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STUDYING PANCREATIC ACINAR CELL DIFFERENTIATION THROUGH TRANSCRIPTION FACTOR REPROGRAMMING

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ABSTRACT

Pancreatic ductal adenocarcinoma is a highly lethal human cancer that originates from acinar cells in the pancreas. Ptf1a is a transcription factor that plays an important role in the development of the pancreas and in the differentiation and maintenance of acinar cells. Ptf1a expression is lost during cancer formation, and deletion of *Ptf1a* in a mouse model of pancreatic cancer results in accelerated cancer progression. In addition, induced expression of Ptf1a in precancerous cells can reprogram them back to normal acinar cells. However, when Ptf1a is expressed alone in fibroblast cells, it is not able to work as a master regulator transcription factor to reprogram fibroblasts into acinar cells. This thesis presents an experiment testing whether Ptf1a needs additional acinar specific transcription factors in order to reprogram fibroblast cells into acinar cells. Our findings show that different acinar transcription factors are required alongside Ptf1a in order to promote expression of different acinar target genes. Combinations with multiple acinar transcription factors tend to be the most successful at inducing expression of target genes. Although multiple acinar-specific target genes were induced, their expression did not reach the levels of normal acinar cells. More research using different transcription factor combinations, chromatin opening drugs, and specifying order of expression may allow us to successfully reprogram fibroblasts to an acinar identity. Our future research hopes to illuminate how Ptf1a works with other acinar transcription factors to establish and maintain acinar cell identity, and potential mechanisms by which this might restrict the formation of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the deadliest cancers, with a 5-year relative survival rate of 34% for those diagnosed in a localized stage, 12% for those in a regional stage, and 3% for those with distant tumor spread, as defined by the American Cancer Society (Siegel et al., 2018). The high mortality of this disease is due to the lack of effective early detection methods and treatments, as early pancreatic cancers often have no symptoms (McGuigan et al., 2018). This results in most patients being diagnosed with cancers that have already spread. Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer, making up 95% of pancreatic cancer cases (*Types of Pancreatic Cancer*, 2018). The cellular origin of PDAC has been debated, and was originally thought to result from ductal cells, due to the ductal characteristics of the cancer cells. In the normal pancreas, ductal cells form vessels that deliver digestive enzymes to the duodenum, while acinar cells synthesize and secrete these enzymes. Further research has found acinar cells are more sensitive to cancer driving mutations, and respond to risk factors such as inflammation of the pancreas, known as pancreatitis (Xu et al.,

2019). We use a mouse model of PDAC in which acinar cells undergo a phenotypic change, acinar-to-ductal metaplasia (ADM), and form precancerous pancreatic intraepithelial neoplasia (PanIN) that can progress into pancreatic cancer (De La O et al., 2008; Krah et al., 2015).

In most PDAC, a mutation is found in the *Kras* oncogene, which drives cancer formation (Waters & Der, 2018). Normally, the Kras protein is only activated in response to growth factors, such as during tissue growth or injury repair, but the oncogenic mutated Kras is constitutively active, and promotes pathways that drive cancerous cell growth (Waters & Der, 2018). Our lab found that coactivation of Kras and Notch, a signaling pathway that is involved in pancreas development, induces PanINs in acinar cells through loss of acinar-specific regulators and reprogramming to a ductal phenotype (De La O et al., 2008). One acinar regulator that is lost during PanIN formation is the transcription factor Ptf1a, a bHLH protein that acts as a transcriptional activator of target genes. Ptf1a is required in early pancreas development for cells to become pancreatic progenitor cells. Without Ptf1a, many cells enter a duodenal fate and become part of the small intestine, and only a rudimentary pancreas is formed (Kawaguchi et al., 2002). Later in development, Ptf1a induces progenitor cells to differentiate into acinar cells specifically (Cleaver et al., 2018). Ptf1a regulates the expression of almost all acinar-specific genes, such as the digestive enzymes and other transcription factors (Hoang et al., 2016).

Our lab has identified Ptf1a as a major suppressor of tumor initiation in acinar cells. We previously found that Ptf1a is severely downregulated or absent in PanINs induced by mutant *Kras* (Krah et al., 2015). In addition, the deletion of *Ptf1a* in a *Kras* mutant background results in rapid ADM, downregulation of acinar-specific genes, loss of acinar cell identity, and accelerated formation of pre-cancerous PanINs (Krah et al., 2015). At the same time ductal cell-specific genes, such as transcription factor Sox9, are upregulated. These phenotypes are consistent with a model in which Ptf1a acts as a "master regulator" of acinar cell identity, and suppresses tumor formation by enforcing normal differentiation.

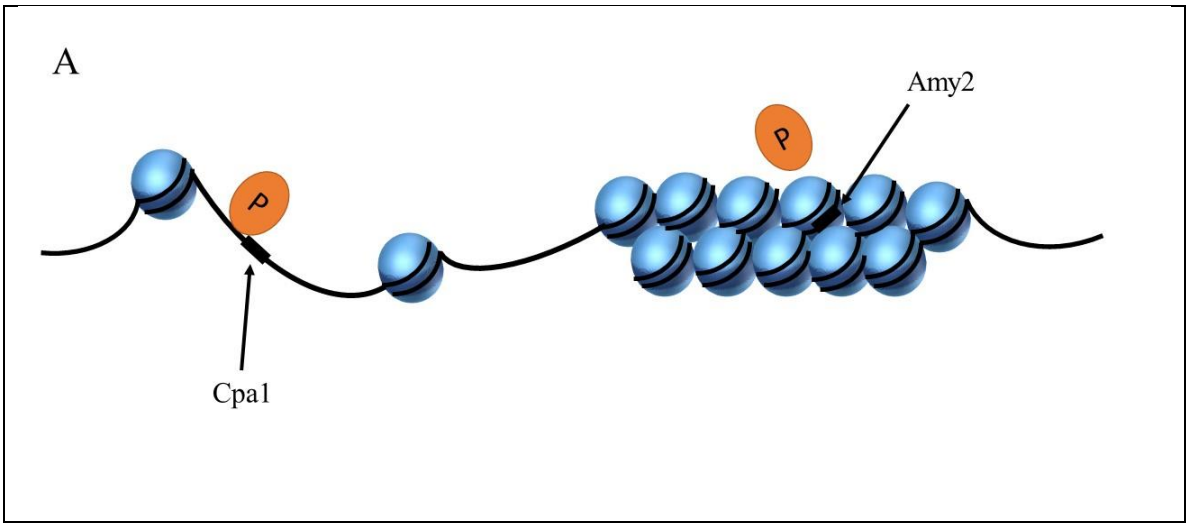
Not only does the absence of Ptf1a lead to pre-cancerous phenotypes, but the presence of Ptf1a can actually halt the progression of tumorigenesis. We recently demonstrated that sustained expression of Ptf1a in Kras mice greatly reduced PanIN formation compared to those mice without Ptf1a expression (Krah et al., 2019). Furthermore, expression of Ptf1a in precancerous PanINs results in partial or complete re-differentiation of the precancerous cells into acini. Our next question to ask was how exactly acinar cells establish and retain their identity, as this previous research suggested the maintenance of acinar cell identity could prevent cancer formation.

We hypothesized that Ptf1a alone might be sufficient to promote an acinar phenotype, analogous to the role of the transcription factor MyoD in skeletal muscle. MyoD was found to be a master regulatory transcription factor when artificially expressing it in multiple cell lines, including NIH3T3 fibroblasts, resulted in transdifferentiation to skeletal muscle, or myogenesis (Weintraub et al., 1989). This study on the ability of MyoD to turn on muscle-specific genes in heterologous cell types inspired further research on how myogenesis was regulated by MyoD and other myogenic bHLH proteins. It appears the differentiation of many cell types may be controlled by specific groups of bHLH proteins, so understanding the mechanisms behind differentiation in one cell lineage could lead to similar advances in others (Tapscott, 2005). Ptf1a is a bHLH protein related to MyoD, which appears to play a central role in acinar cell development and maintenance, so we hypothesized that it may function similarly to MyoD.

My graduate student mentor in the lab designed a reprogramming experiment, in which Ptf1a was inducibly expressed in mouse NIH3T3 fibroblast cells. NIH3T3 are an immortalized but non-cancerous cell line that can be reprogrammed into muscle cells by MyoD (Miner & Wold, 1991). Unpublished results from this experiment revealed that Ptf1a alone is not sufficient

to reprogram fibroblasts into acinar cells. Ptf1a expression was able to turn on its downstream target gene *carboxypeptidase-A1* (*Cpa1*), which encodes a pancreatic digestive enzyme. However, it was unable to induce expression of several other acinar target genes, or any important acinar-specific transcription factors. Thus, although Ptf1a is necessary for acinar identity in vivo, it is not sufficient on its own to completely reprogram a non-acinar cell to an acinar phenotype.

This raised the central question of my thesis: what else does Ptf1a need in order to promote acinar identity? Though Ptf1a does not have the same extensive effect as master regulator MyoD does when expressed in fibroblasts, this does not eliminate the potential for it to act as a master regulator in different conditions. Even the remarkable MyoD is subject to changes in cellular environments. When artificially expressed in hepatocytes, MyoD is not able to induce expression of muscle-specific genes, suggesting that fibroblasts may contain factors that allow MyoD to act as a master regulator, while hepatocytes do not (Schäfer et al., 1990). Ptf1a can act as a master regulator in pancreatic progenitor cells, its continued high expression pushing cells to an acinar fate, possibly because pancreatic progenitors contain the right environment and co-factors that allow Ptf1a to do so. It is plausible that Ptf1a is unable to reprogram fibroblast cells because it does not have physical access to all acinar target genes, which may be embedded in heterochromatin of fibroblast cells (Figure 1A). In addition, Ptf1a is known to bind and activate promoters of target genes together with several partner proteins, some that directly interact with Ptf1a itself and others that may interact indirectly on the DNA (Figure 1B), and which are unlikely to be expressed in all cell types.



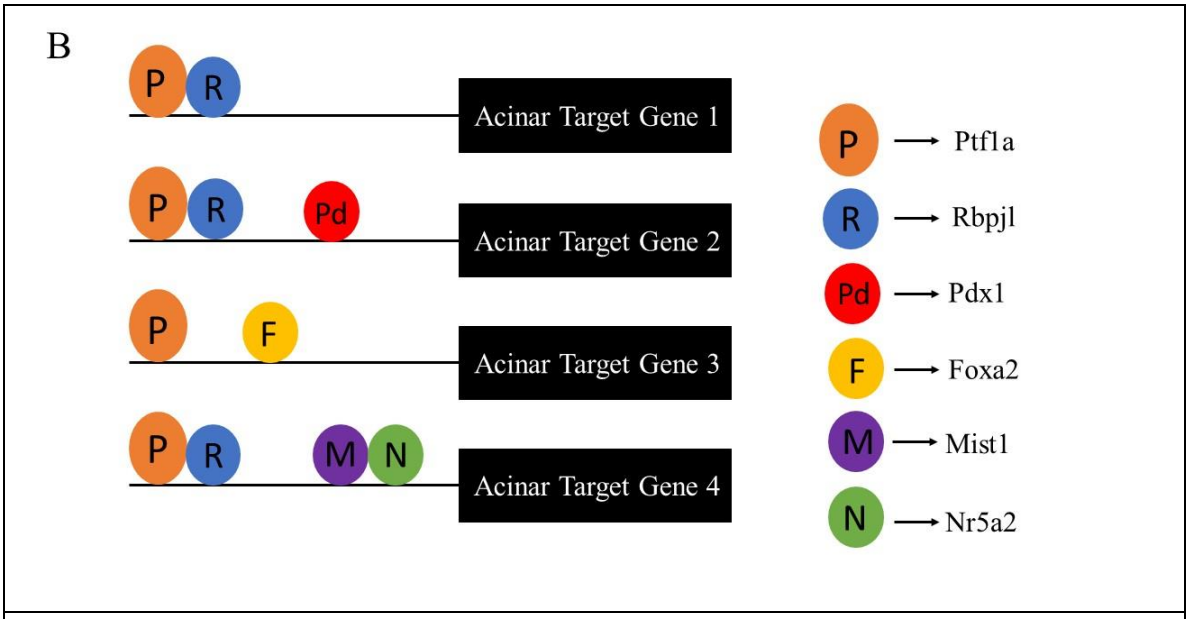


Figure 1. Why Ptf1a is not sufficient to reprogram cells to acinar identity
(A) Ptf1a may not be able to access its target genes due to their location in heterochromatin of fibroblast cells. The location of the *Cpa1* locus in euchromatin renders it accessible to Ptf1a, while *Amylase2*- (*Amy2*) resides in heterochromatin where nucleosomes block access to the gene. (B) Ptf1a may need to interact with other acinar-specific transcription factors on the promoters of specific target genes, in order to induce their transcription.

There are several important transcription factors that may directly or indirectly help Ptf1a induce acinar reprogramming. All these proteins are already known to play important roles in acinar cells:

- In mature acinar cells, **RbpjL** functions as a binding partner for Ptf1a, whereas the related protein Rbpj forms a complex with Ptf1a in embryonic pancreatic progenitors and in other sites of Ptf1a function, such as the brain (Beres et al., 2006; Hori et al., 2008; Masui et al., 2007). Many acinar-specific genes can only be activated to maximal levels by the combination of Ptf1a and RbpjL, referred to as the PTF1-L complex (Masui et al., 2010). During acinar development, expression of RbpjL is activated in pro-acinar cells, and it replaces Rbpj (in the PTF1-J complex), allowing the new PTF1-L complex to bind to the promoters of acinar-specific digestive enzymes (Cleaver et al., 2018).
- In adult pancreas, **Mist1**, also referred to as Bhlha15, is highly expressed specifically in acinar cells as a target of Ptf1a, and is required for maximal expression of many acinar genes, particularly those involved in exocytosis (secretion) and subcellular organization (Cleaver et al., 2018).
- **Nr5a2**, also referred to as LRH-1, is another important acinar transcription factor, and it has been shown that loss of Nr5a2 inhibits normal acinar cell formation, and lowers the expression of Ptf1a in mature acinar cells (Hale et al., 2014). In addition, reduced levels of Nr5a2 in the pancreas can lead to a pre-inflammatory state (similar to pancreatitis, a known risk factor for PDAC) (Cobo et al., 2018). Nr5a2 expression is lost during ADM in a mutant *Kras* background, and loss of Nr5a2 in acinar cells with oncogenic *Kras* causes accelerated ADM and PanIN formation (Figura et al., 2014).

- The transcription factor **Foxa2** (originally referred to as HNF-3β) is ubiquitously expressed in the endodermal organs of the gastrointestinal tract, including the pancreas, and is important for normal pancreas development (Gao et al., 2008). In addition, reporter gene-based studies of pancreas gene regulation suggest that Foxa2 cooperates with Ptf1a to activate the pancreatic *alpha-amylase* gene (*Amy2*) (Cockell et al., 1995).
- **Pdx1** is critical during earlier developmental stages of the pancreas, and embryos without Pdx1 will not form a pancreas at all (Cleaver et al., 2018). It has also been shown that Pdx1 promotes and directly regulates Nr5a2 in the embryonic pancreas (Annicotte et al., 2003). Reporter gene studies have suggested that Pdx1 can cooperate with Ptf1a to regulate the acinar digestive enzyme *Cela1* (*Elastase-1*) (Swift et al., 1998). Given its importance in the early pancreas, and its regulation of acinar-specific Nr5a2, Pdx1 may also be involved in promoting the differentiation of acinar cells directly.

Other experiments have shown it is possible to convert fibroblast cells into other cell types by expressing a combination of transcription factors. One way this has been demonstrated is with the transcription factors Oct4, Sox2, Klf4, and Myc, now known as the "Yamanaka factors," which can convert mouse and human fibroblasts into induced pluripotent stem (iPS) cells (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). From these iPS cells, it is possible to obtain other differentiated cell types by adding cell-specific transcription factors. Rather than going through an intermediate, undifferentiated iPS cell stage using the Yamanaka factors, other approaches have been taken to directly transform fibroblasts into different cell types. For example, introducing specific combinations of transcription factors including Foxa2 (or its related gene Foxa1), HNF1α, HNF4α and Gata4 has been shown to reprogram mouse fibroblasts into hepatocyte-like cells. These induced hepatocytes were able to restore function to damaged livers when transplanted (Huang et al., 2011; Sekiya & Suzuki, 2011). Other examples of successful reprogramming using multiple transcription factors include the transformation of fibroblasts to neurons, fibroblasts to cardiomyocytes, and astrocytes to dopaminergic neurons – the last of which was performed using the same viral vector approach that is used in our own experiments (Addis et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010).

In order to see how other acinar transcription factors cooperate with Ptf1a to promote acinar identity, we established NIH3T3 mouse fibroblast lines with the capacity for conditional expression of Ptf1a alone or in combination with one or more of the transcription factors described above: RbpjL, Mist1, Nr5a2, Foxa2 and Pdx1. In this thesis, I analyze the resulting induced acinar gene expression as a marker of reprogramming for acinar identity. Knowledge gained from this research will help us understand how acinar cells can establish and maintain their identity. This will further provide us with tools to investigate how loss of cell identity contributes to diseases such as PDAC, and how this process could be inhibited or reversed.

METHODS

Cell Lines

We used NIH3T3 and 293T cell lines, which came from American Type Culture Collection (ATCC) and were used for transcription factor overexpression and production of lentiviruses, respectively. These cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM), with 10% FBS and Penicillin/Streptomycin/Glutamine additive at 1x final concentration. NIH3T3 cells infected with the pCLX-pTF-EGFP or pCLX-pTF-Ptf1a lentiviral

vectors (see below) were grown in the presence of 5 ug/ml Blasticidin. Additional details of lentiviral infection are provided below.

Plasmids

We have previously described the generation of conditional lentiviral expression vectors driving either EGFP or Ptf1a (Krah et al 2019), using the pCLX-pTF plasmid backbone (Addgene #45952). To express additional factors, we cloned several cDNAs into the lentiviral pFU-tetO-Gateway plasmid (Addgene #43914): *mCherry, RbpjL, MIST1, Nr5a2, Foxa2,* and *PDX1* (Table 1). Cloning of genes into the pFU-tetO vector was done using Gateway cloning technology (Thermo-Fisher Scientific). In brief, the open reading frames (ORFs) of the genes of interest were amplified using PCR, and were cloned into the pDONR221 vector using BP Clonase, to create "entry clones." Entry clone plasmids were grown in DH5α E. coli, and sequenced to confirm insert identity. ORFs were then transferred into the pFU-tetO-Gateway vector using LR Clonase II. These plasmids were transformed and selected for in OmniMAX cells.

Gene	**NCBI Gene ID**	**Source**
mCherry	N/A	sea anemone (derived artificially)
Foxa2	15376	mouse
PDX1	3651	human
MIST1 (BHLHA15)	168620	human
Nr5a2	26424	mouse
RbpjL	19668	mouse
Table 1. List of inserts cloned into pFU-tetO lentiviral vector.		

Lentiviral Production and Infection

Lentiviruses were made in 293T cells, by transfecting them with the lentiviral vector plasmid (pCLX-pTF or pFU-tetO), the psPAX2 packaging plasmid, and the pCAG4-Eco ecotropic envelope expression plasmid, at a ratio of 3:2:1. We used an ecotropic envelope for increased biosafety, as it restricts infectivity to rodent cells, not human. Virus-containing supernatant was harvested from 293T cells at 72 hours after transfection, and stored at -80°C for later use. For infection, NIH3T3 cells were seeded in 12-well plates, at 30,000 cells per well. The next day, 40 ul of virus was added to a well for each of the viruses that were created. Cells were incubated overnight, and media was changed 24 hours after infection, resulting in stably infected lines. NIH3T3 cells were first infected with the pCLX-pTF-EGFP or -Ptf1a vectors, and selected with Blasticidin to isolate infected cells, as uninfected cells are killed by Blasticidin. Blasticidin-resistant cells were infected in a second round, with pFU-tetO viruses encoding the genes listed in Table 1 alone or in combination.

Transcription Factor Overexpression

Cells were grown in culture media described above. Cells were plated for experiments in 12-well plates, at a density of 100,000 cells/well. 24 hours after plating, current cell media was exchanged for acinar cell media, consisting of Waymouth's Media supplemented with 1% FBS, 0.1 mg/ml Soybean Trypsin Inhibitor, 4 ug/ml dexamethasone, and 1X

penicillin/streptomycin/glutamine supplement (Means et al., 2005). Half of the samples were given acinar media containing 1 ug/ml doxycycline (Dox), to induce expression from the lentiviral vectors. After 48 hours of growth in acinar media with or without Dox, cells were harvested.

Culture and Harvest of Acini

A wild type mouse was anesthetized, euthanized, and dissected. The pancreas was isolated, minced, and transferred into a tube with 5 ml of cold Hank's Buffered Saline Solution (HBSS) and 0.2 mg/ml Collagenase P. This suspension was shaken at 225 rpm at 37°C for about 40 minutes, until clumps were gone and the suspension was cloudy, being checked by eye every 5-10 minutes. 5 ml of cold 5% FBS in HBSS were added to the tube, which was centrifuged at 100x rcf for 3 minutes. Supernatant was removed and the washing process was repeated twice more. The pellet, containing a mixture of cells including intact acinar clusters, was resuspended in HBSS/5% FBS and pipetted through a sterile 100 um mesh, and pelleted again by centrifugation. Acinar clusters were isolated from non-acinar cells and debris by density centrifugation: the pellet was resuspended in 10 ml cold 4% BSA/HBSS and pelleted by centrifugation (100x rcf, 3 min.), and this process was repeated three times total. This density centrifugation procedure eliminates cell debris and enriches for dense acinar clusters. Acinar clusters were then suspended in cold acinar culture media, and counted on a hemocytometer.

RNA Harvest and quantitative RT-PCR (RT-qPCR)

RNA was harvested from cultured pCLX-Ptf1a/pCLX-EGFP cells (after 48 hours of Dox treatment), or from freshly-isolated acinar cells (5,000-10,000 clusters total), using the RNeasy Mini kit (QIAGEN, Germantown, MD), eluting RNA in 30 ul of RNase free water. After determining the RNA concentration using a Nanodrop spectrophotometer, RNA samples were normalized to a concentration of 30 ng/ul. 10 ul (300 ng) of RNA was then converted to cDNA using M-MLV reverse transcriptase and random primers (Thermo-Fisher). cDNA was diluted in a 1:10 ratio with sterile water, and quantitative PCR (qPCR) reactions were set up using PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences, Beverly, MA), with 5 ul cDNA per reaction and oligonucleotide primers at 400 nM final concentration each. qPCR reactions were run on a MyiQ real-time thermal cycler, using the MyiQ Optical System Software (Bio-Rad). Primers used for amplification were identified from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) or using NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table 2 below.

Target	Strand	Sequence	Amplicon Size (bp)
Ppia	Top Bottom	CAGTGCTCAGAGCTCGAAAGT CACCGTGTTCTTCGACATCA	113
Ptf1a	Top Bottom	AGGACAGTCCCGGTAACCA AAAGAGAGTGCCCTGCAAGA	114
Cpa1	Top Bottom	GTCTTCGGCAATGAGAACTTTGT GGAAGGGCACTCGAACATCG	169
Amy2	Top Bottom	GCAAGTGGAATGGCGAGAAGA AGCCAACATAAATCCGACAGC	191

Cela1	Top Bottom	GTGGACACAGTACCGAGGAC CCAGTTGCTTCGGATGAGGG	158
Cela2a	Top Bottom	ACCCCACTTATGAGGTGGAGG AGCACTCGGTAGGTCTGATAG	202
RbpjL	Top Bottom	ACCGTCTGCGGTTACATGG GGTCTTGGCACAACCAAATTC	111
Foxa2	Top Bottom	TCCGACTGGAGCAGCTACTAC GCGCCCACATAGGATGACA	176
Nr5a2	Top Bottom	TTGCCAAATTGACAAAACGCA GGCTCGAATGAGGGCTTTCTT	184
MIST1	Top Bottom	CGGATGCACAAGCTAAATAACG GCCGTCAGCGATTTGATGTAG	128
PDX1	Top Bottom	AGTGGGCAGGCGGCG TCAACATGACAGCCAGCTCC	153

Table 2. List of primers used in qPCR.

Gene expression was analyzed from raw Ct values using the delta-delta-Ct (ΔΔCt) method (Schmittgen & Livak, 2008). Technical duplicate Ct values were averaged, and normalized to the Ct values of the housekeeping gene *Ppia*. These ΔCt values were then normalized to a positive control, this being wild-type mouse acinar cells for all mouse genes, producing ΔΔCt values. The relative expression (with wild-type mouse acinar cell levels set to 1.0) was calculated as $2^{-\Delta\Delta Ct}$. These calculations, analysis, and graphing of relative expression were performed in R.

Western Blotting

Protein lysate was harvested from cell lines after 48 hours of Dox treatment. Samples were heated, loaded onto a gel, and run in a Mini-Cell module using 1X MOPS-SDS running buffer and a protein ladder. Protein gel reagents were from Thermo-Fisher. All samples were loaded with 5 ug protein per well, except for a control sample from 266-6 cells that had 15 ug protein. After running, gels were transferred onto a PVDF membrane using an XCell II transfer module and NuPAGE transfer buffer. Membranes were stained with Ponceau S to ensure proper transfer, were washed with PBT, and pre-blocked in “Blotto” solution (5% nonfat powdered milk in PBS + 0.1% Tween-20 [PBT]) for one hour at room temperature. Membranes were then incubated in primary antibodies in Blotto at 4 degrees Celsius overnight. Primary antibodies used were goat anti-Ptf1a (from Dr. Chris Wright, Vanderbilt University, 1:10,000 dilution), goat anti-Cpa1 (R&D Systems, 1:2,000 dilution), and mouse anti-tubulin (Developmental Studies Hybridoma Bank, 1:50 dilution). Membranes were washed with PBT, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch) diluted 1:10,000, in Blotto for 1 hour at room temperature, and washed again with PBT. Membranes were developed with SuperSignal West Pico ECL reagent, and exposed to X-ray film to detect luminescence signal.

RESULTS

Preliminary experiments in our lab tested the effect of Ptf1a expression in an unrelated cell type in order to ask if Ptf1a could promote expression of any acinar digestive enzymes or other acinar transcription factors when expressed by itself. For these experiments, NIH3T3 fibroblasts were infected with lentiviral vectors that conditionally express either EGFP or Ptf1a in the presence of doxycycline (Dox). Lentiviral expression was induced through treatment with media containing Dox, which binds to an rtTA molecule whose expression is constitutively promoted in the TetO system. The Dox-rtTA complex binds to the TetO promoter upstream of the gene of interest, and only this complex is able to promote expression. Once stable lines were established, infected cells were treated with Dox for 72 hours and harvested for RNA; RNA was reverse transcribed into cDNA, which was amplified for gene targets using PCR. In initial experiments, we performed standard (non-quantitative) PCR and analyzed the products by electrophoresis to detect potential changes in gene expression (Fig. 2).

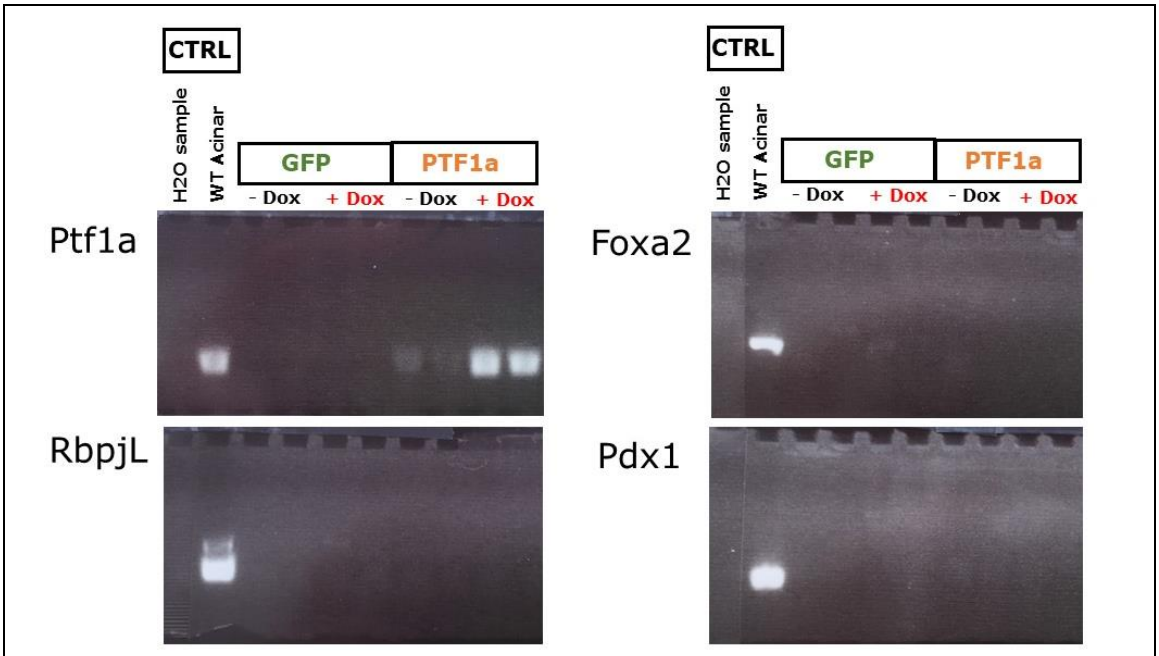


Figure 2. Ptf1a and other acinar transcription factors
Ptf1a expression in NIH3T3 fibroblasts does not result in the expression of other acinar transcription factors.

Our infection of fibroblasts was successful as Ptf1a expression was induced in Ptf1a lines when treated with Doxycycline. Ptf1a was not able to promote expression of other acinar transcription factors, such as *Foxa2* and *Pdx1*, nor was it able to induce expression of its binding partner *RbpjL*. These results might suggest that Ptf1a is non-functional when expressed in NIH3T3 cells, potentially reflecting the presence of inhibitory factors in the heterologous fibroblast environment. We therefore examined expression of acinar-specific digestive enzymes that are normally regulated by Ptf1a (Hoang et al., 2016), including specific members of the amylase (*Amy2*), carboxypeptidase (*Cpa1*), lipase (*Pnlip*) and elastase (*Cela2a*) gene families (Fig. 3).

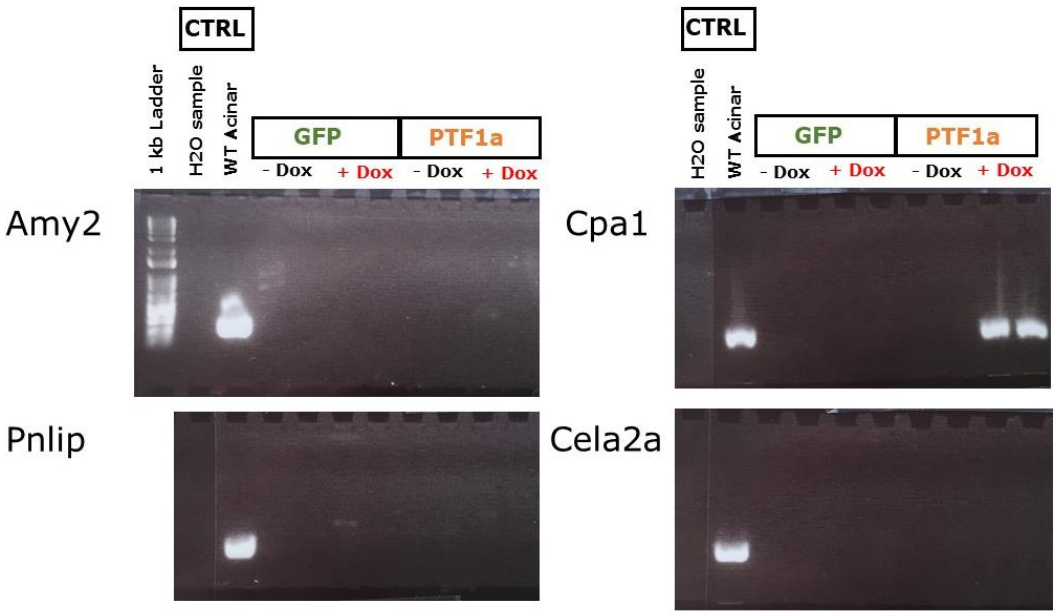


Figure 3. Acinar digestive enzymes
Ptf1a is able to induce expression of the digestive enzyme *Cpa1*, but it cannot promote expression of its other target genes when expressed alone in fibroblasts.

We found that Ptf1a alone was able to promote expression of one of its target genes, *Cpa1*, but all other acinar digestive genes tested were not induced in Ptf1a-expressing fibroblasts. From these and other unpublished results from our lab, we concluded that Ptf1a retained at least some functional activity in fibroblast cells, but that it could not act as a master regulator capable of reprogramming fibroblasts to a complete acinar phenotype. Since Ptf1a alone was not sufficient, we decided to investigate whether Ptf1a required other acinar transcription factors to assist it in inducing expression of acinar target genes.

NIH3T3 cells conditionally expressing either EGFP or Ptf1a were infected with one or more additional lentiviral vectors carrying other transcription factor genes. There were eight conditions used for these infections: six single-factor infections, consisting of *mCherry* (a red fluorescent protein that served as a control for infection, and which does not impact Ptf1a function), *RbpjL*, *MIST1*, *Nr5a2*, *Foxa2*, and *PDX1*, and two combined infections, referred to as RMN (*Rbpjl*, *MIST1*, and *Nr5a2*), and RMNFP (all transcription factors together). This resulted in 16 different cell lines, 8 each with a baseline expression of EGFP or Ptf1a. The lentiviral backbone for EGFP and Ptf1a was pCLX-pTF (Giry-Laterrière et al., 2011), and that used for the second set of transfections was pFU-tetO (Addis et al., 2011). For ease of reference, we refer to the individual cell lines by the combination of pCLX-pTF and pFU-tetO infections, e.g. “GFP-RbpjL” refers to cells first infected with pCLX-pTF-EGFP and subsequently with pFU-tetO-RbpjL.

These cell lines were grown, plated, and the infected transcription factors were expressed in each cell line for 48 hours, after which cells were harvested for RNA. RNA samples were reverse transcribed into cDNA, and used for quantitative PCR (qPCR) to detect levels of gene expression. qPCR data was analyzed in R using the delta-delta-Ct method (Schmittgen & Livak, 2008). qPCR uses a double-stranded DNA-specific fluorescent dye during the amplification process to monitor the accumulation of PCR product. More abundant genes will amplify faster

during the exponential amplification stage, and therefore will cross the threshold of abundance at a lower threshold cycle (Ct) value.

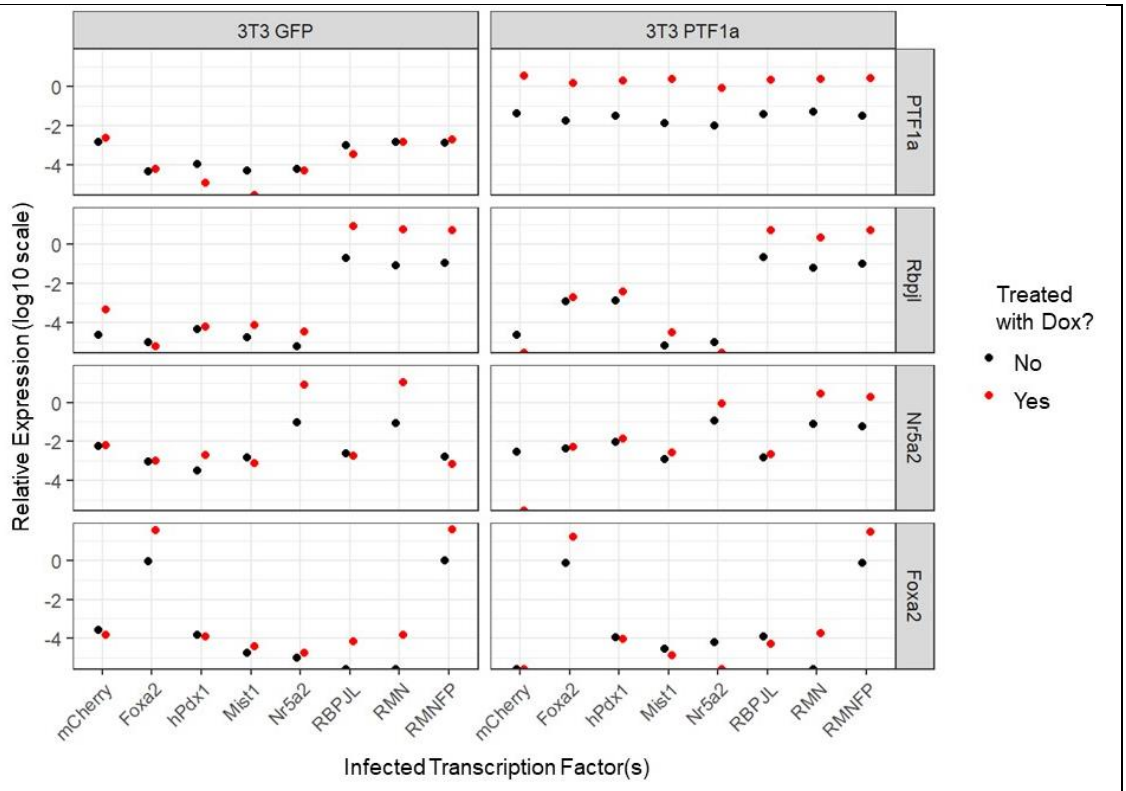


Figure 4. Gene expression levels of infected mouse acinar transcription factors
Log-transformed graph of gene expression (with expression levels in acinar cells originally set to 1.0, i.e. log-transformed to zero on the y-axis), where each horizontal step up represents a 10-fold increase in relative mRNA expression levels. Upon Dox treatment, all infected transcription factors were detected at or above wild type levels, and all were correctly expressed in their infected cell lines excepting the absence of Nr5a2 in GFP-RMN.

We first determined whether our viral infections of mouse transcription factors worked, by performing qPCR against the lentivirally-delivered genes themselves (Fig. 4). In Figures 4 through 6, expression data has been log-transformed, which allows us to see relative changes in gene expression across a wide array of absolute expression levels. Wild-type acinar levels of expression, which were originally normalized to 1.0, are set at zero in these log-transformed graphs. *Ptf1a* was highly expressed in all Ptf1a lines in the presence of Dox, and was not significantly expressed in any GFP line. For all transcription factors, the large gaps in expression between samples treated with Dox in red, and samples not treated in black, show that doxycycline is promoting their expression. The difference was particularly substantial in pCLX-pTF driven Ptf1a, which was induced 100-fold by treatment with doxycycline. All of the pFU-tetO driven factors exhibited moderately high levels of expression without doxycycline treatment, especially in Foxa2 which was expressed near wild-type acinar levels in the absence of Dox. The pFU-tetO factors were induced approximately 10-fold by treatment with doxycycline. This indicates that our pFU-tetO-Gateway vectors are somewhat leaky and these transcription factors may be lowly expressed without Dox treatment. *RbpjL* and *Foxa2* were expressed in all cell lines into which they were introduced. *Nr5a2* was expressed in all of its

infected cell lines except for GFP-RMNFP, which may reflect an error during the infection process. In the case of Nr5a2, GFP-RMNFP is only being used as a control for comparison to Ptf1a-RMNFP, and our error should not really affect the data we are interested in.

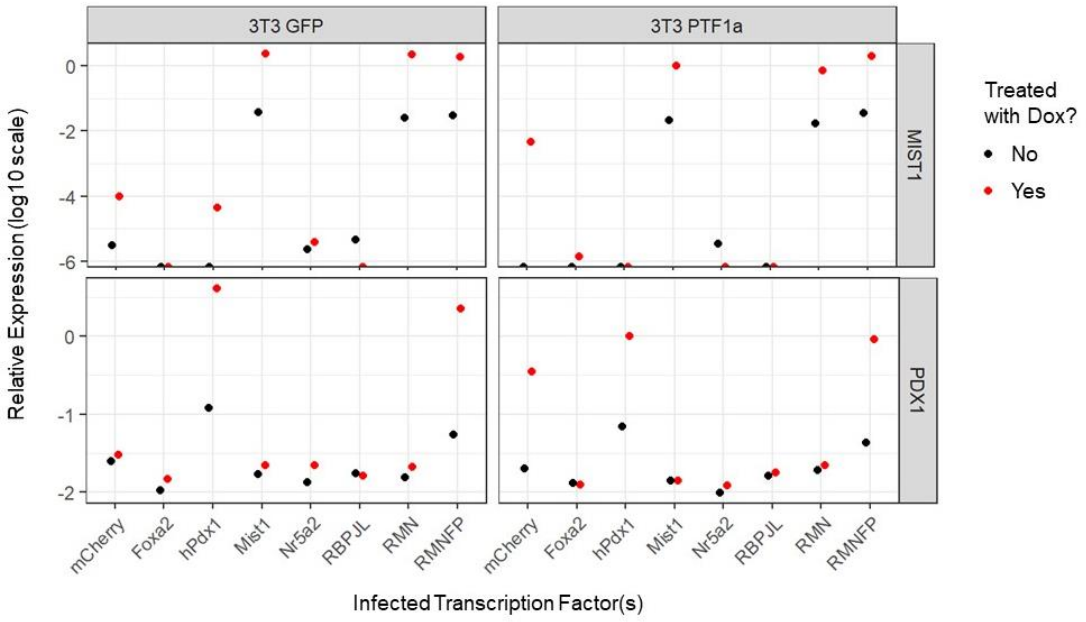


Figure 5. Gene expression levels of infected human acinar transcription factors
Log-transformed graph of gene expression of human MIST1 and PDX1. Note that each horizontal step up is a 10-fold increase in MIST1 but every two steps up is a ten-fold increase in PDX1. Both transcription factors are being correctly expressed in the cell lines they were introduced into.

Two of our infected transcription factors were human and had to be analyzed separately in R due to not being able to use the mouse wild type acinar cDNA as a baseline control for human genes. *MIST1* and *PDX1* were correctly expressed in each cell line they were introduced to, but Ptf1a-mCherry also appears to be expressing *PDX1*, an error that we will keep in mind when analyzing our data.

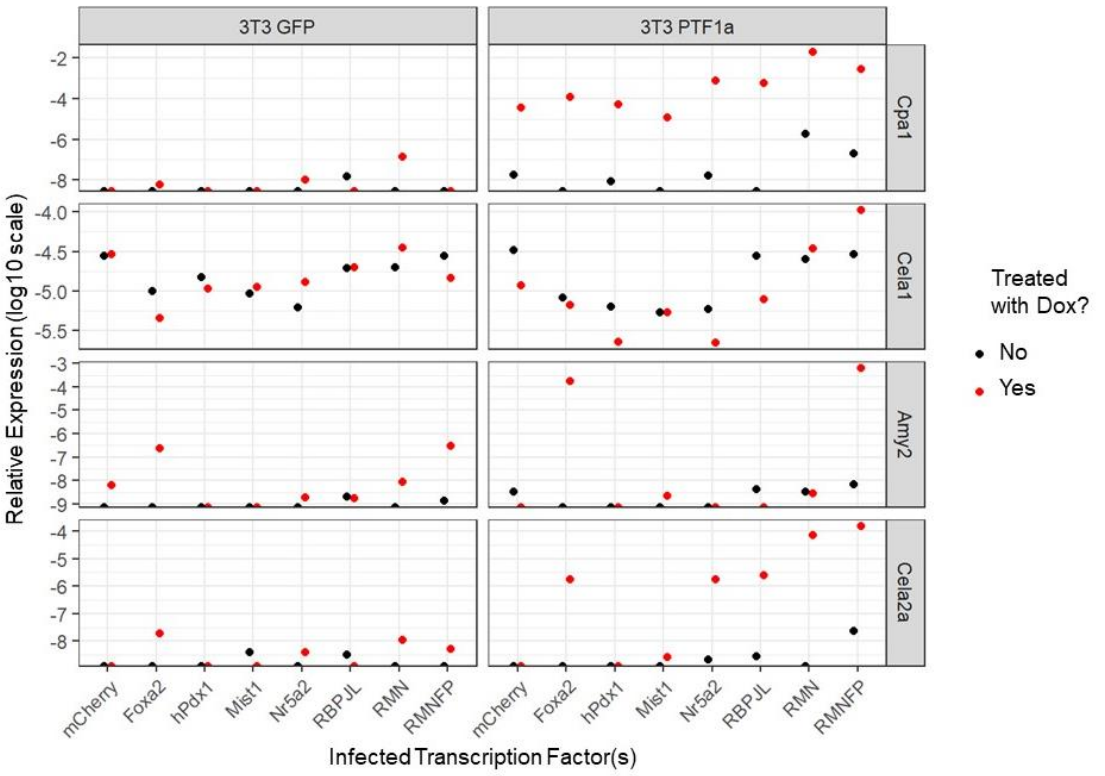


Figure 6. Gene expression levels of acinar digestive enzymes
Different infections of acinar transcription factors boost expression of different acinar target genes, with combination infections RMN and RMNFP being generally the most successful. Expression levels also differ from gene to gene, with *Cpa1* and *Amy2* expression levels being closest to wild type.

Next, we used qPCR to look at expression of digestive enzymes that acinar cells normally express, which are downstream target genes of Ptf1a and indicate acinar cell identity. In the absence of added Ptf1a (3T3 GFP cells), none of the transcription factors alone or in combination produced a significant boost in acinar gene expression (Fig. 6, left). As expected from our non-quantitative RT-PCR preliminary data (Fig. 3), expression of Ptf1a alone (Ptf1a-mCherry) induced *Cpa1* but not *Cela2a* (Fig. 6). Induction of *Cpa1* was further boosted with the addition of other factors, up to 100 times higher than Ptf1a alone in the Ptf1a-RMN combination. This may reflect synergy between RbpjL and Nr5a2, as each of these alone induced a 10-fold increase in *Cpa1*, whereas Mist1 alone did not have an effect. Surprisingly, the further inclusion of Foxa2 and PDX1 (RMNFP) resulted in a reduction of *Cpa1* compared to Ptf1a-RMN.

Cela1 appeared to be very lowly expressed in all fibroblast cell lines, with a slight increase in expression only seen in Ptf1a-RMNFP. *Amylase2* expression was induced only in the Ptf1a-Foxa2 and Ptf1a-RMNFP cells, implying the Ptf1a/Foxa2 combination is necessary and sufficient to promote *Amy2*, with no further boost from other transcription factors. *Cela2a* was expressed when Ptf1a is combined with Foxa2, Nr5a2, or RbpjL, and was most highly expressed in Ptf1a-RMN and Ptf1a-RMNFP combinations.

We also looked at protein expression of Ptf1a and Cpa1 in our single transcription factor-infected cell lines. Transcription factor expression was induced for 48 hours, after which cells were harvested for protein lysate. Proteins were run on a gel, transferred to a membrane, and Western Blotting was performed for Ptf1a and Cpa1, together with alpha-tubulin as a control for protein loading. Lysate from the mouse 266-6 acinar cell carcinoma line was used as a positive control for acinar-specific proteins.

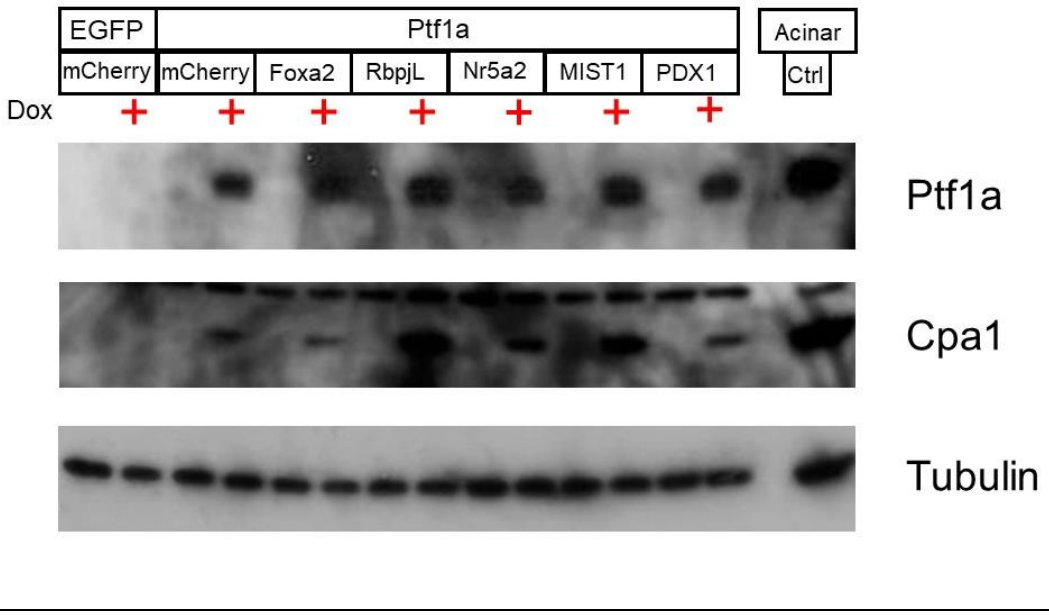


Figure 7. Protein expression of Ptf1a and Cpa1 in single transcription factor infected fibroblast cell lines
266-6 acinar cells were used as acinar controls in the last lane. Expression of Ptf1a is seen in all Ptf1a +Dox samples. Cpa1 is also seen in all Ptf1a +Dox samples, with different band densities. Tubulin serves as a protein loading control.

Ptf1a protein was expressed in all pCLX-pTF-Ptf1a lines when treated with Dox, with apparently similar levels (based on band intensity) across lines (Fig. 7). Cpa1 protein was also expressed in all Ptf1a lines treated with Dox, but with varying intensity. More Cpa1 protein was found in Ptf1a-RbpjL and Ptf1a-MIST1 lines than in other lines. Ptf1a-RbpjL also exhibited increased *Cpa1* mRNA levels, compared to Ptf1a-mCherry (Fig. 6); Ptf1a-MIST1 did not exhibit increased *Cpa1* mRNA, raising the possibility that MIST1 could regulate Cpa1 protein translation or stability. Our alpha-tubulin control was seen at the same intensity in all fibroblast cell lines as expected.

DISCUSSION

Although Ptf1a cannot work alone as a master regulator of acinar identity, it is able to cooperate with other acinar transcription factors to promote expression of some acinar target genes. To our knowledge, no one has ever expressed multiple acinar transcription factors in a heterologous cell type to test their ability to induce acinar-specific gene expression, so this experiment is the first of its kind. In addition, our mRNA and protein expression results indicate that different combinations of acinar transcription factors promote expression of different acinar target genes, which supports the hypothesis depicted in Figure 1B.

Cpa1 expression is highest in Ptf1a-RMN cells, and drops in expression in Ptf1a-RMNFP cells (Figure 6). Thus, the addition of PDX1 and Foxa2 does not appear to be contributing to expression, and in fact may even be slightly inhibiting it. This could be due to Pdx1 and Foxa2 usually being expressed earlier in the pancreatic differentiation process, meaning they are less acinar-specific than the RMN factors (Cleaver et al., 2018). Even within the Ptf1a-RMN combination we can see not all transcription factors are equally contributing to *Cpa1* expression, as Ptf1a-Nr5a2 or -RbpjL alone show higher expression than Ptf1a with other single transcription factors. This same pattern is found in protein expression of Cpa1, although here Ptf1a with Mist1 or RbpjL results in more protein staining than the other single transcription factor cell lines (Figure 7). This difference in Mist1's effect on *Cpa1* mRNA levels versus Cpa1 protein levels may indicate that Mist1 is involved in regulation of Cpa1 protein stability. Ptf1a and Mist1 have been shown to bind to target genes that include secretory protein production, translational regulation, protein processing and folding, and cellular stress response (Jiang et al., 2016). In addition, knockout of Mist1 does not significantly change mRNA levels of Cpa1 (Karki et al., 2015). Therefore, although Mist1 may not be an essential promoter of *Cpa1* transcription, it may be promoting other genes that act in the enhancement of the stability of protein Cpa1.

Cela1 expression may not be induced at all, given that expression levels are extremely low in both GFP and Ptf1a lines. However, there may be a minor (~3-fold) bump in expression in Ptf1a-RMNFP. It is possible that in order to induce *Cela1* expression, another acinar transcription factor not tested in the experiment is needed, or that *Cela1* is regulated by its chromatin state, and under current experimental conditions is inaccessible.

Amylase2 shows a very different pattern of expression than *Cpa1*. *Amy2* expression is not induced at all by Ptf1a alone, but it is promoted both by Ptf1a-Foxa2 and -RMNFP. Clearly Ptf1a in combination with Foxa2 is necessary to promote expression of *Amy2*, while the other acinar transcription factors cannot activate this digestive enzyme even in combination. Interestingly, reporter gene-based studies of the *Amy2* promoter, using the rat acinar carcinoma cell line AR42J, identified adjacent binding sites for Ptf1a and Foxa2/Hnf3β that were both required for *Amy2* expression (Cockell et al., 1995). Our results support a model in which these transcription factors directly cooperate to drive *Amy2* expression.

Among the transcription factors studied in this experiment, Foxa2 is unique as it is considered a "pioneer" transcription factor, meaning it is able to access closed chromatin sites and open them to other factors (Iwafuchi-Doi & Zaret, 2014). Foxa2 has been shown to perform this function in the liver by displacing linker histones and allowing for other liver transcription factors to promote liver gene transcription (Iwafuchi-Doi et al., 2016). It is possible that Foxa2 plays this role in the *Amy2* promotor, opening up chromatin and allowing Ptf1a and itself to induce *Amy2* expression. The pioneer abilities of Foxa have already been used in experiments to convert fibroblasts into hepatocyte-like cells (Huang et al., 2011; Sekiya & Suzuki, 2011), so it would not be surprising to discover Foxa2 playing this role in the induction of acinar-like cells from fibroblasts.

Cela2a is expressed the highest in our Ptf1a-RMN and -RMNFP combinations (Figure 6). As in *Cpa1*, we can also see what transcription factors are contributing to its expression from our single transcription factor infected cell lines, these being Ptf1a-Foxa2, -Nr5a2, and -RbpjL. It appears the synergy between these three factors along with Ptf1a is able to boost expression of *Cela2a* by over 10-fold the amount that any of them can induce on their own.

The maximal expression levels induced by our transcription factor combinations vary by digestive enzyme. *Cpa1* and *Amy2* show the highest maximum expression, up to ~1% of the levels seen in wild-type acinar cells, while *Cela2a* and *Cela1* have lower maximum expression, closer to ~0.01% of acinar levels (Figure 6). None of our digestive enzymes were expressed to

the full level of wild-type acinar cells, so although this experiment reveals important patterns in regulation of acinar identity, it does not represent complete fibroblast-to-acinar cell reprogramming. I will discuss ideas on how to induce higher expression levels of acinar target genes later in this section. It is also important to note that in all of our GFP cell lines, expression of target genes is nowhere near their expression in the respective Ptf1a lines (with the exception of Cela1 where these similar expression levels most likely reflect very low natural expression in fibroblasts). This reinforces our findings that Ptf1a plays a master role in regulation of acinar identity, where its presence is necessary to promote acinar target genes, and even multiple acinar transcription factor combinations such as GFP-RMNFP cannot induce expression without Ptf1a.

There are many potential future directions for this project. We are currently working to repeat these experiments to validate our current data. We will be rerunning qPCR, performing more Western blotting, and possibly conducting immunofluorescence staining. We will also be trying different combinations of transcription factors such as RNF, or RMNF, as RbpjL, Nr5a2, and Foxa2 appear to be contributing to expression of multiple target genes. We can also run qPCR looking at fibroblast specific genes to determine if markers of fibroblast identity are being downregulated while markers of acinar identity are being upregulated. This would help us determine to what degree we are able to reprogram the cells using different transcription factor combinations.

As noted above, we do not induce full expression of any acinar digestive enzymes, and there are a few ways we might be able to increase that expression. Even with the addition of more acinar transcription factors, Ptf1a still may be competing with dense nucleosomes for access to DNA, and we could treat our cells with chromatin-opening drugs to see if this increases target gene expression. One option is the use of the DNA methyltransferase inhibitor 5-Azacytidine, which has been used before in fibroblast reprogramming experiments (Pennarossa et al., 2013). Histone deacetylase (HDAC) inhibitors, which promote the acetylation of histones and opening of chromatin (W. S. Xu et al., 2007) can also be used as a chemical treatment. If expression of an acinar digestive enzyme increases significantly when treated with chromatin opening drugs in addition to acinar transcription factors, this would suggest that the acinar target gene is potentially located within fibroblast heterochromatin. We could also determine if there are any other genes that Ptf1a alone can promote when chromatin is opened and all target genes are presumably accessible.

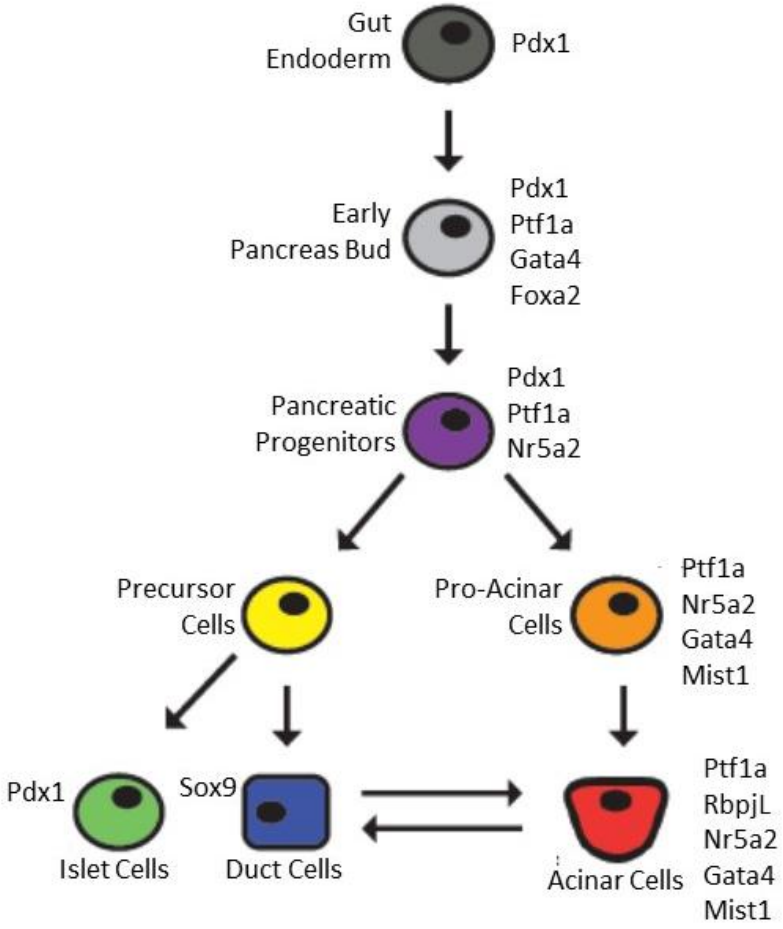


Figure 8. Development of the pancreas and differentiation of pancreatic cell types. The illustrated development of the pancreas, showing how different cell types arise from their progenitors, and what transcription factors are highly expressed at each stage of differentiation.

Another approach that could enhance our reprogramming experiments would be to express pancreatic and acinar transcription factors in a certain order to imitate their order of expression during in vivo pancreas development. We can see in Figure 8 above that transcription factors Foxa2 and Pdx1 are expressed earlier in the differentiation process and are less acinar-specific than factors such as RbpjL, Mist1, and Nr5a2 (Cleaver et al., 2018). Pdx1 is actually later preferentially expressed in islet cells rather than acinar, which may explain why Pdx1 is not as ideal of a transcription factor to promote acinar identity. However, expressing Pdx1 and Foxa2 first in Ptf1a-expressing fibroblast cells, followed by RMN, would emulate their usual expression pattern in pancreatic development. This may allow for more complete reprogramming of fibroblasts to acinar cells, by attempting to take the cells through a progenitor-like stage first. This could be done by infecting *Pdx1* and *Foxa2* into cell lines with a promoter induced by a

drug different than doxycycline. Experimentation could be done to find optimal lengths of time to express each of the "stages" of transcription factors, to promote the highest expression of acinar digestive enzymes.

Ptf1a can work in combination with other acinar transcription factors to promote some of its target genes, although not to wild-type levels of expression. Other combinations of acinar transcription factors, perhaps with different timings of expression or with chromatin opening drugs, may be able to fully reprogram fibroblast cells to an acinar identity. Better understanding acinar differentiation and what transcription factors work with Ptf1a to promote acinar gene expression will provide further insight into how acinar identity is lost during acinar to ductal metaplasia, PanIN formation, and later cancer development. Learning how to promote acinar identity in other cells types could also enable us to design new ways to treat pancreatic cancer through redifferentiation of precancerous or cancerous cells to normal functional acini, or develop superior early cancer-screening techniques by surveying expression levels of key acinar transcription factors. Understanding the cellular identity changes undergone by acini during the development of pancreatic cancer will play an essential role in lowering the mortality rate of this destructive disease.

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