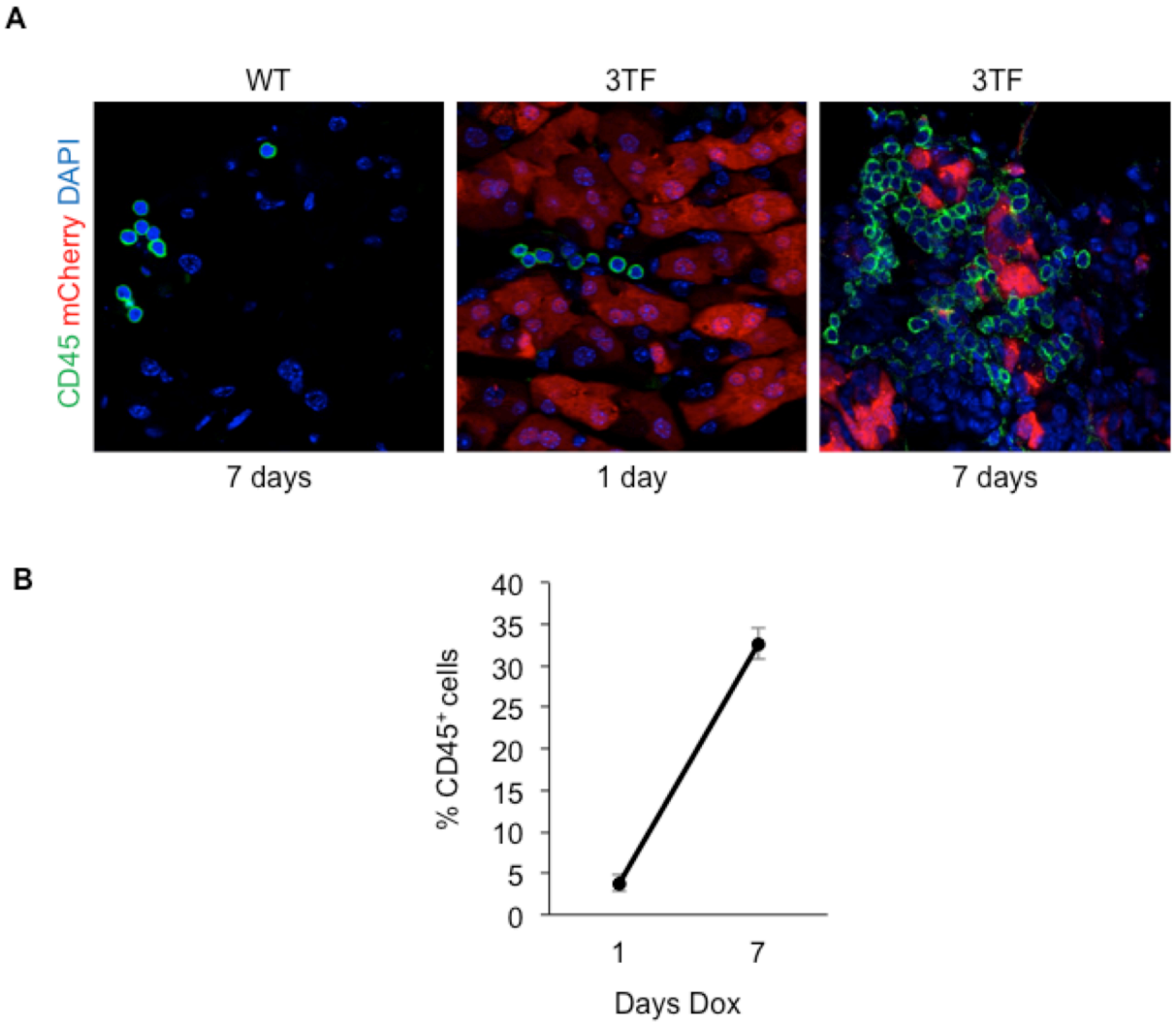


Figure 4.4 Transgene-mediated 3TF expression in pancreatic acinar cells causes an infiltration of immune cells (A) Pancreatic immunofluorescence staining of CD45 from WT mice after 7 days of dox and 3TF mice after 1 and 7 days of dox. (B) Percentage of $CD45^+$ cells among $DAPI^+$ cells. Three mice per time point and over 1,000 $DAPI^+$ cells counted for each mouse. Data are represented as mean $\pm$ SEM.

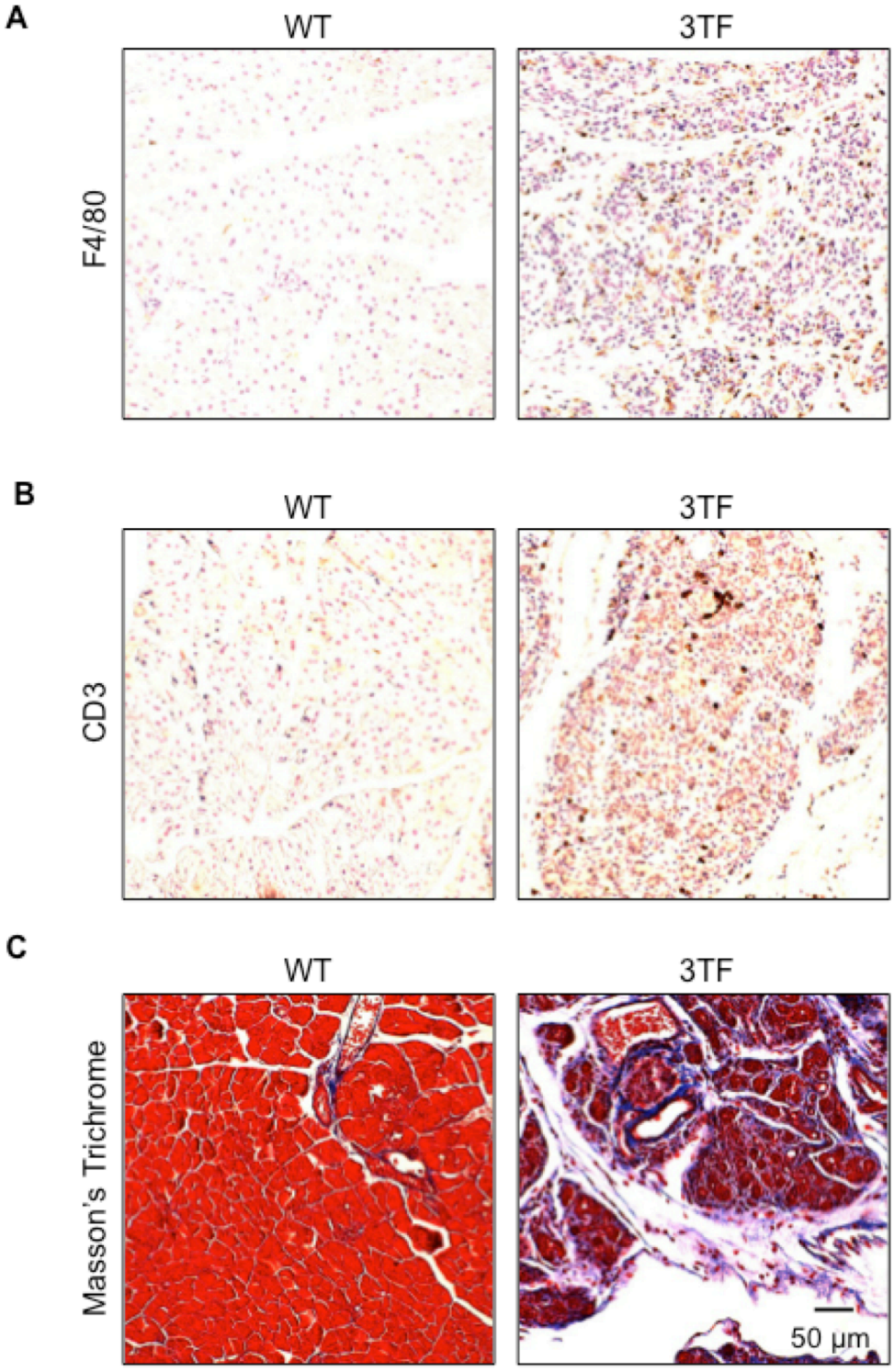


Figure 4.5 Transgene-mediated 3TF expression in pancreatic acinar cells causes a potent inflammatory response. (A) Representative F4/80, (B) CD3, and (C) Masson's trichrome stain of WT and *Ptf1a*$^{rtTA/+}$; *Rosa26*$^{3TF.mCherry/+}$ (3TF) mice at 7 days dox treatment.

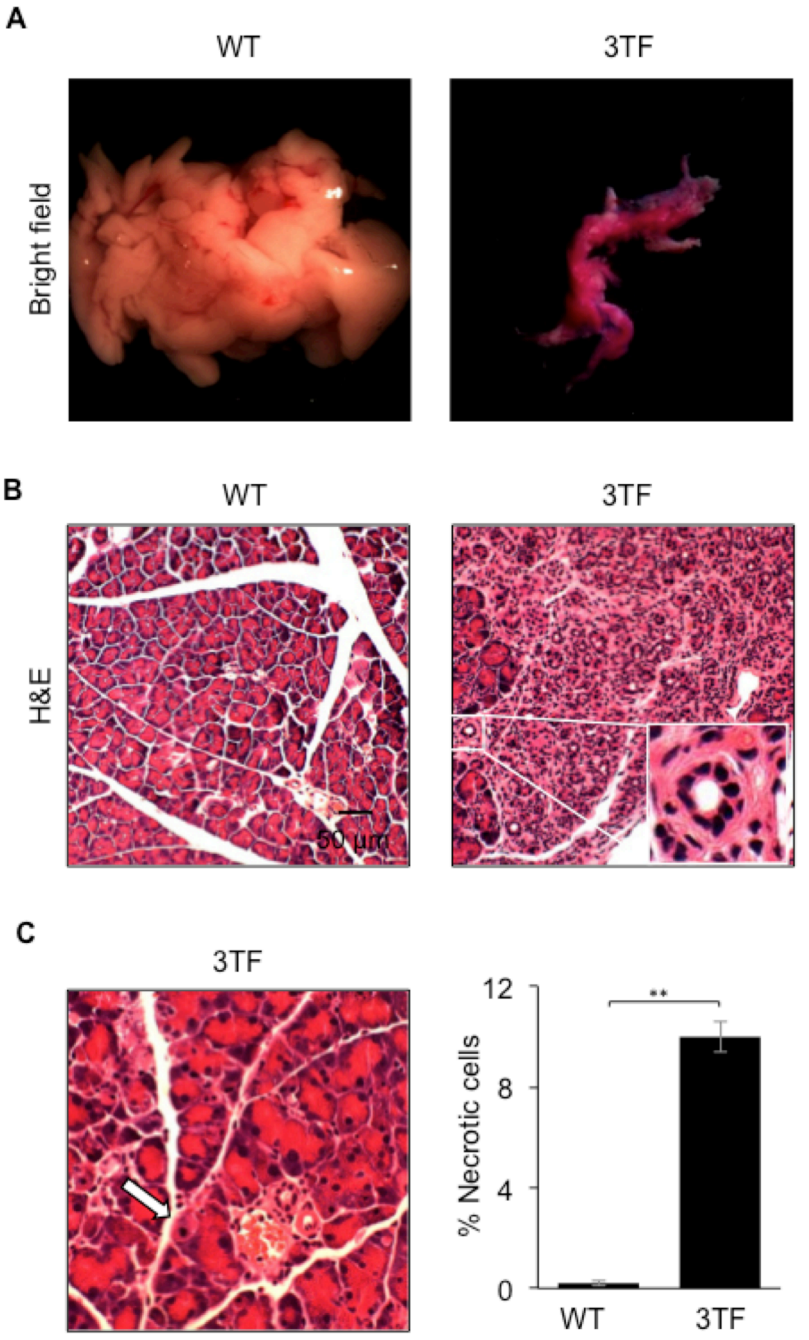


Figure 4.6 Transgene-mediated 3TF expression in pancreatic acinar cells causes Acinar-to-ductal metaplasia. (A) Representative pancreata and (B) hematoxylin and eosin (H&E) staining of WT and 3TF mice after 7 days of dox. Pancreas of 3TF mice is characterized by the presence of abundant tubular complexes (inset). (C) Acinar cell necrosis (arrow) was observed in the pancreata of 3TF mice treated with dox for 2 days. (E) Necrosis was measured on tissue sections stained with H&E from WT mice and 3TF mice treated with dox for 2 days. Necrosis is represented as the percentage of necrotic acinar cells among acinar cells. Three mice per group and over 1,000 acinar cells were counted for each mouse. Data are represented as mean $\pm$ SEM. **$p < 0.001$, student's t-test.

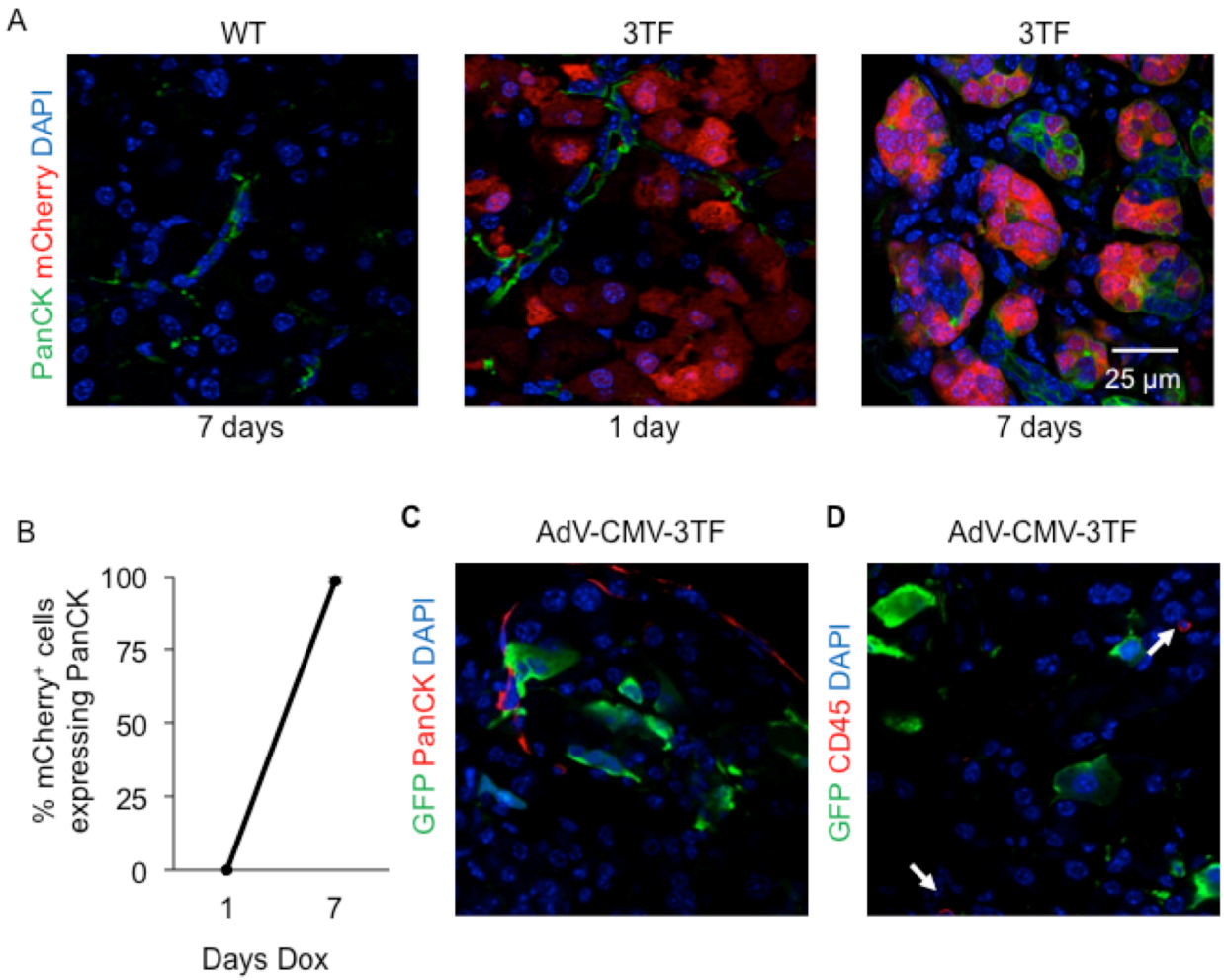


Figure 4.7 Transgene-mediated 3TF expression in pancreatic acinar cells causes the expression of ductal markers. (A) Pancreatic immunofluorescence staining of PanCK, a ductal marker, from WT mice after 7 days dox and 3TF mice after 1 and 7 days of dox. (B) Percentage of PanCK^{+} cells among mCherry^{+} cells. Three mice per time point and over 500 mCherry^{+} cells counted for each mouse. (C) The pancreas of 8 week old *Rag1*$^{-/-}$ mice was injected with 100 µl of a purified AdV-CMV-3TF ($2x10^{10}$ pfu) and an AdV-CMV-GFP ($1x10^{9}$ pfu). Animals were euthanized 7 days later. Representative pancreatic immunofluorescence staining of PanCK (D) and CD45, 7 days post AdV-CMV-3TF, infection indicates that adenoviral delivery of my 3TF construct did not result in a potent inflammatory response or ADM.

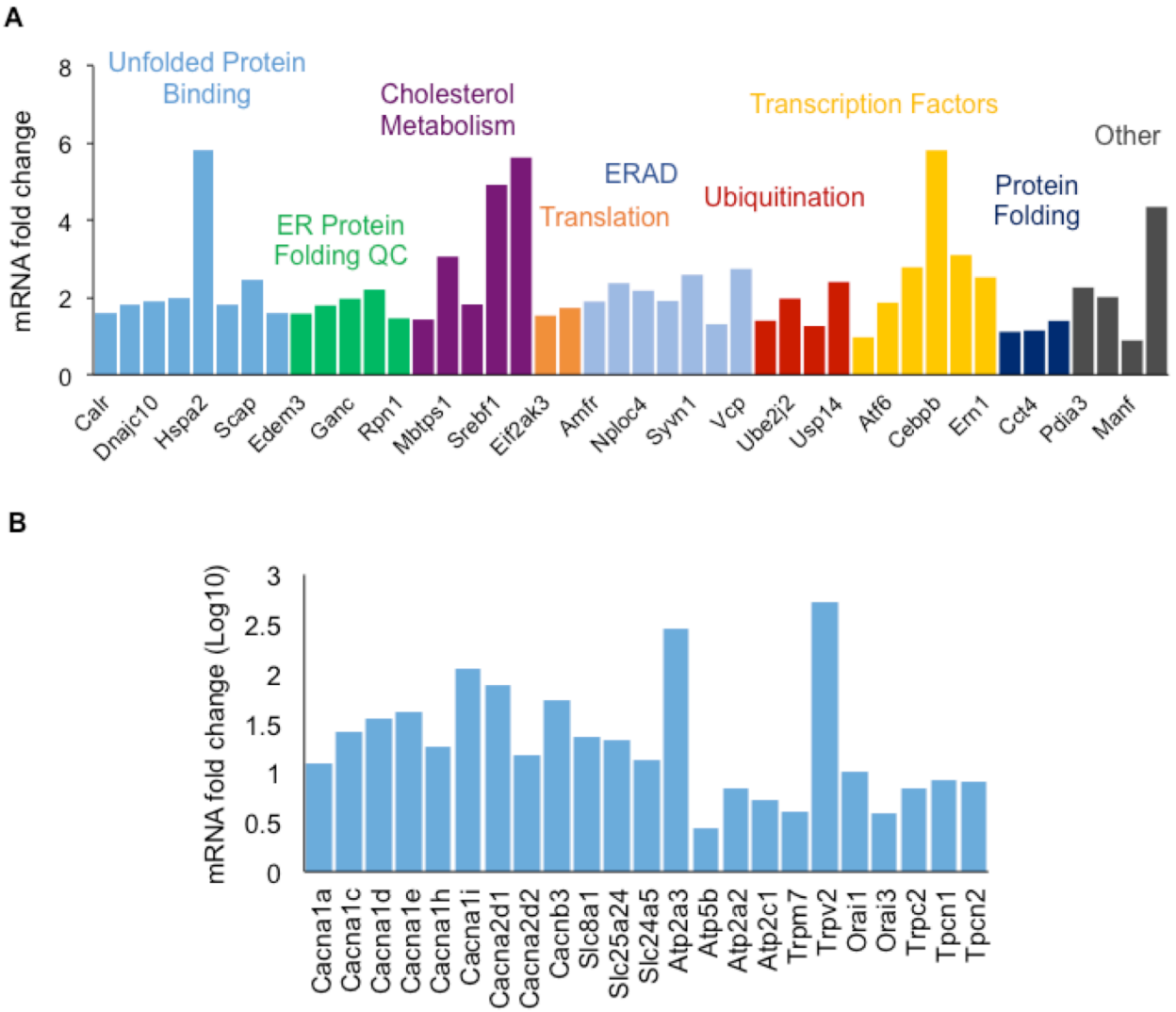


Figure 4.8 Transgene-mediated 3TF-overexpression causes ER stress (A) Fold changes (pseudocounts added) in the expression of key genes recognizing and responding to misfolded protein accumulation in the ER calculated from my RNA-seq data set of 7 day 3TF-induced acinar cells compared to uninduced acinar cells. These mRNAs were found to be differentially expressed by both edgeR and PADE. (B) Fold changes (pseudocounts added) in the expression of Ca^{2+} Channels calculated from my RNA-seq data set of 7 day 3TF-induced acinar cells compared to uninduced acinar cells. These mRNAs were found to be differentially expressed by both edgeR and PADE.

Discussion

By using a novel diallelic transgenic mouse model that expresses 3TF in both a pancreatic acinar- and dox-dependent manner, I found that 3TF expression in acinar cells does not result in new beta-like cells, but instead causes their conversion into duct-like cells due to ADM. Indeed, after 7 days of 3TF expression, many genes that serve to define a mature beta cell, including insulin, were not significantly expressed.

Furthermore, 3TF expression caused acinar cell damage characterized by severe necrosis and a corresponding infiltration of macrophages. While pancreatic acinar cells are an attractive reprogramming target, due to their high degree of plasticity (Kopp et al., 2012; W. Li, M. Nakanishi, et al., 2014; Wallace, Marek, Currie, & Wright, 2009) and similar developmental pathway to beta cells, their function of producing and secreting digestive enzymes may pose challenges. Indeed, premature enzyme activation is a major contributor to pancreatitis, and under inflammatory conditions, acinar cells can undergo ADM. While in some instances inflammation promotes cellular reprogramming, such as the reprogramming of fibroblast to iPSCs (Lee et al., 2012) or hepatocytes to beta-like-cells (A. Y. Wang, Ehrhardt, Xu, & Kay, 2007), in the pancreas, inflammation may prevent A→β reprogramming. It is possible that the conversion of acinar cells into a duct-like, progenitor cell takes precedence, especially since ADM may serve to protect the organ in the face of damage by preventing the continued production and activation of digestive enzymes that would likely result in continuing pancreatic damage.

It is unclear as to why robust 3TF expression causes pancreatic inflammation and ADM. It is possible that a high concentration of 3TF causes a pathological increase in the $[Ca^{2+}]_i$ in acinar cells, which triggers the premature activation of pancreatic zymogens such as trypsinogen, and activation of the ER stress response mechanism (Muili et al., 2013; Raraty, Petersen, Sutton,

& Neoptolemos, 1999; Sah et al., 2014). Indeed, inspection of my RNA-seq data revealed a marked increase in both the expression of genes encoding voltage-gated and other Ca^{2+} channels and of genes involved in the activation of the unfolded protein response after 7 days of robust 3TF-transgene expression. Ca^{2+} plays a central role in controlling the secretion of digestive enzymes in pancreatic acinar cells and a rise in $[Ca^{2+}]_i$ is a key event in the pathogenesis of pancreatitis with elevated $[Ca^{2+}]_i$ levels causing premature trypsin activation, necrosis, activation of inflammatory genes, and activation of the ER stress response (J. Li et al., 2014; Sah et al., 2014).

Widespread metaplastic changes resulting from tissue inflammation have not been reported when adenoviruses are used to introduce the reprogramming factors into the pancreas of *Rag1*$^{-/-}$ normoglycemic mice (Cavelti-Weder et al., 2016). I speculate that under normoglycemic conditions similar metaplastic conversions that were observed with transgene-mediated 3TF-expression are not observed due to the lower infection efficiency of the adenovirus. This results in fewer 3TF-expressing cells, thereby lowering the overall inflammatory potential. In addition, adenoviral delivery may result in lower levels of 3TF-expression compared to transgene-mediated expression, which may also prevent acinar cell damage and the ensuing inflammation. In any case, it is clear that the dynamics of A→β reprogramming are more complex than previously thought, and that a better understanding of the many variables that are involved will be essential prior to testing the feasibility of A→β reprogramming in humans.

CHAPTER 5

RAG1 AND ADENOVIRAL INFECTION DOES NOT INFLUENCE THE OUTCOME OF TRANSCRIPTION FACTOR-MEDIATED ACINAR CELL REPROGRAMMING

Introduction

Viral-mediated delivery of 3TF to the pancreas of *Rag1*$^{-/-}$ mice has been shown by Zhou et. al to reprogram pancreatic acinar cells into insulin-secreting beta-like cells (Zhou et al., 2008). However, using a diallelic transgenic mouse model, I have found that transgene-mediated expression of 3TF does not result in the production of new beta-like cells. Instead, 3TF expression causes a marked immune response and ADM. Two obvious differences between my experimental design compared to that of Zhou et al. was the use of a transgene to express 3TF, opposed to a viral vector, and the presence of *Rag1* (Zhou et al., 2008). On account of this, I tested whether one or both of these experimental variables might account for the divergent reprogramming outcome observed and have found that neither *Rag1* nor viral infection influenced the reprogramming outcome. In this chapter, I will discuss my findings on the role of *Rag1* and adenoviral infection on the outcome of 3TF-mediated A→β reprogramming.

Rag1

Rag1 is an enzyme essential for the maturation of B- and T-cells, and its deletion results in the complete absence of both T- and B-cells (Mombaerts et al., 1992). Since T-cells are important in controlling the early innate immune response and regulating macrophage polarization, deletion of *Rag1* also alters the innate immune response resulting in an increase of

M1 macrophage functionality as well as a reduced M2 macrophage profile (Chan, Pek, Huth, & Ashkar, 2011). Indeed, macrophages form a heterogeneous population of cells within the mononuclear phagocyte system and are potent immune regulators playing an important role in tissue remodeling and homeostasis. Furthermore, in the face of pancreatic damage, M1 macrophages can drive ADM (Liou et al., 2013). Initially, I believed that use of immunodeficient mice would be unnecessary when using a transgene, not a virus, to express 3TF. However, after being unable to reprogram acinar cells into new beta–like cells in $Rag^{+/+}$ mice, and observing a cellular infiltrate of macrophages after inducing 3TF transgene expression, I hypothesize that the altered immune response in $Rag1^{-/-}$ mice facilitates A→β reprogramming.

Adenoviral infection

Reprogramming methods routinely utilize adenoviral vectors, which allow for transient expression of exogenous genes without integration into the host genome (Stadtfeld, Nagaya, Utikal, Weir, & Hochedlinger, 2008). In some settings, the use of a viral vector is required for successful reprogramming (Lee et al., 2012; A. Y. Wang et al., 2007; Zaldumbide et al., 2012). Indeed, viral infection and adenoviral regulatory proteins, such as E4-ORF3, have been shown to activate host immune response pathways resulting in epigenetic changes that improve the binding of the reprogramming factors to their targets (Lee et al., 2012; Zaldumbide et al., 2012). These findings support the notion that an open chromatin state improves reprogramming efficiency. Acinar cells are littered with repressive histone modifications and compared to alpha and beta cells, exocrine cells exhibit many more genes marked with the repressing H3K27me3 histone modification (Bramswig et al., 2013). Indeed, the addition of chromatin modifying agents that alter the chromatin structure to a more "open" state has also been shown to improve

reprogramming efficiency of iPSCs (Huangfu et al., 2008; Mali et al., 2010; Shi et al., 2008). Thus, I hypothesis that adenoviral infection alters the epigenetic landscape of acinar cells in a manner that enables successful 3TF-mediated A→β reprogramming.

Results

The presence or absence of *Rag1* or adenovirus infection does not alter the reprogramming outcome

Given that new beta cells were observed only when 3TF was expressed by an adenoviral vector, but not from a transgene, I performed several experiments to exclude a role for two experimental variables that might have accounted for this divergent reprogramming outcome. The first experimental variable was the presence or absence of *Rag1* (Zhou et al., 2008), a gene required for V(D)J recombination in B and T cells (Mombaerts et al., 1992). *Rag1* null mice were used in the adenoviral expression studies to prevent clearance of the transcription factor-expressing viruses whereas the transgene-based expression studies utilized $Rag1^{+/+}$ animals since viral clearance was not an issue. Since it is possible that the lack of mature B and T lymphocytes in $Rag1^{-/-}$ mice might modulate the inflammatory response and allow reprogramming to occur, I crossed the *Rag1* null allele into my diallelic mice to derive $Ptf1a^{rtTA/+}$; $Rosa26^{3TF.mCherry/+}$; $Rag1^{-/-}$ mice and then treated these mice with dox for 7 days. Pancreas immunostaining for CD3 showed that T cells were indeed absent (**Figure 5.1A**). However, despite the absence of *Rag1*, many F4/80$^+$ cells were observed in the pancreas after 7 days of dox treatment (**Figure 5.1B**), and similar to the $Rag1^{+/+}$ mice, many cytokeratin$^+$ tubular complexes were formed (**Figure 5.1C and 5.2A**) that expressed ghrelin (**Figure 5.2C**). Masson's trichrome staining again revealed extensive fibrosis (**Figure 5.2B**) and, more importantly, there were no cells that co-

expressed mCherry and insulin (**Figure 5.2D**). Thus, these findings showed that the presence or absence of *Rag1* has no substantive role in determining the reprogramming outcome when I used 2.0 mg/ml dox to induce 3TF expression.

The second experimental variable was the presence or absence of adenoviral infection. Since it has been reported that viral infection and the presence of adenoviral regulatory proteins could improve reprogramming efficiency (Lee et al., 2012; A. Y. Wang et al., 2007; Zaldumbide et al., 2012), I simultaneously induced 3TF expression for 7 days using 2.0 mg/ml dox while also injecting a GFP-expressing adenovirus directly into the pancreas (**Figure 5.3A, B**). Once again, and despite examining over 600 mCherry and GFP co-expressing cells, no cells were seen that also expressed insulin (**Figure 5.3D**). Instead, many of the mCherry and GFP co-expressing cells continued to express ghrelin (**Figure 5.3C**). These findings enabled us to exclude the host immune response to viral infection as having a major role in the divergent reprogramming outcome when using 2.0 mg/ml dox to induce 3TF expression.

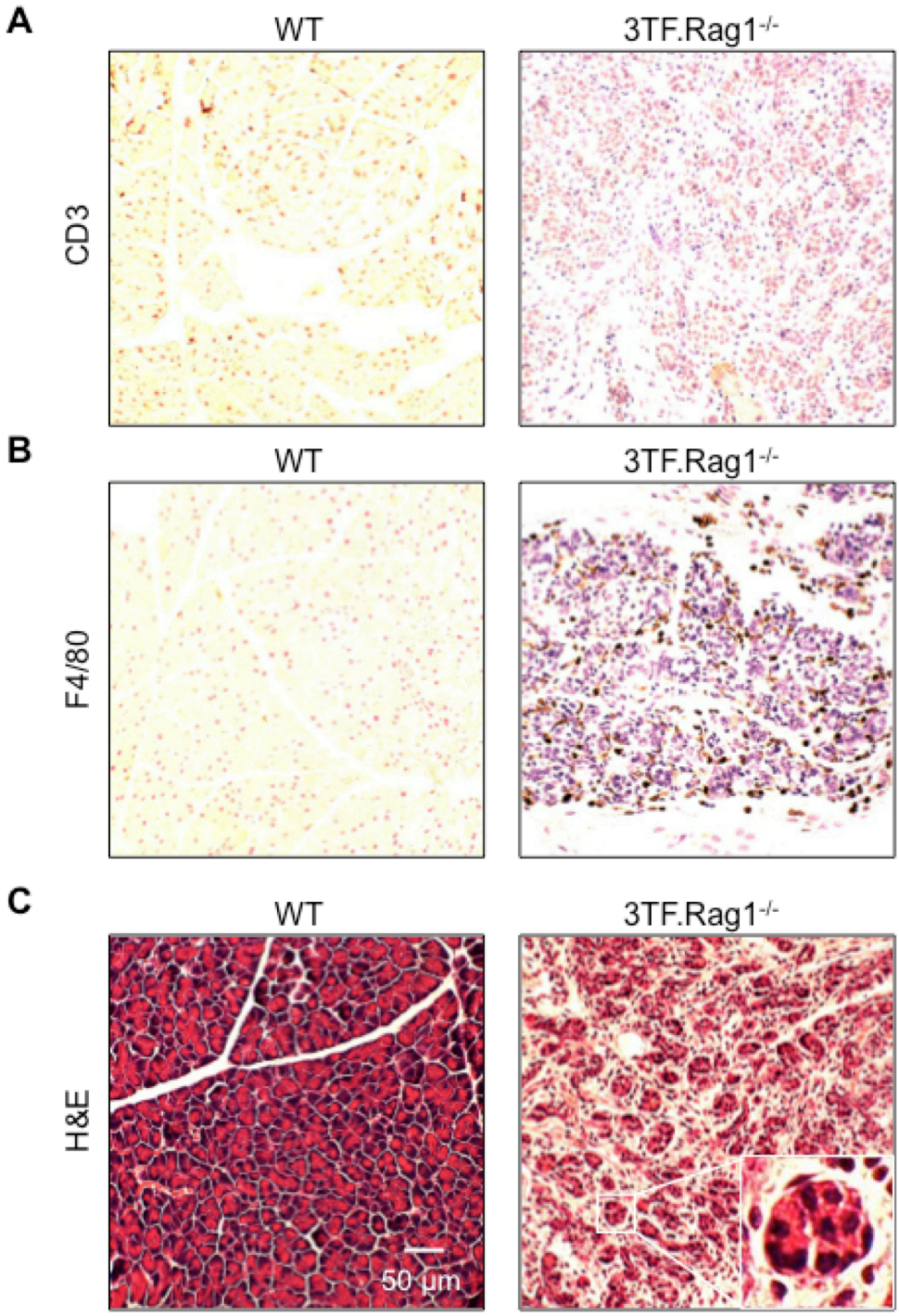


Figure 5.1 Rag1 deletion does not prevent macrophage infiltration or pancreatic histological changes. (A) Representative CD3 stain of WT and *Ptf1a*$^{rtTA/+}$; *Rosa26*$^{3TF.mCherry/+}$; *Rag1*$^{-/-}$ (3TF. Rag1$^{-/-}$) mice at 7 days dox (2.0 mg/ml) treatment. No CD3^{+} cells are observed in WT or 3TF.Rag1$^{-/-}$ mice. (B) Representative F4/80 stain of WT and 3TF. Rag1$^{-/-}$ mice at 7 days dox treatment. (C) H&E staining of WT and 3TF. Rag1$^{-/-}$ mice after 7 days dox administration. Pancreas of 3TF. Rag1$^{-/-}$ mice is characterized by the presence of abundant tubular complexes (inset).

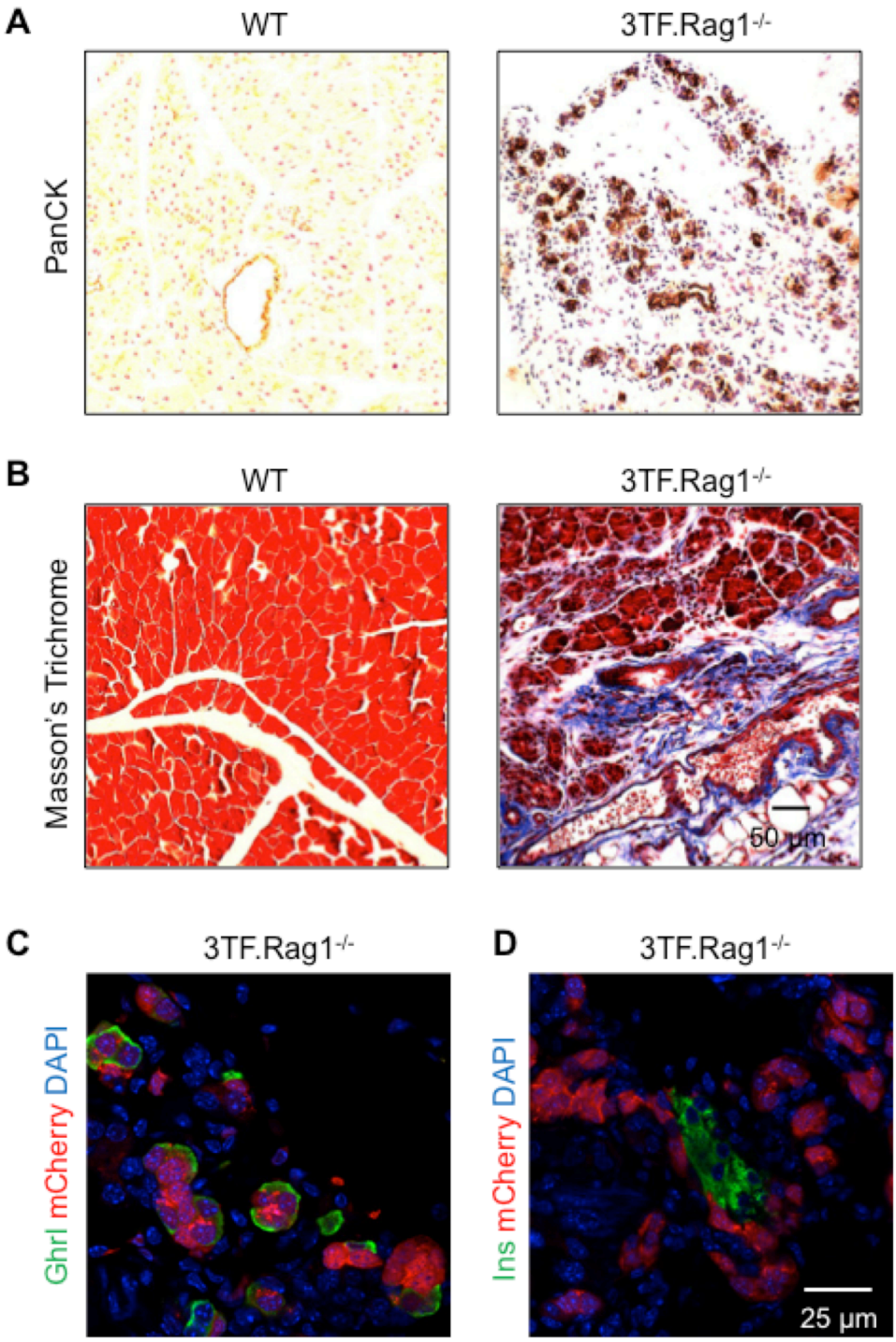
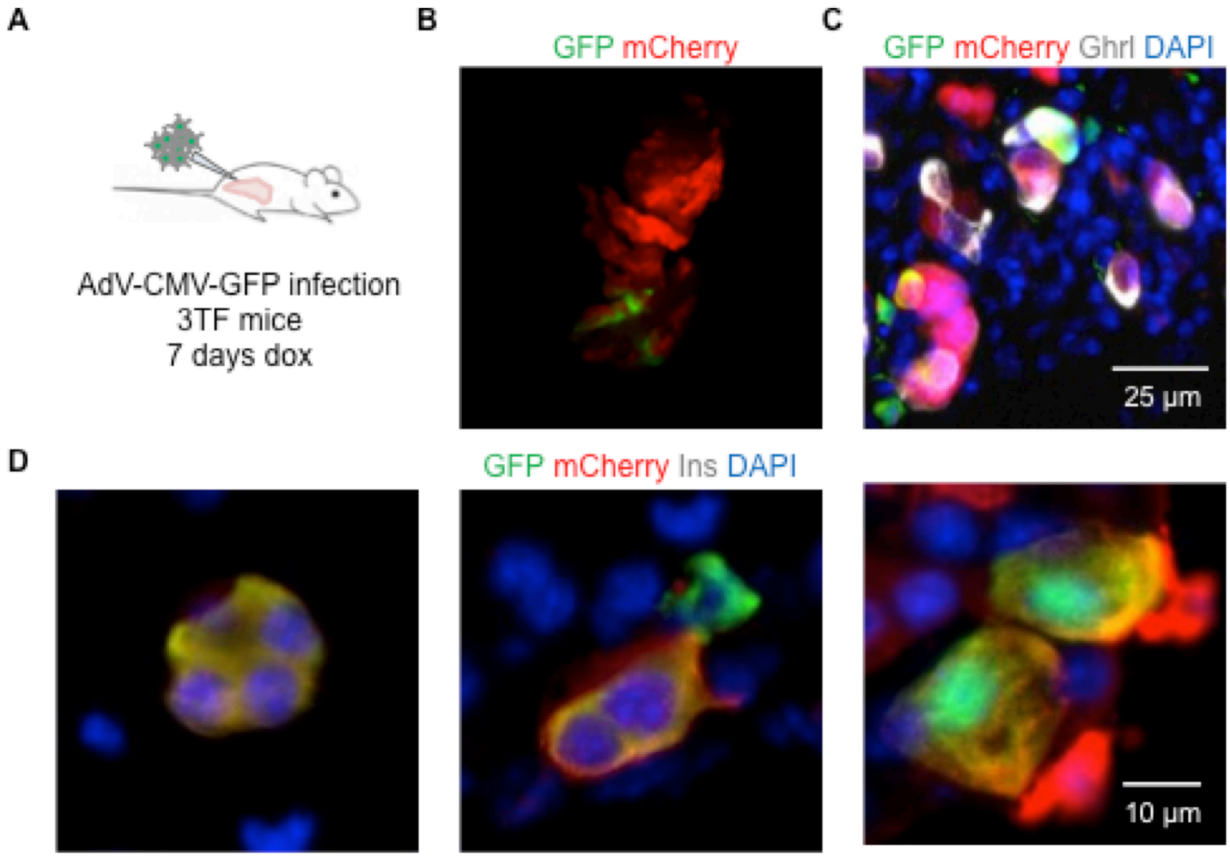


Figure 5.2 Rag1 deletion does not alter the outcome of reprogramming. (A) Representative PanCK stain and (B) Masson's trichrome stain of WT and 3TF. Rag1$^{-/-}$ mice at 7 days dox (2.0 mg/ml) treatment. (C) Pancreatic immunofluorescence ghrelin staining of 3TF. Rag1$^{-/-}$ mice at 7 days dox treatment. Multiple mCherry^{+} cells co-express ghrelin. (D) Pancreatic immunofluorescence insulin staining of 3TF. Rag1$^{-/-}$ mice at 7 days dox treatment. No mCherry^{+} cells that co-express insulin were observed despite examining over 1,000 mCherry^{+} cells.



GFP⁺mCherry⁺ cells	615
GFP⁺mCherry⁺insulin⁺ cells	0

Figure 5.3 Adenoviral infection is not required for 3TF-mediated A→β reprogramming. (A) Schematic to test if the adenoviral capsid or viral infection is required for 3TF-mediated A→β reprogramming. 6- 8 week old 3TF mice were injected with 100 µl of AdV-CMV-GFP ($1x10^9$ pfu) then administered dox (2.0 mg/ml) for 7 days. (B) Representative pancreas of 3TF mice infected with an AdV-CMV-GFP and administered 2.0 mg/ml of dox at 7 days. Successful pancreatic injection of the AdV-CMV-GFP is demonstrated by GFP fluorescence. (C) Pancreatic immunofluorescence ghrelin staining of 3TF mice at 7 days of dox treatment and AdV-CMV-GFP infection. Multiple $mCherry^+$ cells co-express ghrelin and the GFP-expressing adenovirus. (D) Pancreatic immunofluorescence insulin staining of 3TF mice at 7 days of dox treatment and AdV-CMV-GFP infection. No $mCherry^+$ cells that co-express insulin and GFP were observed despite examining over 600 $mCherry^+/GFP^+$ cells.

Discussion

Two differences between my experimental design compared to that of Zhou et al. was the use of a transgene to express 3TF, opposed to a viral vector, and the presence of *Rag1 (Zhou et al., 2008)*. Thus, it is possible that one or both of these experimental variables might account for the divergent reprogramming outcome observed. In some settings, viruses are required for successful reprogramming by activating host immune response pathways that ultimately result in epigenetic changes that improve the binding of the reprogramming factors to their targets (Lee et al., 2012). Adenoviral regulatory proteins, such as E4-ORF3, were also proposed to alter chromatin accessibility for reprogramming factors (Zaldumbide 2012). However, when I simultaneously expressed 3TF by dox administration and infected the pancreas of my biallelic mice with a GFP-expressing adenovirus, none of the 3TF-expressing acinar cells that had been infected with the adenovirus co-expressed insulin. While this experiment does not fully exclude a role of viral infection, it suggested that the lack of A→β reprogramming in the transgenic mouse model was due to some other factor when using 2.0 mg/ml dox to induce 3TF expression. Similarly, since T cells modulate innate immune responses and regulate macrophage polarization (Chan et al., 2011), I considered the possibility that the use of *Rag1*$^{-/-}$ mice by Zhou et al. might account for the differences in experimental outcome (Zhou et al., 2008). However, when I examined the effect of *Rag1* null background on the reprogramming outcome, despite the absence of T cells in the pancreas of dox-treated mice, I continued to observe ADM and the absence of A→β conversion. Thus, while I do not fully exclude the possibility that *Rag1* might influence reprogramming efficiency, this variable alone was unable to account for the divergent reprogramming outcome when I used 2.0 mg/ml dox to induce 3TF expression. This finding is consistent with a recent report showing that acinar cells take on a sustained duct-like appearance

thereby impairing pancreatic regeneration in *Rag1*$^{-/-}$ mice (Folias, Penaranda, Su, Bluestone, & Hebrok, 2014).

CHAPTER 6

ATTENUATING INFLAMMATION PROMOTES ACINAR TO BETA CELL REPROGRAMMING

Introduction

The original goal of this study was to explore how the combined expression of *MafA, Pdx1,* and *Neurog3* causes A→β reprogramming using a transgene-based system. However, I found that transgene-mediated expression of these three factors in pancreatic acinar, in marked contrast to prior studies using adenoviruses, did not result in the production of new, insulin-secreting cells but instead causes a potent inflammatory response and the generation of pancreatic duct-like cells. This observation led me to explore some of the variables that may differ between viral and transgene-based expression of 3TF. Indeed, widespread metaplastic changes resulting from tissue inflammation have not been reported when adenoviruses are used to introduce the reprogramming factors. I speculate that under normoglycemic conditions similar metaplastic conversions are not observed due to the lower infection efficiency of the adenovirus. This results in fewer 3TF-expressing cells, thereby lowering the overall inflammatory potential. In addition, adenoviral delivery may result in lower levels of 3TF-expression compared to transgene-mediated expression, which may also prevent acinar cell damage and pancreatic inflammation. Thus, I hypothesized that reprogramming-induced inflammation causes ADM thereby preventing A→β reprogramming. I used two approaches to attenuate inflammation. First, I lowered the level of dox, reasoning that the robust expression of 3TF was principally responsible for the inflammatory response. Second, I used the macrophage toxin gadolinium

chloride ($GdCl_3$) to attenuate inflammation during 3TF-medieated reprogramming. I found that successful transcription-mediated reprogramming of acinar cells into beta-like-cells can be disrupted by both inappropriate levels of the reprogramming factors and pancreatic inflammation. Too high factor expression causes pancreatic inflammation and ADM diverting the acinar cell to a duct-like cell while too low factor expression does not induce transcriptional changes. In this chapter, I discuss my studies investigating the role of inflammation on the outcome of A→β reprogramming.

Pancreatic inflammation and ADM

Damaged pancreatic acinar cells release pro-inflammatory signals that lead to macrophage infiltration and activation (Forsmark, 2013). These recruited macrophages can induce pancreatic acinar cells to either undergo apoptotic or necrotic cell death, or, if the cells survive, ADM (Liou & Storz, 2015). The precise mechanisms mediating ADM are still under investigation. Recently, it has also been shown that M1-polarized macrophages are responsible for initiating and driving ADM, and that this metaplastic conversion is mediated through macrophage-secreted cytokines such as tumor necrosis factor and chemokines such as CCL5/RANTES, which activate NFκB (Liou et al., 2013). It has also been shown that both the EGF receptor ligand TGF-α and activating Kras mutations promote ADM (De La et al., 2008; Means et al., 2005; Miyamoto et al., 2003). ADM has also been associated with the activation of both Notch and STAT3 signaling pathways (Corcoran et al., 2011; Liou et al., 2015). Indeed, both pathways are upregulated in PDAC, so it is not surprising that these pathways have been found to drive ADM. In addition, both pathways are important in establishing cell fates. Notch signaling regulates multiple cell fate decisions during pancreatic development and maintains a

pool of undifferentiated, progenitor-like cells (Murtaugh, Stanger, Kwan, & Melton, 2003) while STAT3 is important in regulating embryonic cell fate (H. Chen et al., 2015). Moreover, Notch signaling, by inducing expression of the downstream transcription factor *Hes1*, has been shown to promote duct cell over endocrine cell commitment during development (Shih et al., 2012). Furthermore, Protein Kinase D1 has been shown to act downstream of TGF-β and Kras and to induce Notch signaling (Liou et al., 2015) and thus suggested to be involved in mediating ADM.

Pancreatic macrophages

Macrophages are a highly versatile cell type with a diverse range of functions important for tissue homeostasis and injury responses. These diverse biological activities are mediated by the phenotypically distinct subpopulations of macrophages that develop in response to inflammatory mediators they encounter in their microenvironment (Murray & Wynn, 2011). Macrophage subsets differ in their receptor expression, effector function, and cytokine and chemokine production (Murray & Wynn, 2011). To date, the two major populations characterized are the so-called classically-activated M1 macrophages and the alternatively-activated M2 macrophages. M1 macrophages secrete pro-inflammatory cytokines and have antimicrobial functions whereas M2 macrophages are anti-inflammatory and contribute to tissue remodeling and repair (Sica & Mantovani, 2012).

Little is known about the specific role of activated macrophages in pancreatic inflammation except that these cells secrete potent immune system modulators that regulate tissue remodeling in order to maintain organ-wide homeostasis. However, their role during pancreas development is better defined. During development, $F4/80^{+}$ macrophages are found in the pancreas where they contribute to expansion of the endocrine cell mass with macrophage-

deficient mice displaying a reduced beta cell mass, abnormal postnatal islet morphogenesis, and impaired pancreatic cell proliferation (Banaei-Bouchareb et al., 2004). After birth, the number of F4/80^{+} macrophages decreases, with adult pancreatic resident macrophages characterized by a mixed macrophage profile with the majority being F4/80 negative (Geutskens, Otonkoski, Pulkkinen, Drexhage, & Leenen, 2005).

Pancreatic macrophages are best known for their pathogenic roles in the progression of T1 and T2 diabetes, and for this reason, are generally thought to be detrimental for pancreatic beta cell survival and function (Tesch, 2007; Yoon & Jun, 2005). However, pancreatic macrophages have also been shown to protect beta cells during pancreatic inflammation, and have been implicated in beta cell repair and regeneration in mouse models of pancreatitis (Brissova et al., 2014; Tessem et al., 2008; Van Gassen et al., 2015; Xiao et al., 2014)

Results

Reducing the level of 3TF expression lowers the coinciding immune response promoting A→β reprogramming

Given that pancreatic inflammation was associated with transgenic expression of 3TF, I explored the effect of varying 3TF expression by treating the diallelic mice with a 10- and 100-fold lower concentration of dox for 7 days. I reasoned that robust 3TF expression may be responsible for acinar cell damage and the subsequent ADM, thereby preventing successful A→β reprogramming. Both groups (mice treated with a 10- and 100-fold lower concentration of dox) exhibited lower levels of mCherry fluorescence, consistent with 3TF expression also being reduced (**Figure 6.1A, C**). I also observed that fewer acinar cells expressed mCherry as the

concentration of dox was lowered. Indeed, when I used a 10-fold lower concentration of dox to induced 3TF expression, there was over a 60% reduction in the number of acinar cells expressing 3TF after 1 day of dox administration ($78 \pm 2.5\%$ vs. $30 \pm 9.9\%$; n=3). In addition, after 7 days of dox treatment, the pancreas in both groups was nearly normal in size (**Figure 6.1A**), CD45 staining was reduced (**Figure 6.1B, D)**, and cytokeratin staining was either reduced or absent (**Figure 6.2A, B**). Most importantly, mice treated with the 10-fold lower concentration of dox (0.2 mg/ml) exhibited many $mCherry^+$ cells that co-expressed low levels of insulin (**Figure 6.2C, D**). On the other hand, mice given the lowest concentration of dox (0.02 mg/ml) had no $mCherry^+$/ $insulin^+$ co-expressing cells nor any $mCherry^+$ cells that co-expressed chromogranin A or ghrelin (**Figure 6.3A-D**). Instead, the $mCherry^+$ cells in these animals continued to express amylase at 7 days (**Figure 6.3E, F**), indicating that they had undergone very little, if any, A→β reprogramming within the one-week experimental timeframe. Taken together, these findings indicate that A→β reprogramming depends on the level of 3TF expression. When it is too low, no reprogramming occurs. Conversely, when it is too high, a potent inflammatory response occurs which diverts the reprogramming outcome to an ADM-like phenotype.

Macrophage depletion permits A→β reprogramming

I next sought to determine whether the inflammatory response itself might be responsible for the divergent reprogramming outcome. Since the majority of immune cells present after 7 days of 3TF expression were macrophages and since macrophages are involved in mediating ADM (Liou et al., 2013), I administered $GdCl_3$, a macrophage toxin (Jankov et al., 2001), both prior to and during dox treatment. The depletion of macrophages in the pancreas was confirmed by immunostaining for F4/80 (**Figure 6.4A, B**). Strikingly, the pancreata of mice treated with

$GdCl_3$ were nearly normal in both size and appearance (**Figure 6.4C, D**). Animals administered $GdCl_3$ did not exhibit either the formation of tubular complexes or the diffuse cytokeratin-staining that characterized the diallelic mice not administered $GdCl_3$ (**Figure 6.4E, F** and **6.5A,B)** nor did they exhibit fibrosis (**Figure 6.5C**). More importantly, approximately 6% of the $mCherry^+$ cells, after 7 days of dox treatment, were found to co-express insulin (**Figure 6.6A, B**). Cell counting after 7 days of treatment revealed approximately 650,000 new beta-like cells per animal (**Table 6.1**). Interestingly cell counting also revealed a decrease in the number of cells expressing mCherry at 7 days of dox.

To further confirm that macrophage depletion promotes 3TF-mediated A→β reprogramming, RT-qPCR was performed on FACS-sorted $mCherry^+$ cells. Indeed, both *Ins1* and *Ins2* mRNA were increased after 7 days of dox in mice administered $GdCl_3$ (**Figure 6.6C**). These findings suggest that the overly robust expression of 3TF triggers a potent inflammatory response, mediated by macrophages, that prevents A→β reprogramming.

To assess the function of the newly generated beta-like cells, mice were rendered diabetic by administration of the beta cell toxin streptozotocin (STZ), treated with $GdCl_3$ to attenuate inflammation, then robust 3TF expression was induced with 2.0 mg/ml dox. Within two days, the diabetic mice began to exhibit an improvement in blood glucose concentration, and at 6 days, their blood glucose concentrations were indistinguishable from untreated control animals (**Figure 6.7A**). Furthermore, by day 7 of dox, two mice died with low blood glucose levels (86 and 72 mg/dl). Removal of dox at day 7 was followed immediately by a worsening of glycemic control and reversion to a fully diabetic state within a few days. Interestingly, intraperitoneal glucose tolerance tests (GTT) performed after 7 days of dox treatment revealed two different patterns of response in the five mice treated. Two mice appeared to be glucose responsive

whereas the other three mice, which were hypoglycemic at the beginning of GTT, lacked glucose-sensitive insulin secrection (**Figure 6.7B**). In any case, these findings indicate a sufficient number of new beta-like cells were produced to rescue STZ-induced diabetes and that the production of insulin by these cells was dox-dependent. In addition, these results suggest the presence of at least two different groups of reprogrammed beta cells, one group that is glucose responsive and a second that may constitutively secrete insulin in a non-glucose responsive manner.

Finally, I sought to determine whether I could increase the efficiency of 3TF-mediated A→β reprogramming by simultaneously lowering the level of dox and reducing inflammation by depleting macrophages with $GdCl_3$. Interestingly, while the dual treatment preserved pancreatic mass and histology (**Figure 6.8A-C**), prevented abnormal cytokeratin staining (**Figure 6.8D-F**), and further decreased tissue inflammation (**Figure 6.8G-I**), it did not increase the reprogramming efficiency (**Figure 6.6 D, E**).

Table 6.1 Estimated number of new beta-like cells produced

Estimated number of new beta-like cells	
Cells in Pancreas [1]	57.5×10^6
$mCherry^+$ Cells*	10.9×10^6
New beta-like cells**	6.5×10^5

[1] See Ref (Dore et al., 1981)

*19% of the cells present in the pancreas after 7 days of dox treatment (2 mg/ml) are $mCherry^+$

** 6% of the $mCherry^+$ cells express insulin after 7 days of dox (2 mg/ml)

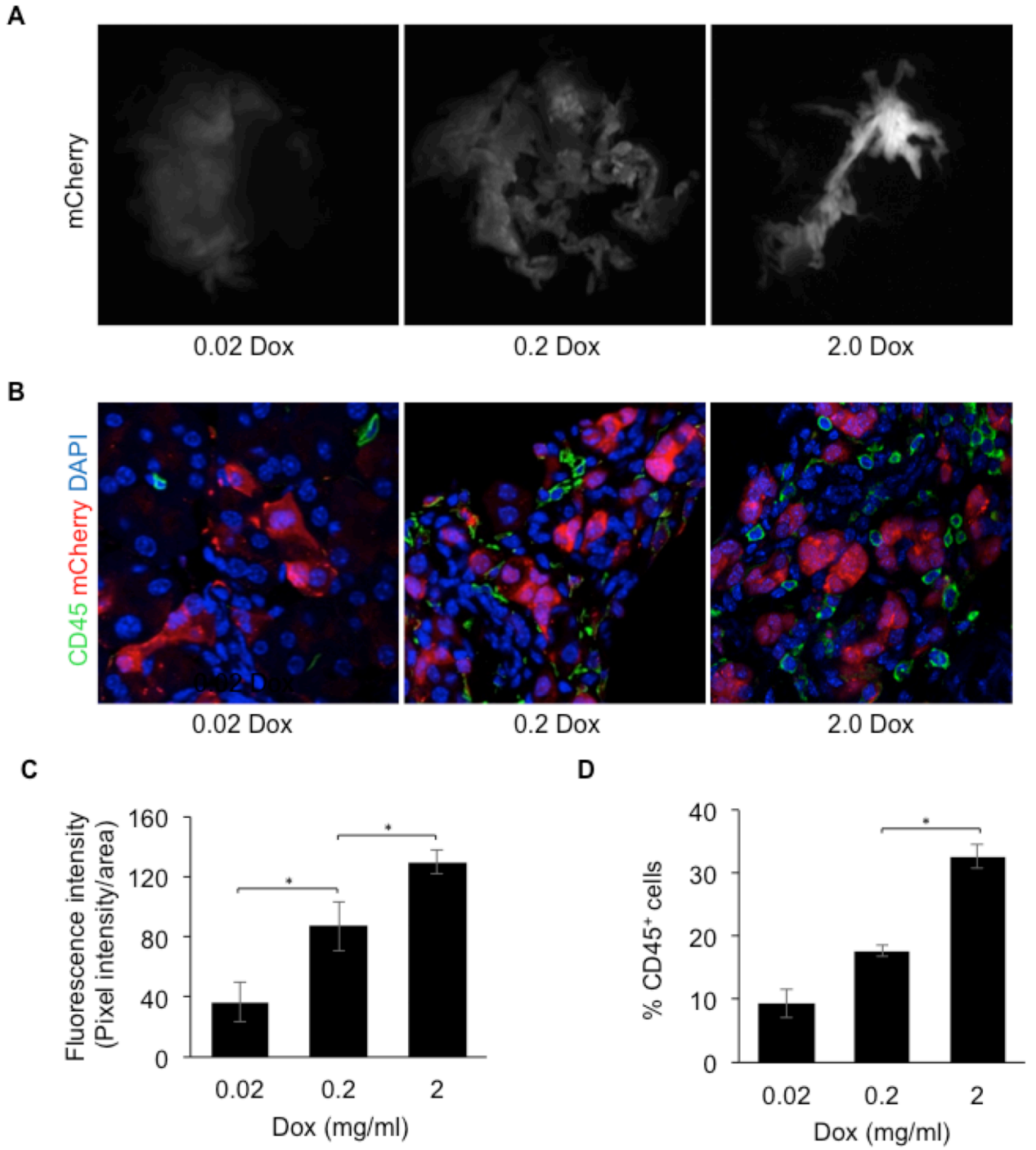


Figure 6.1 Reducing 3TF expression attenuates inflammation. (A) mCherry fluorescence in the pancreas of 3TF mice administered either 0.02, 0.2, or 2.0 mg/ml of dox for 7 days. (B) Pancreatic immunofluorescence CD45 staining of 3TF mice administered varying concentrations of dox at 7 days. (C) Quantification of fluorescence intensity per pancreas area. Data are represented as mean ± SEM. *$p < 0.05$, student's t-test. (D) Percentage of $CD45^+$ cells among $DAPI^+$ cells. Three mice per time point and over 1,000 $DAPI^+$ cells counted for each mouse. Data are represented as mean ± SEM. *$p < 0.05$, student's t-test

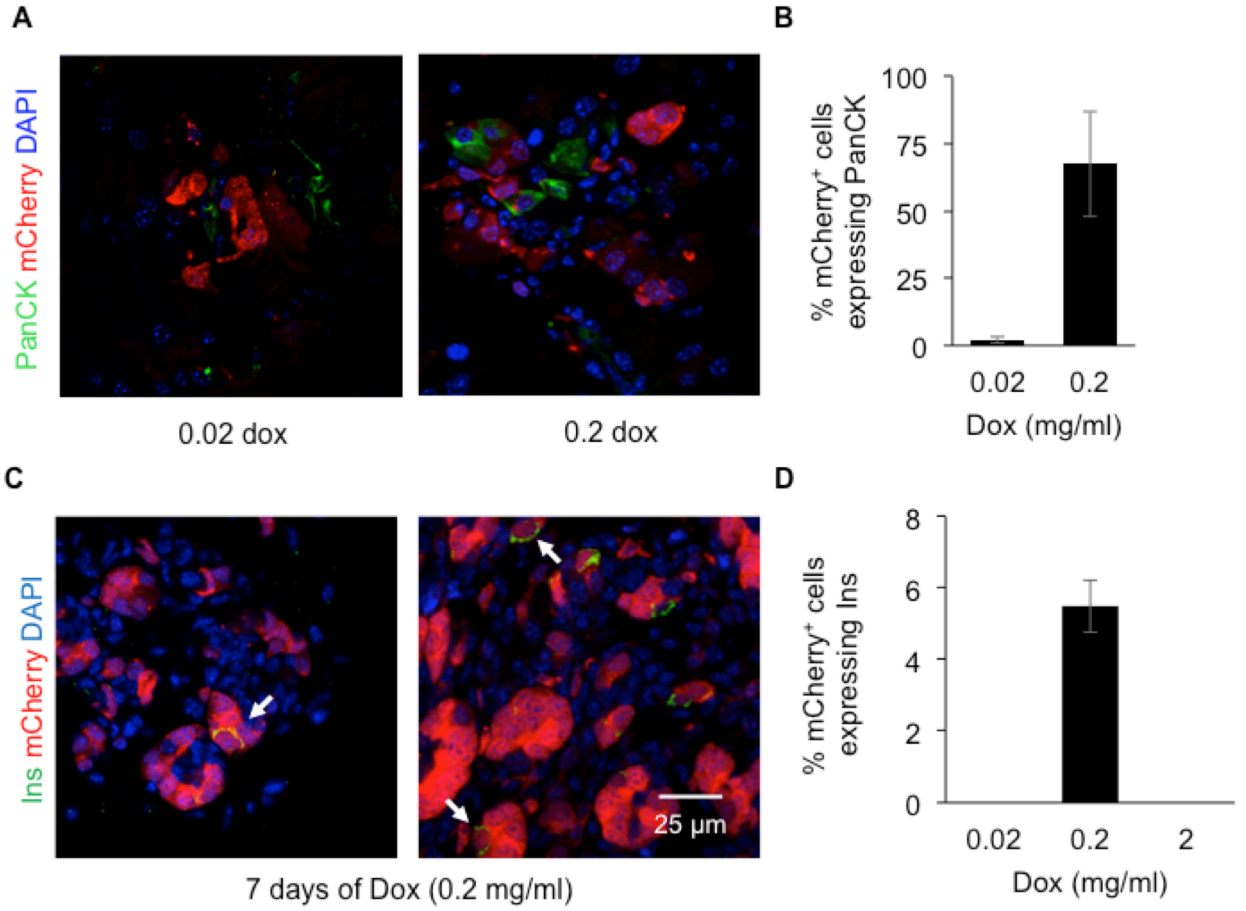


Figure 6.2 Reducing 3TF expression attenuates inflammation and promotes A→β reprogramming. (A) Immunostaining for PanCK in pancreata of 3TF mice administered varying concentrations of dox for 7 days. (B) Percentage of PanCK$^+$ cells among mCherry$^+$ cells. Three mice per time point and over 500 mCherry$^+$ cells counted for each mouse. Data are represented as mean ± SEM. (C) Pancreatic immunofluorescence insulin staining of 3TF mice administered 0.2 mg/ml of dox for 7 days. mCherry$^+$ cells that co-expressed insulin (arrows) were observed. (D) Percentage of insulin$^+$ cells among mCherry$^+$ cells. Three mice per time point and over 500 mCherry$^+$ cells counted for each mouse. Data are represented as mean ± SEM.

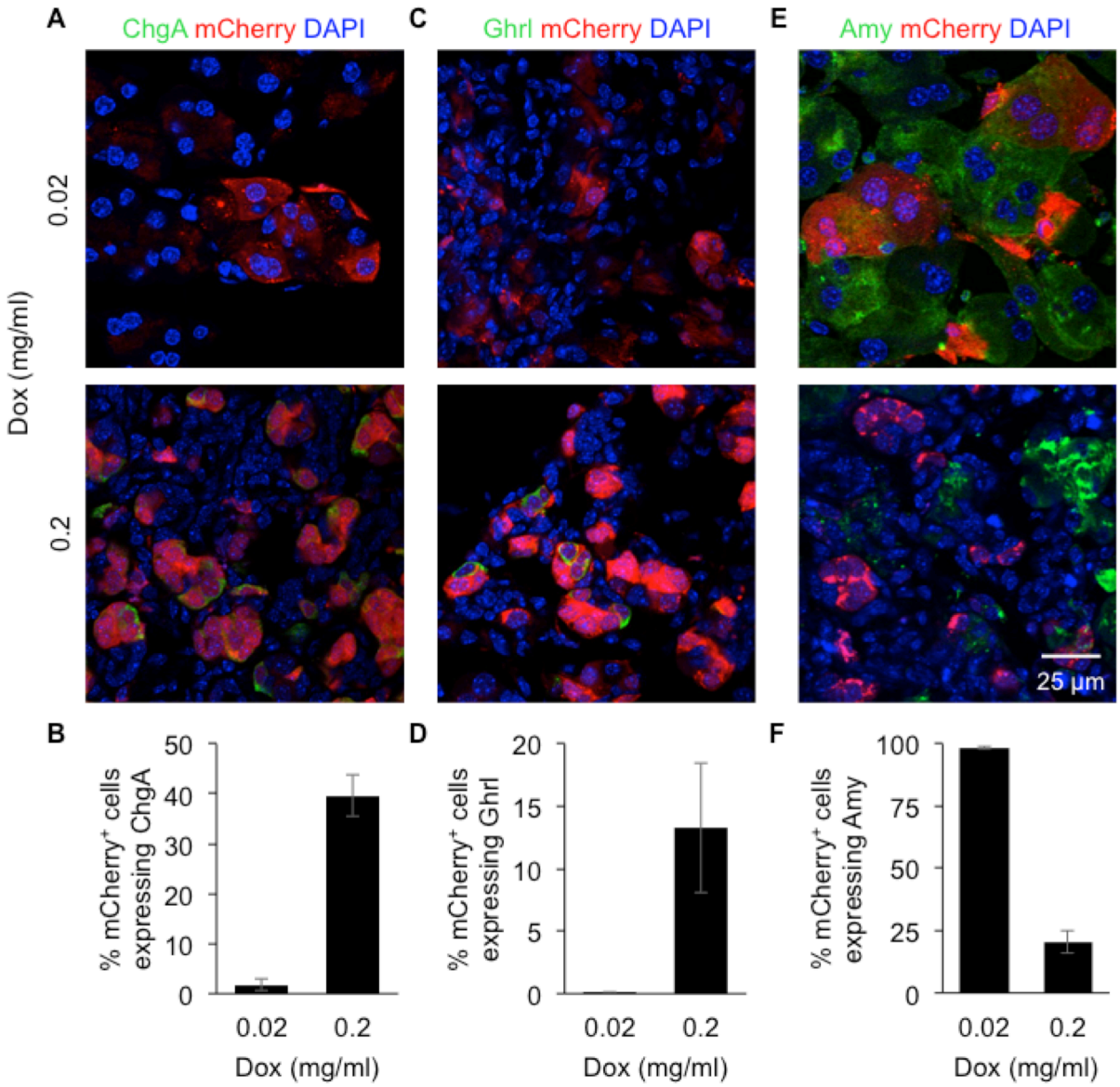


Figure 6.3 The magnitude of 3TF expression affects the reprogramming outcome. (A) Immunostaining for chromogranin A in pancreata of 3TF mice administered varying concentrations of dox for 7 days. (B) Percentage of chromogranin A^+ cells among $mCherry^+$ cells. Three mice per time point and over 500 $mCherry^+$ cells counted for each mouse. Data are represented as mean ± SEM. (C) Immunostaining for ghrelin in pancreata of 3TF mice administered varying concentrations of dox for 7 days. (D) Percentage of $ghrelin^+$ cells among $mCherry^+$ cells. Three mice per time point and over 500 $mCherry^+$ cells counted for each mouse. Data are represented as mean ± SEM. (E) Pancreatic immunofluorescence staining of amylase in pancreata of 3TF mice administered varying concentrations of dox for 7 days. (F) Percentage of $amylase^+$ cells among $mCherry^+$ cells. Three mice per time point and over 500 $mCherry^+$ cells counted for each mouse. Data are represented as mean ± SEM.

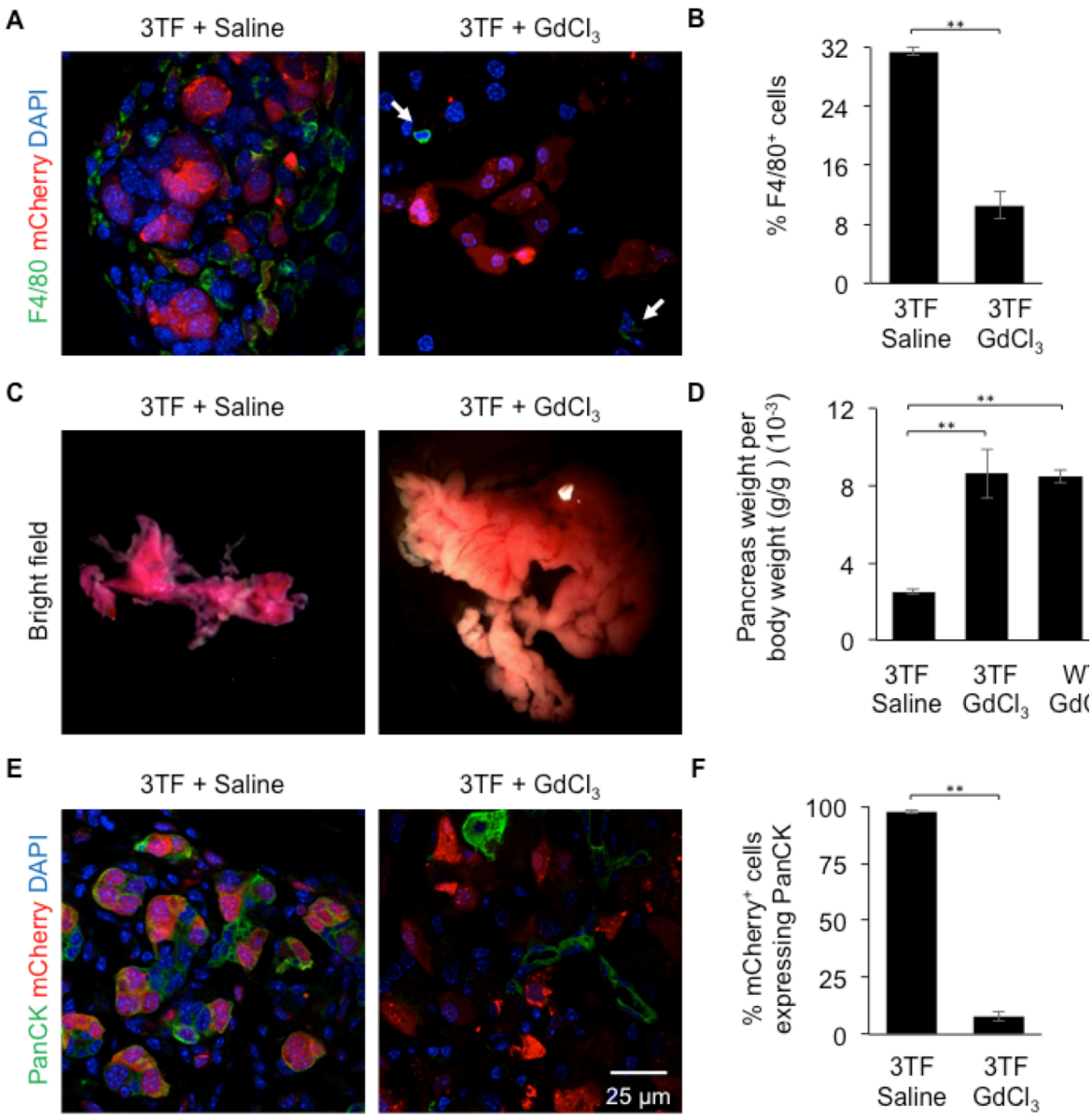


Figure 6.4 Macrophage depletion preserves pancreatic mass and architecture. (A) Pancreatic immunofluorescence F4/80 staining of 3TF mice administered either saline or $GdCl_3$ after 7 days dox. Arrows indicate $F4/80^+$ cells. (B) Percentage of $F4/80^+$ cells among $DAPI^+$ cells at 7 days dox. Three mice per time point and over 1,000 $DAPI^+$ cells counted for each mouse. Data are represented as mean ± SEM. **$p < 0.001$, student's t-test. (C) Representative pancreata from 3TF mice given either saline or $GdCl_3$ after 7 days dox. (D) Pancreatic weight per body weight of 3TF mice given either saline or $GdCl_3$ and WT mice given $GdCl_3$ after 7 days dox. Data are represented as mean ± SEM. **$p < 0.001$, student's t-test. (E) Pancreatic immunofluorescence staining of PanCK of 3TF mice administered either saline or $GdCl_3$ at 7 days dox. (F) Percentage of $PanCK^+$ cells among $mCherry^+$ cells at 7 days dox. Three mice per time point and over 500 $mCherry^+$ cells counted for each mouse. Data are represented as mean ± SEM. **$p < 0.001$, student's t-test.

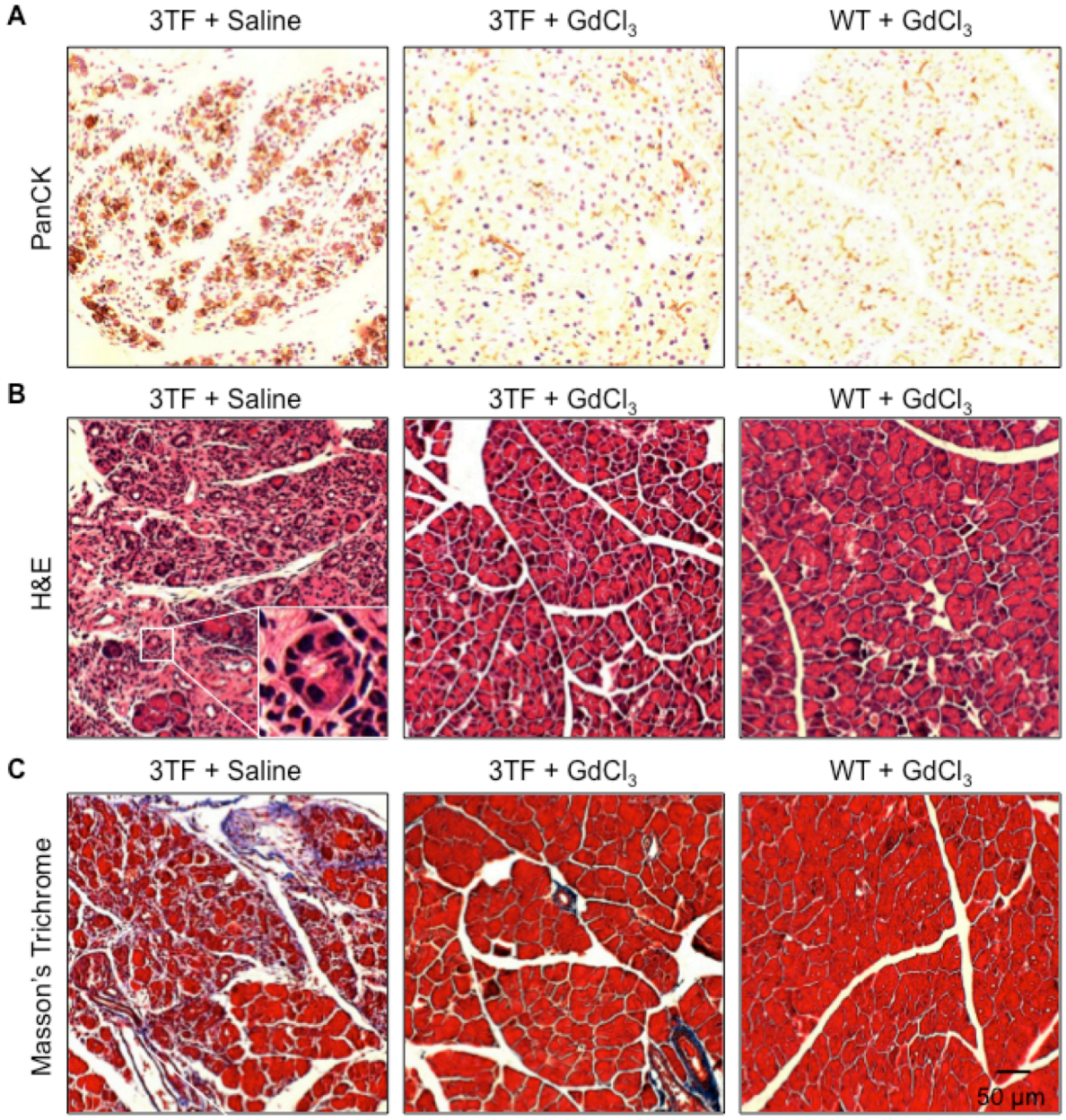


Figure 6.5 Macrophage depletion prevents ADM. (A) Representative pancreatic PanCK staining of 3TF mice administered either saline or $GdCl_3$ and WT mice administered $GdCl_3$ at 7 days dox. (B) Representative H&E staining of 3TF mice administered either saline or $GdCl_3$ and WT mice administered $GdCl_3$ at 7 days dox. Tubular complexes (inset) observed in pancreas of 3TF mice administered saline. (C) Representative Masson's trichrome stain of 3TF mice administered either saline or $GdCl_3$ and WT mice administered $GdCl_3$ at 7 days dox.

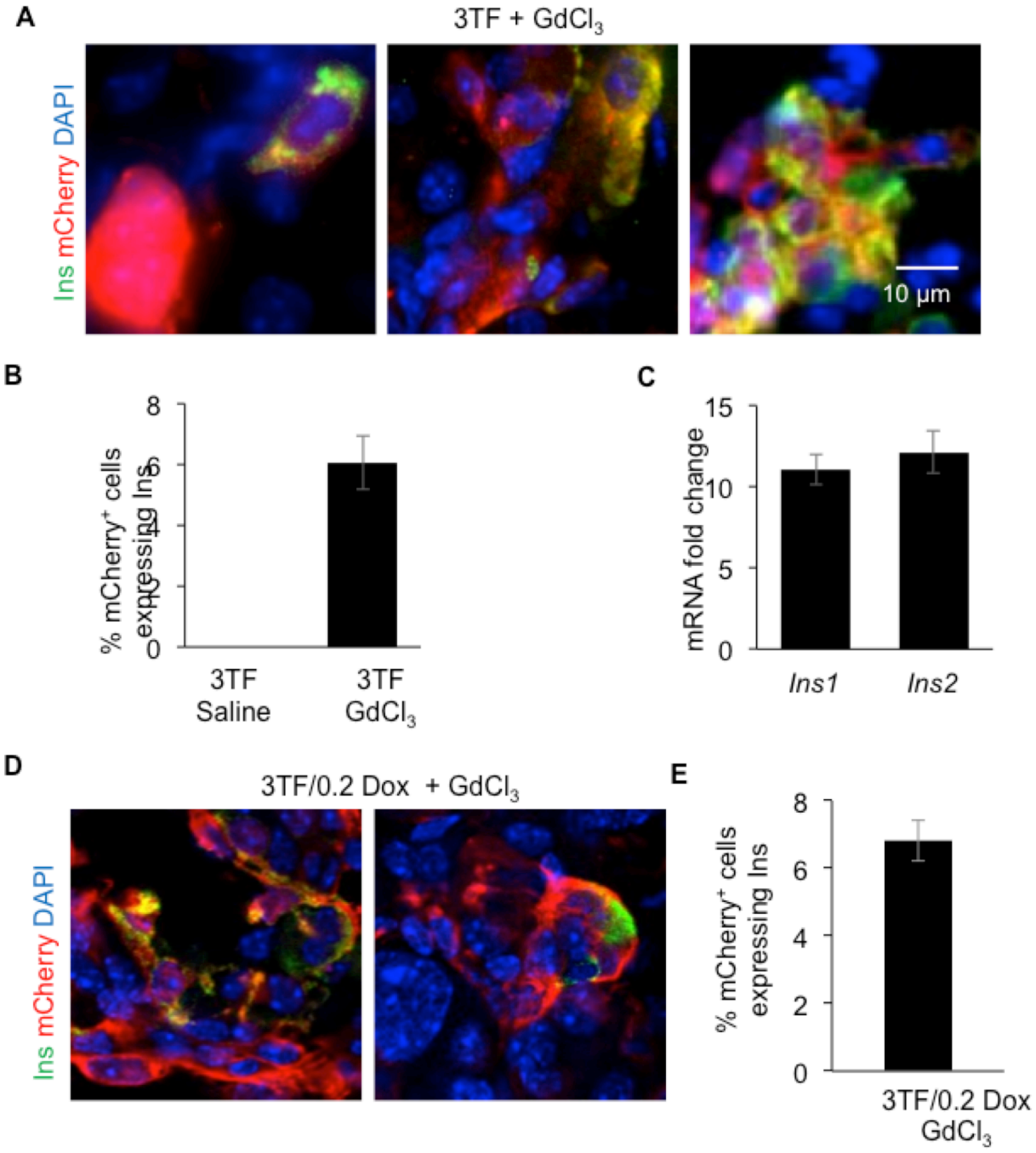


Figure 6.6 Macrophage depletion promotes A→β reprogramming. (A) Pancreatic immunofluorescence insulin staining of 3TF mice administered $GdCl_3$ at 7 days dox. (B) Percentage of Insulin$^+$ cells among mCherry$^+$ cells at 7 days of dox. Three mice per time point and over 1,000 mCherry$^+$ cells counted. Data are represented as mean ± SEM. (C) RT-qPCR analysis of *Ins1* and *Ins2* expression in 3TF mice given either $GdCl_3$ after 7 days of dox. Fold change calculated against mRNA expression in uninduced acinar cells. Data are represented as mean ± SEM. (D) After 7 days of dox, a few scattered insulin$^+$ cells that expressed mCherry were observed. (E) Percentage of insulin$^+$ cells among mCherry$^+$ cells. Three mice per time point and over 1,000 mCherry$^+$ cells counted. Data are represented as mean ± SEM.

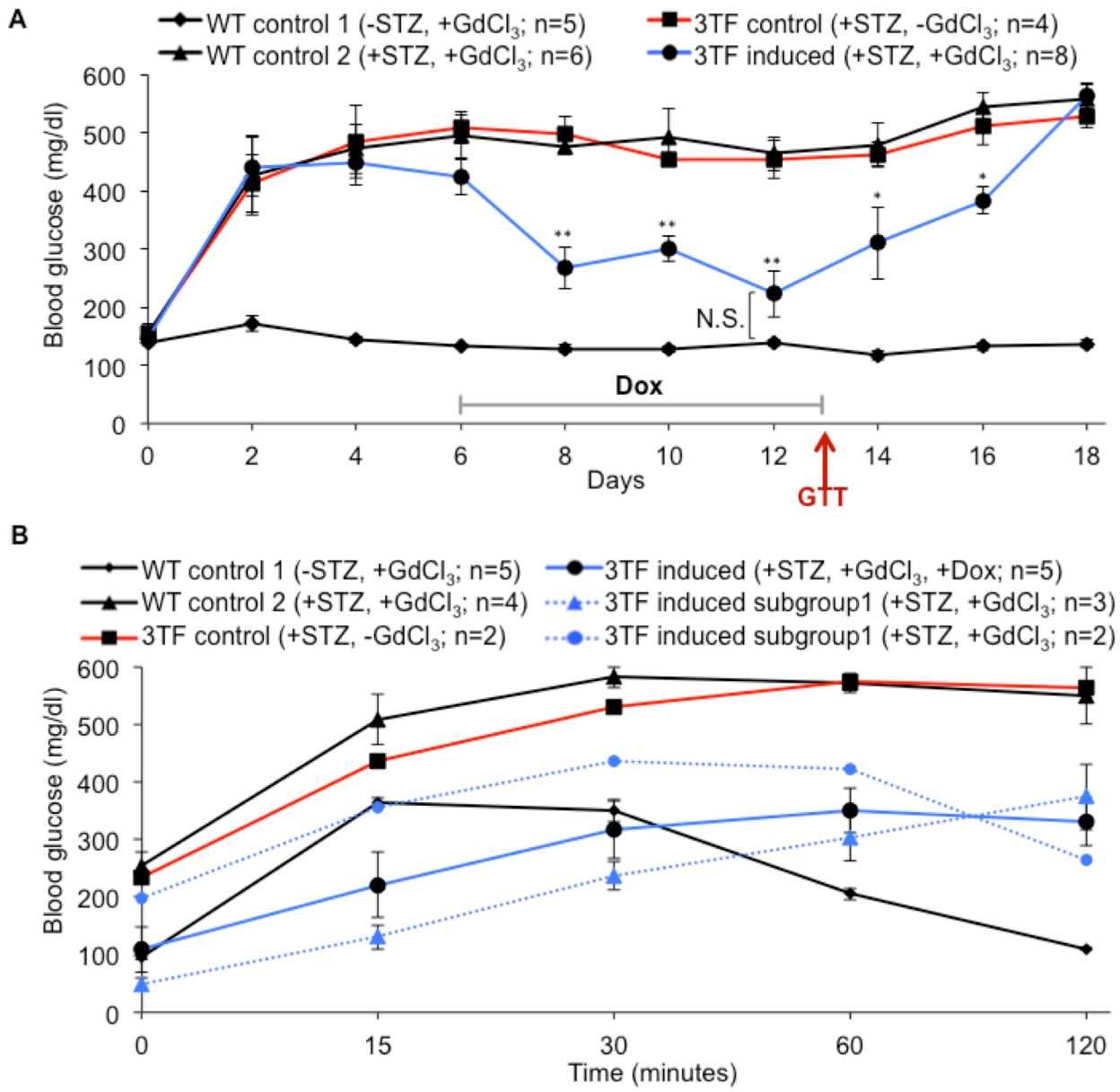


Figure 6.7 New beta cells rescue STZ-induced diabetes. (A) Mice were administered STZ on day 0 to induce diabetes and dox was administered for 7 days (day 6-13). Blood glucose was measured every other day. ● 3TF mice administered $GdCl_3$, STZ, and dox; ■ 3TF mice given STZ and dox but not administered $GdCl_3$; ▲WT mice administered $GdCl_3$, STZ, and dox (control); ♦ 3TF mice administered $GdCl_3$ and dox but not STZ (control). Significance calculated against 3TF mice not given $GdCl_3$. (B) GTT was performed on day 13. (C) Two groups of reprogrammed beta cells observed: glucose responsive (n=2) and non-glucose responsive (n=3).

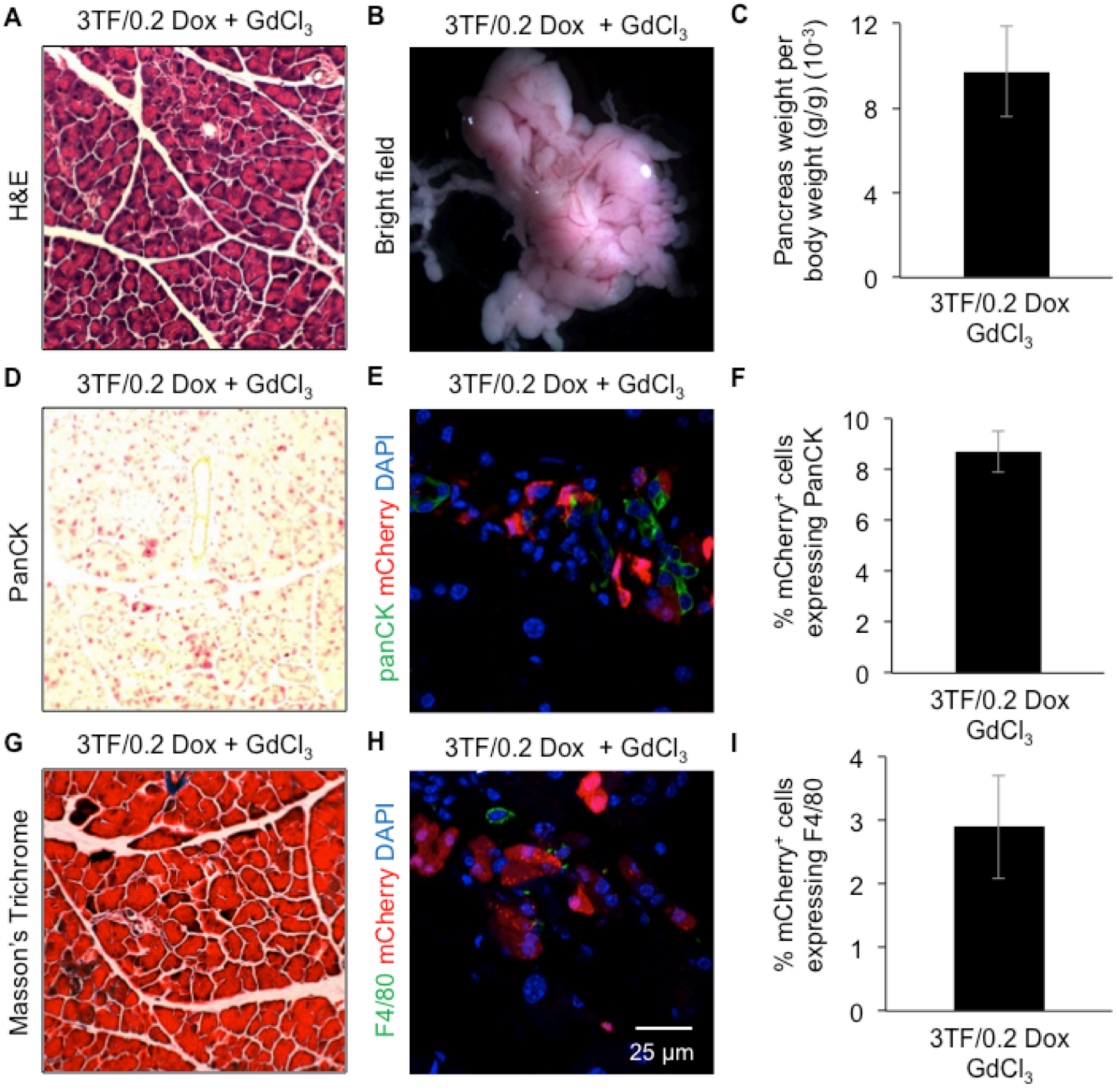


Figure 6.8. Simultaneously lowering the concentration of dox and depleting macrophages does not further increase A→β reprogramming. 3TF mice were simultaneously administered a low concentration of dox (0.2 mg/ml) and injected with the macrophage toxin $GdCl_3$ for 7 days. (A) Representative H&E staining and (B) pancreata from mice reveals that this dual treatment preserves pancreas histology and mass. (C) Pancreatic weight per body weight. Data are represented as mean ± SEM, n=3. (D) Pancreatic PanCK staining and (E) immunofluorescence staining of PanCK reveals that this dual treatment prevents ADM. (F) Percentage of $PanCK^+$ cells among $mCherry^+$ cells at 7 days of dox. Three mice per time point and over 500 $mCherry^+$ cells counted per mouse. Data are represented as mean ± SEM. (G) Representative Masson's trichrome stain and (H) immunofluorescence staining of F4/80 reveals that this dual treatment reduces inflammation. (I) Percentage of $F4/80^+$ cells among $DAPI^+$ cells. Three mice per time point and over 1,000 $DAPI^+$ cells counted. Data are represented as mean ± SEM.

Discussion

By analyzing a novel diallelic transgenic mouse model that expresses 3TF in both a pancreatic acinar- and dox-dependent manner, I found that the overly robust expression of 3TF in acinar cells does not result in new beta-like cells, but instead causes their conversion into duct-like cells. This finding led me to discover that the magnitude of transcription factor expression as well as tissue inflammation greatly affects the reprogramming outcome. Indeed, only when the expression of 3TF or the resulting inflammatory response was attenuated did I observe the production of new beta-like cells from pre-existing acinar cells.

My results suggest that when the concentration of 3TF is too high, pancreatic acinar cell stress and damage occurs which causes cytokine release, macrophage infiltration, and ADM, thereby preventing A→β reprogramming. I suggest that efficient A→β reprogramming requires a coordinated series of events whereby acinar cells cease zymogen production, delaminate and migrate to the surrounding mesenchyme, then cluster into new vascularized islets. For these many cellular changes to occur without inducing necrosis, acinar cells may need time to cease the production and secretion of tissue-digesting enzymes or time to establish a new endocrine cell-like state of internal homeostasis. The pace of such events may be directly influenced by the concentration of the reprogramming factors with high 3TF expression causing reprogramming to occur at a rate that exceeds the ability of the cell to undergo an orderly transition from one cell state (acinar) to another (endocrine), leading to cellular damage. Indeed, when I reduced the level of 3TF expression, not only did this diminish the inflammatory response but it also resulted in the production of new beta-like cells. Such a conclusion is consistent with previous studies that have shown the importance of factor level in achieving successful reprogramming (Carey et al., 2011; Tonge et al., 2014).

I also found that inflammation, in particular macrophages, plays a major role in determining the success of 3TF-mediated A→β reprogramming. By using $GdCl_3$ to deplete macrophages, I was able to blunt the inflammation induced by 3TF expression and prevent ADM, thereby enabling the acinar cells to be reprogrammed into beta-like cells. However, the mechanisms whereby macrophages are able to block A→β reprogramming are unclear. It has been reported that macrophage-secreted cytokines mediate ADM through activation of NFκB and STAT3 (Liou et al., 2013). Thus, it is possible that NFκB or STAT3 alter signaling pathways that are necessary for A→β reprogramming. Given that STAT3 signaling was recently shown to be required for cytokine-mediated A→β conversion (Baeyens et al., 2013), it is possible that NFκB signaling plays a very different role, inhibiting A→β reprogramming by the induction of ADM. Inspection of my RNA-seq data revealed an increase in many NFκB targets after 7 days of robust 3TF-transgene expression (data not shown), further supporting the role of NFκB signaling in mediating AMD and thereby enabling successful A→β reprogramming. Not only is NFκB implicated in ADM (Liou et al., 2013), it is also markedly increased in the early pathogenesis of pancreatitis and pharmacological inhibition of NFκB results in an amelioration of the disease. Indeed, activated NFκB induces the transcription of many genes involved in inflammatory and apoptotic responses including cytokines, chemokines, immune receptors, and adhesion molecules (H. Huang et al., 2013).

Furthermore, I estimate that approximately 650,000 new beta-like cells are produced, on average, in response to administration of 2.0 mg/ml dox and $GdCl_3$. While this number of cells is about a third of the approximately 2 million beta cells in an average mouse pancreas (Dor et al., 2004), it is sufficient to transiently rescue STZ-induced diabetes. However, the ability of these new beta-like cells to stably secrete insulin in a glucose-dependent manner is not established

after only 6 days of 3TF treatment since the removal of dox at 7 days caused a quick reversion to a diabetic state. These findings are consistent with prior studies that used adenoviral delivery of the same three transcription factors in which two months were required for the reprogrammed acinar cells to adopt a DNA methylation and transcriptional profile similar to that of endogenous beta cells (W. Li, C. Cavelti-Weder, et al., 2014). Thus, my findings support the notion that an extended exposure to the three reprogramming factors is necessary for acinar cells to adopt both the epigenetic and transcriptional profile of an endogenous beta cell.

CHAPTER 7

NOVEL MOUSE MODELS FOR THE TETRACYCLINE-DEPENDENT EXPRESSION OF *PDX1* AND *NEUROG3*

Introduction

In 2008, Zhou *et al.* reported that forced expression of *Pdx1, Neurog3*, and *MafA,* using adenoviruses, converts pancreatic acinar cells into new beta-like cells (Zhou et al., 2008). In order to determine the specific roles of *Neurog3* and *Pdx1* in the reprogramming process, the Magnuson lab developed tetracycline-inducible mouse models in which the expression of the single factors could be specifically induced in pancreatic acinar cells. A recent study, using adenoviruses, has begun to tease out the specific role of each of the three factors during A→β reprogramming. This study showed that co-infection of mouse pancreas with two different adenoviruses carrying *Neurog3* and *MafA* converts pancreatic acinar cells into both glucagon- and somatostatin-expressing cells and that infection of mouse pancreas with *Neurog3* alone converts acinar cells into only somatostatin-expressing cells. Surprisingly, *Mafa* or *Pdx1* alone did not induce the formation of any hormone positive cells. These studies suggested that *Neurog3* is responsible for establishing the genetic endocrine state in acinar cells at the onset of reprogramming by suppressing acinar fate-regulators and activating endocrine genes and that *Mafa* and *Neurog3* work together to suppress delta cell-specification ensuring the formation of beta-like cells (W. Li, M. Nakanishi, et al., 2014). Preliminary studies utilizing my mouse models have shown that transgene-mediated expression of *Neurog3* causes pancreatic inflammation and, similarly, transgene-mediated expression of *Pdx1* also results in pancreatic

inflammation, a finding that corroborates a report by Miyatsuka et al. showing that transgene-mediated, expression of *Pdx1,* in pancreatic cells, causes pancreatic inflammation and ADM (Miyatsuka et al., 2006).

In this chapter, I discuss the derivation and preliminary validation of mouse models that express either *Neurog3* or *Pdx1* and my preliminary findings using these mouse models.

The reprogramming factors

Pdx1, Neurog3, and *MafA*, which in combination reprogram acinar cells into new beta-like cells (Zhou et al., 2008), all play critical roles in beta cell development and function. *Pdx1*, a homeodomain transcription factor, is required for the embryonic development of the pancreas and beta cell function with homozygous deletion of *Pdx1* resulting in pancreatic agenesis (Offield et al., 1996). *Pdx1* is first detected at embryonic day 8.5 in dorsal and ventral buds of the foregut endoderm (Guz et al., 1995). The *Pdx1*-expressing epithelium gives rise to all three lineages of the pancreas (acinar, endocrine and duct) (Gu et al., 2003). *Pdx1* is required both for the primary transition of the pancreas, a phase involving the branching and expansion of the developing pancreatic epithelium, and the secondary transition, a period of rapid exocrine and endocrine differentiation (Hale et al., 2005). Its expression eventually becomes restricted to beta cells where it plays a key functional role. Although *Pdx1* haploinsufficient mice develop normally, beta cell function is compromised resulting in impaired glucose tolerance and glucose-stimulated insulin secretion (Brissova et al., 2002). Indeed, *Pdx1* activates insulin as well as many genes involved in insulin biosynthesis and secretion (Khoo et al., 2012).

Neurog3, a basic helix-loop-helix transcription factor, is required for *Pdx1*-expressing pancreatic MPCs to enter the endocrine lineage and *Neurog3*$^{+}$ cells contribute to all endocrine

cells of the mature pancreas (Gradwohl et al., 2000; Gu et al., 2002). Mice carrying a targeted disruption of *Neurog3* fail to generate pancreatic endocrine cells and die 1–3 days postnatally (Gradwohl et al., 2000). Furthermore, forced expression of *Neurog3* is sufficient to drive precursor cells to an endocrine fate (Apelqvist et al., 1999). In addition, direct targets of *Neurog3* include genes critical for endocrine differentiation such as *NeuroD1, Pax4, Insm1*,and *Nkx2.2* (H. P. Huang et al., 2000; Smith et al., 2003; Watada et al., 2003).

While *MafA* has no known role in beta cell development, it is vital for beta cell maturation and function. Beta cell development is unaffected in *MafA*-deficient mice. However, as these mice age, they display glucose intolerance and develop diabetes (C. Zhang et al., 2005). Furthermore, *MafA* is crucial for the functional maturation process as nascent beta cells acquire glucose-responsive insulin secretion (Aguayo-Mazzucato et al., 2011). Indeed, direct targets of *MafA* include genes critical for beta cell function, including glucose sensing, vesicle maturation, and Ca^{2+} signaling (H. Wang et al., 2007).

Results

Design and validation of mouse alleles

To investigate the specifics role of two individual factors, *Neurog3* and *Pdx1,* in A→β reprogramming, the Magnuson lab developed two alleles (*Rosa26*$^{Neurog3.CFP}$ and *Rosa26*$^{Pdx1.YPF}$) that after interbreeding with Ptf1a^{rtTA} allow for the tetracycline-dependent expression of either *Neurog3* or *Pdx1* in pancreatic acinar cells (**Figure 7.1A**). When adult mice containing both the *Ptf1a*^{rtTA} and *Rosa26*$^{Neurog3.CFP}$ alleles were given 2.0 mg/ml dox in their drinking water for 1 day, CFP was observed in the pancreas but no other visceral organs, and CFP was observed only in the pancreatic acinar cells (**Figure 7.2A**). Similarly, treatment of

Ptf1a$^{rtTA/+}$; *Rosa26*$^{Pdx1.YFP/+}$ mice with 2.0 mg/ml dox for 1 day resulted in the expression of YFP in the pancreas but no other visceral organs, and YFP was observed only in the pancreatic acinar cells (**Figure 7.2B**).

In order to determine the transcriptional changes associated with single factor overexpression, RNA-seq was performed on FACS-purified CFP^{+} cells from *Ptf1a*$^{rtTA/+}$;*Rosa26*$^{Neurog3.CFP/+}$ mice and FACS-purified YFP^{+} cells from *Ptf1a*$^{rtTA/+}$;*Rosa26*$^{Pdx1.YFP/+}$ mice after 1 and 7 days of dox. Furthermore, to determine if the overexpression of either *Neurog3* or *Pdx1* causes pancreatic inflammation, as is seen with 3TF overexpression, I stained pancreata for inflammatory cells using CD45, a pan-leukocyte marker. Interestingly, I observed a potent immune infiltration in response to either *Neurog3* or *Pdx1* overexpression (**Figure 7.3).**

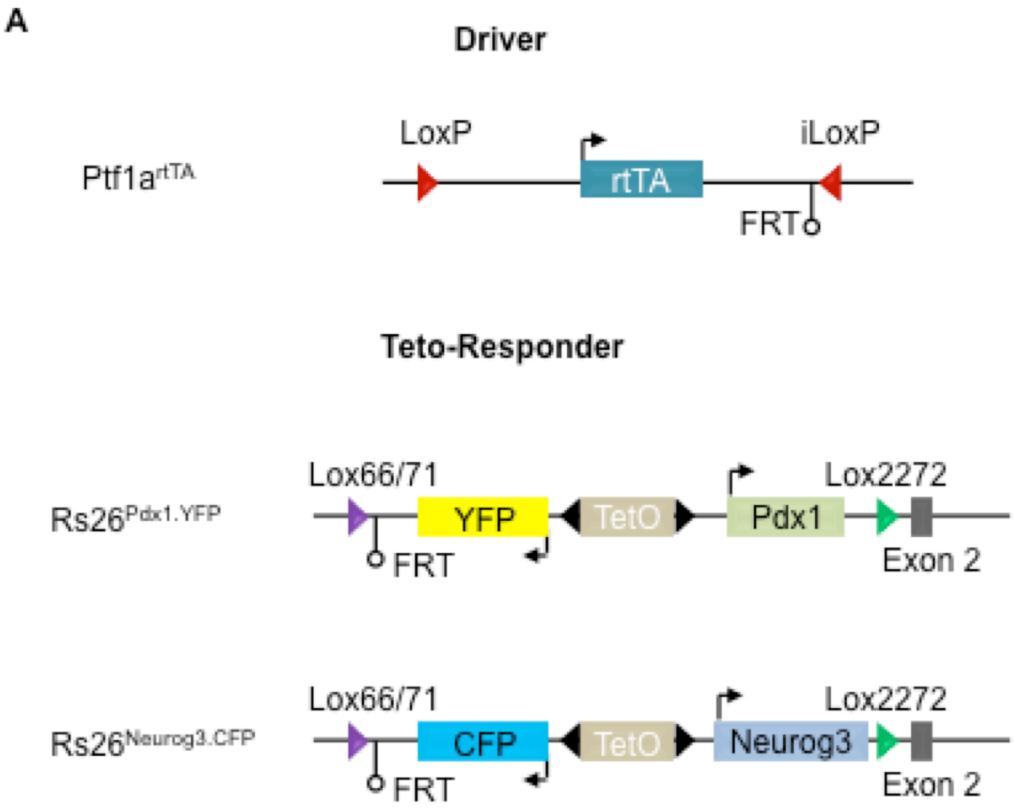


Figure 7.1 Design of mouse alleles for the dox-dependent expression of either *Neurog3* or *Pdx1* in pancreatic acinar cells. (A) *Rosa26*$^{Neurog3.CFP}$ and *Rosa26*$^{Pdx1.YFP}$alleles were generated by recombinase-mediated cassette exchange. When either allele is interbred with *Ptf1a*^{rtTA}, the two alleles resulted in dox-inducible expression of either *Neurog3* or *Pdx1* in a pancreatic acinar cell-specific manner.

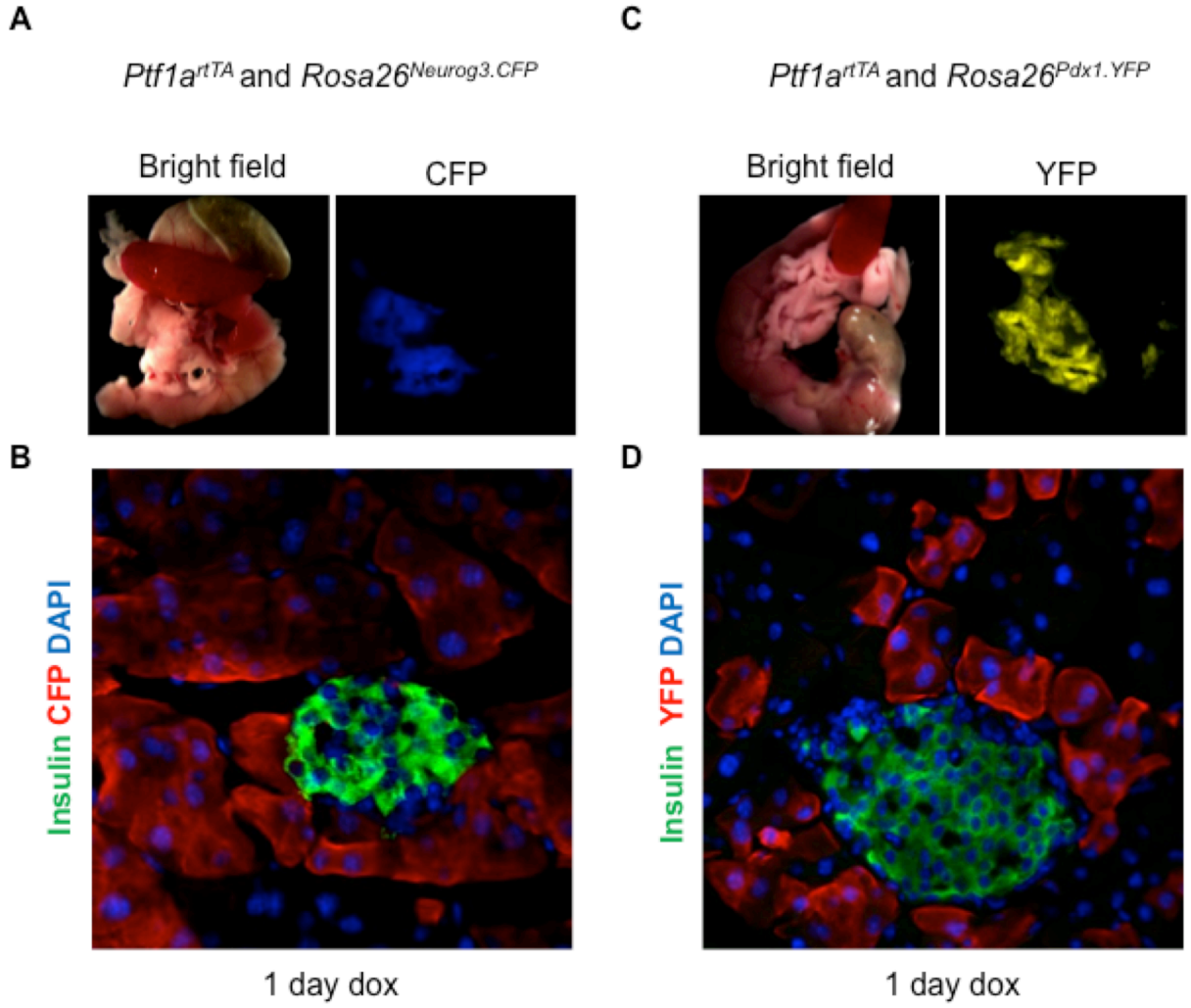


Figure 7.2 Validation of mouse alleles for the dox-dependent expression of either *Neurog3* or *Pdx1* in pancreatic acinar cells. (A) CFP expression was visible after administering 2.0 mg/ml of dox for 1 day (n=5) to *Ptf1a*^{rtTA}; *Rosa26*$^{Neurog3.CFP}$ mice. CFP fluorescence was restricted to the pancreas of dox treated mice and was not observed in other tissues. (B) Dox-inducible, acinar cell-specific expression of CFP was confirmed with immunofluorescence analysis. Pancreas sections stained with insulin and CFP showed that the two proteins were not co-localized. (C) YFP expression was visible after administering 2.0 mg/ml of dox for 1 day (n=4) to *Ptf1a*^{rtTA}; *Rosa26*$^{Pdx1.YFP}$ mice. YYFP fluorescence was restricted to the pancreas of dox treated mice and was not observed in other tissues. (B) Dox-inducible, acinar cell-specific expression of YFP was confirmed with immunofluorescence analysis. Pancreas sections stained with insulin and YFP showed that the two proteins were not co-localized.

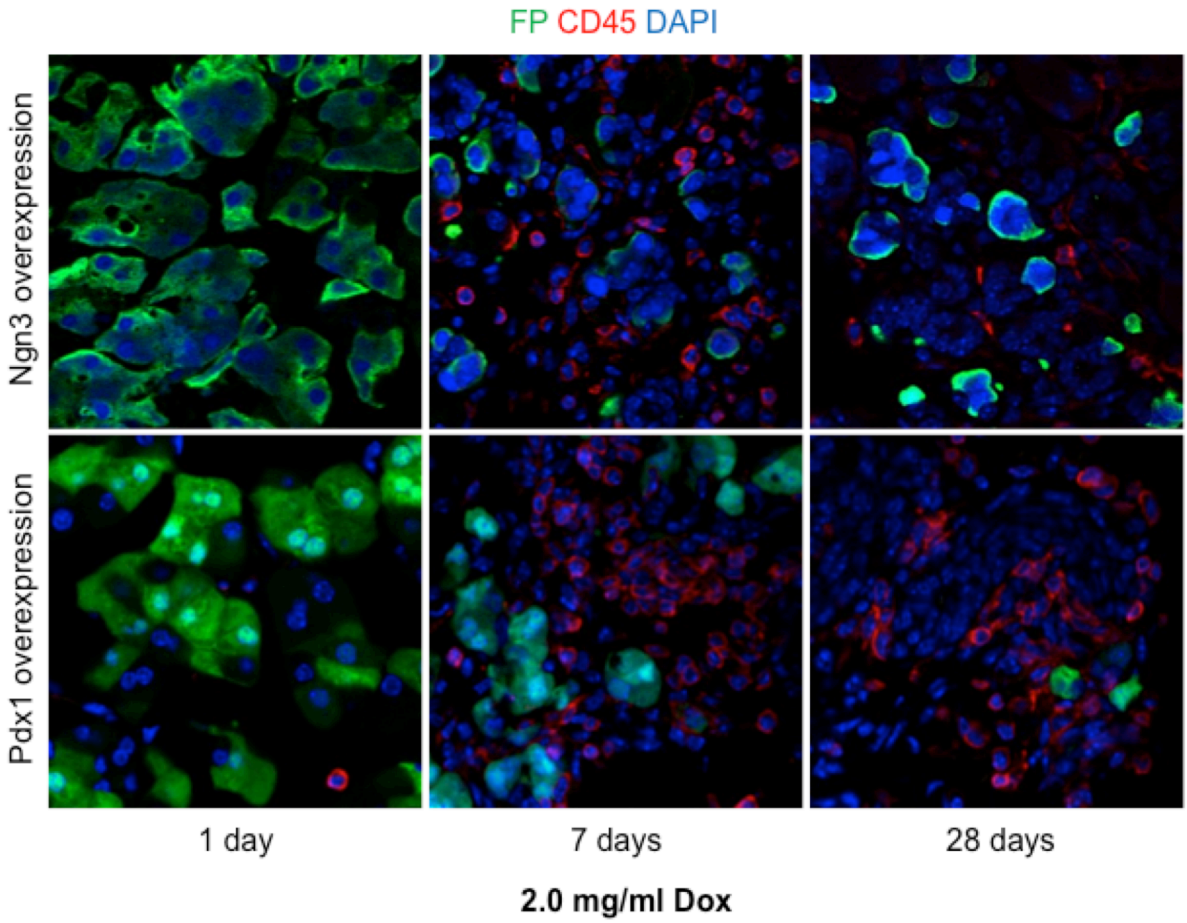


Figure 7.3 Single factor overexpression causes pancreatic inflammation. Pancreatic immunofluorescence staining of CD45, fluorescent protein (FP), and DAPI from mice that overexpress either *Neurog3* (Ngn3) or *Pdx1* in the pancreatic acinar cells after 1, 7, and 28 days of dox.

Discussion

In order to determine the specific roles of *Neurog3* and *Pdx1* in the 3TF-mediated reprogramming of acinar cells into insulin-secreting beta-like cells, the Magnuson lab developed tetracycline-inducible mouse models in which the expression of the single factors could be expressed specifically in pancreatic acinar cells. While adenoviral-mediated delivery of *Neurog3* has been shown to convert acinar cells into somatostatin-expressing cells and adenoviral-mediated delivery of *Pdx1* did not induce the formation of any hormone positive cells (W. Li, M. Nakanishi, et al., 2014), my studies showed that transgene-mediated delivery of either *Neurog3* or *Pdx1* resulted in pancreatic inflammation and ADM.

My previous findings, discussed in Chapter 4, showed that high levels of 3TF-transgene expression induced by 2.0 mg/ml of dox causes acinar cell necrosis resulting in marked inflammation and ADM. Only when inflammation is attenuated, either by reducing the intensity of 3TF expression or by depleting macrophages, did the production of new beta-like cells occur. Thus, it is possible that the high levels of either *Pdx1* or *Neurog3* expression alone also cause acinar cell stress and necrosis, which triggers a potent inflammatory response and ADM thereby masking their reprogramming potential. To truly investigate the role of the single factors, it may also be necessary to attenuate the inflammatory response.

Nonetheless, inflammation of the pancreas confers signifigant risk for PDAC (Siegel et al., 2013). However, the signaling mechanisms underlying ADM are largely undefined. My mouse models, which all result in a potent inflammatory response and ADM, could potentially allow us to elucidate the mechanisms underlying ADM.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Diabetes is a global epidemic affecting over 350 million individuals worldwide. Among the two most prevalent types, T1D is characterized by the autoimmune destruction of beta cells and T2D arises from either insufficient insulin synthesis or insulin resistance leading to hyperglycemia. Currently the most effective treatment for T1D has been lifelong insulin therapy. While, transplantation of whole pancreas and isolated beta cells have proven effective (Shapiro et al., 2006) the shortage of cadaver pancreases, have lent strong motivation to the search for new sources of beta cells. A promising strategy to produce beta cells is the reprogramming of other terminally differentiated cells into beta cells (Bonner-Weir & Weir, 2005; Pagliuca & Melton, 2013).

Studies over the past decade have established that the identity of pancreatic cells is much less firmly fixed than once thought, and that cells within this organ can undergo specific fate conversions in response to pancreatic ductal ligation (Pan et al., 2013), cytokine treatment (Baeyens et al., 2013), severe hyperglycemia (Thorel et al., 2010), and expression of transcription factors (Zhou et al., 2008). These discoveries have stimulated interest in learning more about specific reprogramming events in order to understand their biological nature, and to be able to exploit these natural processes to generate new pancreatic beta-like cells from other non-beta cell types. Such knowledge, if adequately understood and developed, may enable the physiological regeneration of beta cells in patients with T1D (Bramswig et al., 2013; W. Li, M. Nakanishi, et al., 2014; Thorel et al., 2010; Zhou et al., 2008).

Of the various pancreatic cell types that might be targeted for reprogramming, acinar cells are especially appealing since they are highly abundant, exhibit transcriptional plasticity (W. Li, M. Nakanishi, et al., 2014; Puri et al., 2015; Ziv et al., 2013), and are derived from a common progenitor cell during pancreatic organogenesis (Gu et al., 2002). Two different strategies for the *in vivo* reprogramming of acinar cells into beta-like cells were recently described. In the first, Zhou et al. used adenoviruses that express three pancreas-specific transcription factors (*MafA, Pdx1, and Neurog3)* to convert pancreatic acinar cells into new insulin-secreting cells (Zhou et al., 2008). In the second, Baeyens et al. showed that transiently administered epidermal growth factor and ciliary neurotrophic factor promoted the conversion of pancreatic acinar cells into new beta-like cells (Baeyens et al., 2013). However, the precise mechanisms enabling or limiting this cellular conversion are not understood. Indeed, the factors and experimental variables that promote, limit, or modify cellular reprogramming *in vivo* remain largely to be defined. In the case of transcription factor mediated reprogramming, some of the variables may include the use of a virus (A. Y. Wang et al., 2007), concentration and stoichiometry of the transcription factors (Carey et al., 2011), the presence or absence of an inflammatory response (Lee et al., 2012), and hyperglycemia (Cao, Tang, Horb, Li, & Yang, 2004). Thus, for an *in vivo* beta cell restorative therapy to ever become clinically feasible, we need a better understanding of the factors that promote or inhibit specific cellular identity conversions, and the physiological effects that inducing such conversions might inevitably cause.

Using a novel mouse model that expresses 3TF in both a pancreatic acinar cell- and dox-dependent manner, my thesis work has been focused on studying the factors that modulate acinar to beta cell conversion and the physiological effects that such conversion induces. I have discovered that the outcome of transcription factor-mediated acinar to beta-like cellular

reprogramming is dependent on both the magnitude of 3TF expression and on reprogramming-induced inflammation. My findings indicate that the overly robust expression of 3TF in acinar cells induces pancreatic inflammation which blocks A→β reprogramming and results, instead, in the production of new duct-like cells (**Figure 8.1**). Only when inflammation is attenuated, either by reducing the intensity of 3TF expression or by depleting macrophages, does the production of new beta-like cells occur. The new beta-like cells were able to reverse streptozotocin-induced diabetes 6 days after inducing 3TF expression but failed to sustain their function after removal of the reprogramming factors.

Thus, I propose that when the concentration of 3TF is too high, pancreatic acinar cell stress and damage occurs, thereby causing cytokine release, macrophage infiltration, and ADM, which prevents A→β reprogramming. I further suggest that efficient reprogramming requires a coordinated series of events whereby acinar cells cease zymogen production, delaminate and migrate to the surrounding mesenchyme, then cluster into new vascularized islets. For these cellular changes to occur without causing cellular damage and inducing inflammation, acinar cells may need time to cease the production and secretion of tissue-digesting enzymes before delamination from the pancreatic duct.

The pace at which reprogramming occurs is likely influenced by the concentration of the reprogramming factors. If 3TF expression is too high, and reprogramming occurs at a rate that exceeds the ability of the cell to undergo an orderly transition from one cell state to another, acinar cell damage may occur, preventing the formation of new beta-like cells. Indeed, when I reduced the level of 3TF expression, the inflammatory response was attenuated and new beta-like cells were produced. Conversely, if 3TF levels are too low, no reprogramming occurred, at least within a one-week timeframe.

I have also found that the presence of inflammatory macrophages within the pancreas greatly influences the outcome of 3TF-mediated acinar cell reprogramming. While depleting T- and B-cells during reprogramming does not prevent ADM or allow for the production of new beta-like cells, the depletion of macrophages, by the administration of $GdCl_3$, prevented ADM, thereby enabling the acinar cells to be reprogrammed into beta-like cells. The mechanisms involved in the macrophage-dependent blockage of A→β reprogramming remain unclear, but it has been reported that macrophage-secreted cytokines mediate ADM through activation of NFκB and STAT3 (Liou et al., 2013). While the RNA-Seq data suggest that inflammation enhances signaling through NFκB, STAT3 signaling was recently shown to be required for cytokine-mediated A→β conversion (Baeyens et al., 2013). Thus, it is possible that NFκB and STAT3 signaling oppose each other, with STAT3 signaling promoting A→β reprogramming and NFκB signaling impairing reprogramming by causing ADM.

Furthermore, I estimate that approximately 650,000 new beta-like cells are produced, on average, in response to administration of 2.0 mg/ml dox and $GdCl_3$. While this number of cells is about a third of the approximately 2 million beta cells in an average mouse pancreas (Dor et al., 2004), it is sufficient to transiently rescue STZ-induced diabetes. Furthermore, adenoviral delivery of the reprogramming factors has been reported to result in 40-50% of infected acinar cells being converted to new beta-like cells (W. Li, C. Cavelti-Weder, et al., 2014). While I expected that the transgenic delivery of the factors would further improve reprogramming efficiency, I found the opposite with only 6% of 3TF-expressing cells expressing insulin after 7 days. This suggests that there are additional variables that distinguish adenoviral- and transgene-mediated reprogramming, such as the dynamics of 3TF-expression. While use of the Tet-On system has the distinct advantage of allowing us to simultaneously turn on or off 3TF expression

and to vary the amount of 3TF expression, it does not allow stable expression of the reprogramming factors over an extended time. My use of the *Ptf1a* gene to drive expression of rtTA, while being a straightforward means of achieving acinar-cell specificity, has the limitation that *Ptf1a* expression is extinguished as acinar cells are converted into new beta cells. This limitation can only be overcome with a more complicated transgene design.

Widespread metaplastic changes resulting from tissue inflammation have not been reported when adenoviruses are used to introduce the reprogramming factors into the pancreas of normoglycemic mice (Cavelti-Weder et al., 2016). I speculate that similar metaplastic conversions are not observed due to the low infection efficiency of the adenovirus, which results in fewer acinar cells expressing the reprogramming factors, thereby avoiding triggering widespread pancreatic inflammation and allowing for more of the virally-infected cells to be reprogrammed. However, despite the highly penetrant expression of 3TF in my transgene-based model (nearly 80% of acinar cells at 2.0 mg/ml dox), I could only achieve the visible reprogramming of 6% of the 3TF-induced cells. Even so, I was able to produce over twice the number of new insulin-positive cells (650,000 versus 245,000 ± 32,000) that were reported when using an adenovirus (W. Li, M. Nakanishi, et al., 2014). This very marked difference in experimental outcome is likely due to my inability to fully suppress pancreatic inflammation when using a high dose of dox to express 3TF in the maximum number of acinar cells.

My findings indicate that a better understanding of the role of transcription factor concentration, duration of expression, and the number of cells affected, will be essential for translating findings in mice to humans. However, due to the physiological role of acinar cells, whose function is to produce enzymes for the digestion of proteins, complex carbohydrates, lipids, and nucleic acids, and thus pose a very real potential for pancreatic autodigestion, much

consideration must be taken on the safety and efficacy of employing the acinar cell as a reprogramming target.

In any case, the very marked differences in experimental outcomes compared to those achieved using an adenovirus suggest that both the dynamics and secondary effects of A→β reprogramming are complex. However, by exploring the outcomes achieved with a transgene-based reprogramming model, a better understanding of some of the many variables that will need to be understood to be able to safely and efficiently reprogram acinar cells in humans has been obtained.

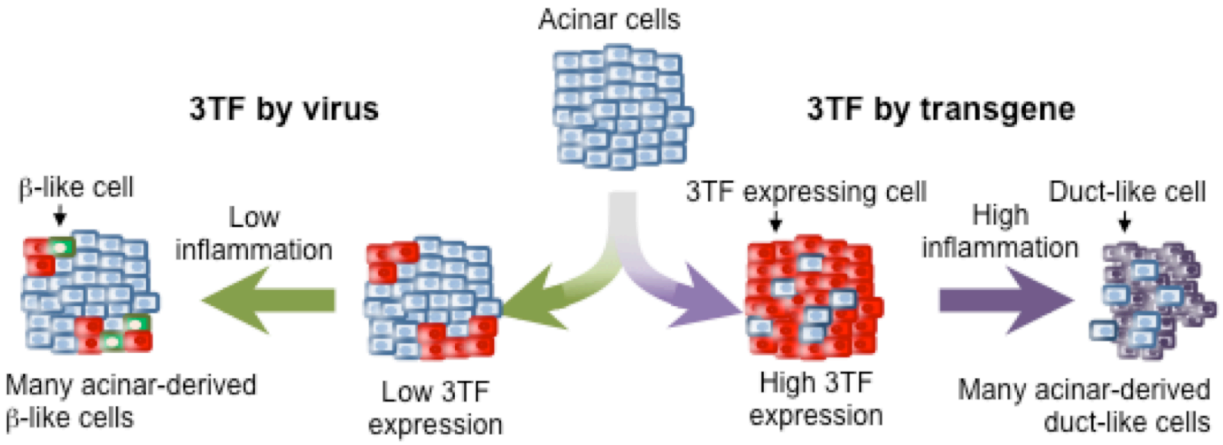


Figure 8.1 Model of divergent 3TF reprogramming. Adenoviral delivery of 3TF to the pancreatic acinar cells of *Rag1*$^{-/-}$ mice is relatively inefficient resulting in only a few pancreatic acinar cells expressing 3TF. Low levels of inflammation permit A→β reprogramming. Transgene expression of 3TF in the pancreatic acinar cells of *Rag1*$^{+/+}$ mice is very efficient resulting in many acinar cells expressing 3TF and high levels of 3TF expression. Rapid reprogramming causes ER stress, a rise in $[Ca^{2+}]_i$, and cell death, triggering a potent inflammatory response that results in ADM, thereby blocking A→β reprogramming.

Future directions

Why do high levels of 3TF expression lead to pancreatic inflammation and macrophage infiltration?

It is unclear as to why robust expression of 3TF in a large portion of acinar cells causes acute pancreatic inflammation. Efficient A→β reprogramming may require a coordinated series of events and robust expression of 3TF may disrupt these events, thereby causing pancreatic inflammation. Indeed, given that profound transcriptional changes are required for an acinar cell to become a beta cell, it is easy to imagine a loss of cellular homeostasis if the transition from one cell type to another occurs too rapidly. Moreover, the loss of cellular homeostasis could be further compounded by the disruption in tissue morphology, something that might result in the inappropriate secretion of proteolytic enzymes within the pancreas itself. It is also possible that a high concentration of 3TF causes a pathological increase in the $[Ca^{2+}]_i$ in acinar cells, which triggers the premature activation of pancreatic zymogens such as trypsinogen, and activation of the ER stress response mechanism (Muili et al., 2013; Raraty et al., 1999; Sah et al., 2014). Indeed, in acinar cells, a rise in $[Ca^{2+}]_i$ has been associated with ADM and is known to cause activation of inflammatory genes and the ER stress response (Sah et al., 2014). Furthermore, inspection of the RNA-seq data revealed a marked increase in both the expression of genes encoding voltage-gated and other Ca^{2+} channels and of genes involved in the activation of the unfolded protein response after 7 days of robust 3TF-transgene expression. However, future studies will need to be conducted to test whether high concentration of 3TF causes a pathological increase in the $[Ca^{2+}]_i$ in acinar cells.

What are the effects that activated macrophages have on signaling pathways in 3TF-expressing acinar cells?

My studies show that high levels of 3TF-transgene expression in pancreatic acinar cells results in a potent inflammatory response and ADM within 7 days of dox administration at 2.0 mg/ml. Macrophage infiltration is observed as early as 2 days and persists for up to 28 days. Furthermore, I have shown that depletion of macrophages prior to and during 3TF-mediated reprogramming attenuates the ADM phenotype and promotes successful A→β reprogramming. However, it is not understood how macrophages affect the outcome of A→β reprogramming. Indeed, the cytokines secreted or the signaling pathways they activate within 3TF-expressing acinar cells, which result in ADM and the prevention of A→β reprogramming is unkown. While it has been reported that macrophage-secreted cytokines mediate ADM through activation of NFκB signaling (Liou et al., 2013) and inspection of the RNA-seq data revealed an increase in many NFκB targets in acinar cells after 7 days of robust 3TF-transgene expression, future studies profiling the recruited macrophages to determine their polarization state and use of pharmacological approaches to accurately assess the signaling pathways that become activated within acinar cells in response to inflammation need to be conducted.

Optimize the efficiency of 3TF-induced A→β reprogramming

Two models for the reprogramming of fibroblasts to iPSCs have been proposed. An "elite" model posits that only a subset of cells are capable of being reprogrammed whereas a "stochastic" model posits that all cells have the potential to be reprogrammed (Yamanaka, 2009). Others have suggested that the reprogramming process can also be considered "deterministic" with fixed latency periods or "stochastic" with different latency periods (Hanna et al., 2009).

Determining the mechanism by which acinar cells are reprogrammed into new beta cells is important since, if it occurs by an elite mechanism, efforts should be directed to identifying and producing more elite cells. On the other hand, if it is stochastic, attention should be on improving the reprogramming protocol. At present, the highest A→β conversion efficiency I have achieved is approximately 6% even though mCherry and 3TF are expressed in over 70% of the acinar cells. This is considerably less than the 40% reprogramming efficiency reported by Li et al. (W. Li, C. Cavelti-Weder, et al., 2014) using an adenoviral vector that simultaneously expresses all three reprogramming factors. Since I hypothesize that *A*→β reprogramming is likely to be a stochastic process, and since expression of the 3TF-expressing transgene is highly penetrant, I expect to surpass the reported efficiencies when an optimal concentration of 3TF is identified and when I am able to fully suppress pancreatic inflammation. Future studies will need to be conducted to determine the optimal concentration of factors required for efficient reprogramming.

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