In vitro direct reprogramming of mouse biliary epithelial cells towards an insulin-producing state

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Abstract

Type I diabetes is an autoimmune disease resulting in the destruction of beta cells, the cell type in the body that produces insulin in the pancreas. Direct reprogramming can enable respecification of non-beta cells to produce insulin. The reprogramming vector, Ad-PNM, that expresses the genes Pdx1, Ngn3, and Mafa has reprogrammed non-beta cells of the pancreas and liver to produce insulin and restore normoglycemia in diabetic mice in vivo. Another possible approach is in vitro reprogramming with the same vector using cultured liver cells. Specifically, in this project biliary epithelial cells were isolated from mice, examined for Sox9 expression and treated with Ad-PNM to induce reprogramming.
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Introduction

1. Type I diabetes etiology, approaches to a cure

Diabetes mellitus is a disease that results from an inability to maintain blood glucose homeostasis. This condition can occur because the body does not produce insulin (Type I) or because the body produces insulin, but cells are no longer responsive to insulin (Type II). The lack of insulin production in Type I diabetes (T1D) is caused when the immune system progressively kills beta cells in the pancreas. This progressively leads to diabetes because the only mature cell type in the human body known to produce insulin is the pancreatic beta cell.

Currently, there is no long-term cure for T1D. While diet and exercise also help manage T1D, exogenous insulin injections are required to regulate blood glucose levels. Most patients self-monitor glucose levels, measure and inject insulin (or an insulin-like compound) themselves; use an automated or programmable pump to regulate slower releases of insulin; and practice diet regimens.

Cures for type I diabetes could come from resolving the malfunctioning immune response or from introducing a permanent insulin-producing entity. Despite the advances in immunology in the late 20th century, preventing beta cell destruction is currently a less feasible approach than one compensating for lack of insulin. Thus far, the most successful attempts at a cure for T1D involve allogeneic transplants of whole pancreas and purified islet transplants (Type 1 Diabetes, 2014).

Successful islet transplants can stabilize a diabetic patient’s condition. However, allogeneic islet transplants are currently not a permanent solution: 50% of patients aged 18-35 remained insulin independent 1 year after successful engraftment, and 50% of patients aged 35-<50 remained insulin independent 2 years after engraftment in North American, Australian, and European patients (CITR Seventh Annual Report, 2011). Transplanted patients most often become insulin dependent because the immune system will recognize the donor islets as non-self and attack them despite the regime of immunosuppressive medication that accompanies the allogenic transplant.

T1D not only reduces quality of life, it also reduces the patient’s lifespan. Historically, individuals with T1D had reduced life expectancy of nearly 25 years, although recent studies have projected that current young adult T1D patients will live only 10-15 years less than their non-diabetic peers (Nainggolan, 2013). Despite recent clinical improvements in injectable
insulins, islet transplants and medical devices such as insulin pumps and continuous glucose monitors, a permanent restoration of insulin production would restore the personal freedom and quality of life to the 30,000 people diagnosed every year in the US (Type 1 Diabetes Facts, 2015).

2. Brief overview of liver development
The development of endodermal organs is highly conserved in vertebrates. In mice, after formation of the three germ layers (ecto-, endo-, mesoderm), the endoderm undergoes spatial rearrangements resulting in three endodermal subregions including the fore-, mid-, and the hindgut (Zorn, 2008). In mice this definition occurs by E8. The lung, liver, pancreas, and gall bladder all develop from the foregut region of the endoderm.

In liver development, precursor cells (shown by lineage tracing studies) that commit to the hepatic lineage are initially surrounded by mesoderm stromal cells that include cardiac mesodermal cells. After changes in gradients of Wnt and Fgf4 signalling, these surrounded cells begin to express the homeobox family transcription factor, Hhex, and decrease laminin (Lam) expression and secretion (Zorn, 2008). Decreasing laminin secretion allows the progenitor cells increased mobility, allowing them to migrate away from contact with cardiac mesoderm. Enclosed in a new pocket of mesodermal cells, the region in which the newly migrated cells reside is termed the Septum Transversum (ST). At this point (E9.5 in mouse development), the newly relocated cells proliferate and along with contact-dependent signaling from the new mesoderm they alter their transcriptional programs, defining them as hepatoblasts (Zorn, 2008).

Once the hepatoblasts have proliferated to sufficient density and have formed defined outgrowths of cells, termed “chords”, the liver bud is said to have formed (Zorn, 2008). This time has been determined as E10.5 in mouse development, and coincides with the infiltration of hematopoietic cells. As the hepatoblasts continue to develop the architecture of the liver they express notable protein markers such as Epithelial Cell Adhesion Molecule (Epcam), α-fetoprotein (Afp), and E-cadherin (Ecad) (Zorn, 2008).

Around E13 hepatoblasts in the liver bud express genes found in adult hepatocytes as well as bile duct cells, such as hepatocyte nuclear factor 4 (Hnf4), albumin (Alb); and cytokeratin 19 (Ck19), Hnf1β, respectively. By E14 differential expression patterns can be
seen histologically. Hepatoblasts most distal to portal veins do not express Sox9, and increase expression of genes such as Alb, Cebpa, Hnf1a, Hnf4a. These cells are considered committed to the hepatocyte lineage as time progresses they develop longer hepatic chords and mature to hepatocytes (Zorn, 2008).

Hepatoblasts most proximal to portal veins will maintain Sox9 expression and have lower expression of hepatocyte-specific genes. Such single cell layers that line the stroma of portal veins are termed a ductal plate. Ductal plate hepatoblasts notably express osteopontin (Opn), Epcam, Ck19 in addition to Sox9. Previously, cells identified in this location were considered committed to the bile duct/cholangiocyte lineage (Zorn, 2008).

At approximately E18 in mice (a few days before birth), distinct populations of hepatocytes and cholangiocytes are detectable with architecture similar to that observed in the livers of adult animals. This maturation of sheets of cells near ductal plates ultimately develops into the intrahepatic hepatocyte-surrounded bile duct tracts throughout the lobes of the liver (Figures 2, 3, 4).

3. Developmental relatedness of pancreas and liver

The liver and pancreas develop from the definitive foregut endoderm and their precursor populations diverge during mid-gestation in mice. Their divergence is largely due to relative proximity to the cardiac mesoderm and ST as well as the notochord. After the nascent organs have accumulated significant mass, liver and pancreatic precursor cells begin differentiating toward more specialized cell types recognized in the mature animal, and it is
at this time that Sox9 expression is detected in each organ during late gestation. Sox9 expression was long thought to define ductal plate cells as committed bile duct cells in the liver. Sox9, Hnf1b, and Ngn3 co-expression is observed in cells that later become pancreatic endocrine and pancreatic ductal cells. Because of the similar Sox9 expression in late gestation, and the common foregut endodermal origin of the liver and pancreas, it is plausible that Sox9 expression could be an important factor in converting a liver cell program to a pancreatic cell program and vice versa.

4. Basic liver biology

The liver is known to be a highly regenerative organ as well as metabolically active organ. Hepatocytes comprise the liver parenchyma and make up the 80% of organ volume (Miyajima, A., Minoru, T., & Itoh, T., 2014). Another other major cell type of endodermal origin in the liver is the biliary epithelial cell (BEC) or bile duct cell (BDC). Mesoderm-derived cells include endothelial cells lining the vasculature and sinusoidal endothelial cells lining the fenestrations of the basolateral side of hepatocytes, hepatic stellate cells, lymphacytic pit cells and Kupffer cells (lumenal macrophages) (Tanaka, M., Itoh, T., Tanimizu, N., & Miyajima, A., 2011).

As polarized epithelial cells, hepatocytes take up various compounds (nutrients, toxins, metabolites, etc) through their basolateral side. Blood flows along their basolateral side after entering the liver from the small intestine and from whole body circulation; these blood supplies enter via the portal vein and hepatic artery respectively. The flow of blood along the basolateral side of hepatocytes (as well as that of BECs) moves from the portal vein and hepatic artery towards the central vein. In contrast, the flow of bile (the main product secreted by hepatocytes) flows along the apical side of hepatocytes and moves toward bile ducts (Figure 2). Before bile is transported through the tubular biliary network, bile duct cells will absorb and secrete ions to modify the bile that enters the gall bladder (Yang et al., 2010). The region in which extensions of the portal vein, hepatic artery, and intrahepatic bile ducts are clustered is called a portal triad.
Figure 2. Activity within liver lobule. Blood enters the liver through extensions of the hepatic artery and portal vein and a central vein collects blood that runs along hepatocytes. Hepatocytes metabolize and secrete various compounds from and to the blood supply as well as produce bile that is secreted and flows toward the bile ducts. Adapted from http://www.daviddarling.info/encyclopedia/L/liver.html

Figure 3. Liver lobule structure. A central vein is equidistant from 6 portal triads that contain extensions of the portal vein, hepatic artery and bile duct tracts. Adapted from http://mkk.szie.hu/dep/aeet/tanweb/liver/liver0/liver.html
5. Rationale for reprogramming to “beta-like” cells

Stem cells are cells that have the ability to 1) self-renew indefinitely and 2) have the capacity to differentiate (adopt a more specialized phenotype). Stem cells exist along a spectrum, with regards to mammalian species, the zygote is the first cell of any organism. The zygote is totipotent in that it can become all cells of the embryo and the extra-embryonic tissue that facilitates growth of the embryo inside the uterus. At the other end of the spectrum is an adult stem cell, such as the satellite cell, which can only replace a few cell types, e.g. muscle fibers and more satellite cells.

Study of totipotent cells was documented as early as the 1880s (De Robertis, 2006). However, most recent studies focused on human pluripotent stem cells are based on the work with embryonic stem cell (ESC) lines initiated by Jamie Thomson and induced pluripotent stem cells (iPSCs) reported by Shinya Yamanaka and colleagues. Work with pluripotent stem cells (cells that can differentiate into any cell type of the embryo) has introduced new potential approaches to cure diseases that arise from dysfunctional cell activity: whether disease-associated cells are aged, metabolically abnormal, or even selectively destroyed by the immune system. The idea behind such cures is that the ability to control the end phenotype of a multipotent cell provides a reserve population of ‘healthy’ cells that can

Figure 4. Vessel organization within the liver. Branches of the hepatic artery, hepatic vein, portal vein, and bile duct extend throughout the lobes of the liver. Adapted from http://profmohammadali.com.bd/pioneerhepa.php
displace or replace a disease-causing population. This healthy reserve population can be
directed through identity change toward a more differentiated, specialized cell type of
interest.

In changing the identity of any particular cell the overarching questions become: what
is the starting cell type and what is the end goal? One answer has been to start with
undifferentiated, pluripotent cells that are either embryonic stem cells or iPSCs. The end goal
has often been the differentiated cell type that is damaged in a disease, such as a neuron,
hepatocyte or cardiomyocyte (Shi, Y., Kirwan, P, & Livesey, F. J., 2012; Cai et al., 2012;
Lian et al., 2013).

Another approach to changing cell identity has been directly reprogramming a mature,
differentiated cell type into another mature, differentiated cell type without a stable
progenitor (e.g. pluri- or multipotent) intermediate. Advantages may include shorter timelines
and less cell loss in the process of manipulating the population; these aspects are particularly
useful for implementing the product as a cell-based therapy. Direct reprogramming has been
demonstrated in vivo as well as in vitro using virally delivered transgenes and transgene-free,
chemical/protein combinations (Richard et al., 2011; Masuda et al., 2013).

Direct reprogramming has also been implemented in the development of a T1D cure.
Ideally, cells from the same patient or from a donor would be converted to a stable cell type
that produces insulin to replace that critical function of beta cells that is most damaging in
Type I diabetes. There are several potential starting cell types that are related to beta cells,
including other endocrine cells of the pancreatic islet: alpha, delta, and pancreatic-
peptide (PP-) cells (Steiner et al., 2010). Due to the embryonic formation of foregut
endoderm organs, the cells of the gall bladder and liver, in addition to non-beta cells of the
pancreas, all provide developmentally relevant cells for possible reprogramming into a stable
insulin-producing cell.

Using transcription factors to reprogram cells may require relevance to an organ’s
developmental biology. For re-specification towards a beta cell identity, the most successful
candidates have been Pancreatic-Duodenal Homeobox 1 (Pdx1), Neurogenin 3 (Ngn3), and
V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A (Mafa). Pdx1 drives
the early pancreatic program in the early pancreatic epithelium, it induces expression of
Insulin, Glut2, Nkx6.1, Nkx2.2. Ngn3 is expressed during neuronal and pancreatic
development. *Ngn3* expression promotes pancreatic progenitor cells commit to non-exocrine lineages. *Mafa* is expressed in mature beta cells and is considered critical to beta cell identity and insulin expression.

In 2008, Zhou et al. showed that infecting mouse pancreatic tissue with 3 separate adenoviruses encoding *Pdx1*, *Ngn3*, and *Mafa* sequences was effective in converting amylase+CK19−insulin−exocrine cells to insulin+c-peptide+ beta-like cells *in vivo* (Zhou et al., 2008).

Akinci et al. (2012) cloned the genes for *Pdx1*, *Ngn3* and *Mafa* into a single adenovirus vector, referred to as Ad-PNM (*Figure 5*). Tail-vein injection of Ad-PNM into NOD-SCID mice localized Ad-PNM infection to liver cells and was found to produce ectopic ducts containing insulin+, c-peptide+ cells (Banga et al., 2012). Furthermore, the Slack laboratory found that injection with Ad-PNM was also able to stably reverse diabetes for 4 months in treated NOD-SCID mice.

Newport Green dye was used to isolate insulin-containing cells from whole liver single cell suspensions 12 weeks after Ad-PNM administration (Banga et al., 2012). Newport Green is a fluorescent molecule that has an affinity for zinc which is necessary to form insulin granules in beta cells. After fluorescence activated cell sorting (FACS) for Newport Green dye, the group conducted a RT-PCR-based expression study. This showed more than fifteen-fold increases in *Insulin1*, *Insulin2*, and endogenous *Pdx1* expression in Newport Green+ cells compared to the relative expression of these genes in Newport Green− cells. A ten-fold difference could be seen for beta cell-specific transporter, *Slc30a8*, endoderm marker, *Sox17*, and five-fold difference for *Glut2*, among other beta cell and endocrine-specific genes. The expression analysis demonstrated transcriptional changes present in the cells as a result of the Ad-PNM-reprogramming.

![Figure 5. Ad-PNM construct. *Pdx1*, *Ngn3*, and *Mafa* sequences were cloned into one single stranded vector. 2A peptide sequences ensure 3 independent peptide sequences are produced from one polypeptide. Adapted from Banga et al. (2012)](image-url)
The Slack group’s data suggested that using a gene therapy construct like Ad-PNM could provide a Type I diabetes therapy using non-pancreatic starting cell types. The liver is a healthy, highly regenerative organ that can serve as an autologous or allogeneic starting cell source.

The Slack group also examined the starting identity of reprogrammed cells. Immunostaining of sections from mice sacrificed at 12 weeks post infection demonstrated ectopic ductal cells were absent of the hepatocyte protein albumin and expressed the bile duct cell marker, cytokeratin 19 (Ck19). Immunostaining further indicated that insulin$^+$ ectopic ductal cells were often also Sox9$^+$. The group then used Cre-mediated lineage tracing to label Sox9$^+$ ductal plate cells in Sox9-CreERT2;mTmG reporter mice injected with tamoxifen at E15.5. Histological sections of adult mice showed that the labelling in these experiments was highly specific to bile duct cells of the liver and immunostaining of these sections showed that some of the lineage-labelled ($mGFP$-expressing) bile duct cells continue to express Sox9 in the adult mouse (Banga et al., 2012).

Additional information about the reprogramming process came from experiments using the peroxisome-proliferator-activated receptor $\alpha$, $\gamma$ (PPAR$\alpha$, $\gamma$) agonist, WY14643 (Banga et al., 2014). The Slack group administered Ad-PNM via tail vein injections into immunocompetent mice with a CD1 background. WY14643 was added to the animals’ chow for 4 days. After gene delivery the total time before sacrifice was either 1 or 6 weeks.

1 week after Ad-PNM administration, hepatocytes and ductal cells were observed to be insulin$^+$, WY14643 treatment resulted in EdU labelling of hepatocytes and bile duct cells, and EdU incorporation was associated with Sox9-expressing cells (Banga et al., 2014). 6 weeks after Ad-PNM administration, insulin$^+$ cells included only those within ductal structures, expressing bile duct and beta cell markers such as Ck19, EpCAM, and c-peptide, respectively.

Despite a lack of immunohistochemical data demonstrating co-expression of Sox9 and Insulin or Sox9 and Ad-PNM genes in the same cells at 1 week post infection, the above results suggested that successful reprogramming with Ad-PNM is increased with actively cycling, Sox9-expressing, periportal ductal cells. Due to the low frequency of insulin$^+$ ductal cells 6 weeks post-infection, it appears that only rare cell populations of the periportal bile duct region will stably express Insulin after Ad-PNM infection.
Collectively, the data with the NOD-SCID mice and CD1 mice given WY14643 suggest that periportal ductal cells and hepatocytes can be reprogrammed after virus administration, however cells that undergo stable reprogramming after 6 and 12 weeks have a ductal morphology. Additionally, reprogramming efficiency is influenced by active cell cycling. The possible origin of cells that stably express Insulin and other endocrine genes at 6 and 12 weeks post viral administration include a bipotent resident Sox9+ progenitor cell, cycling hepatocytes or BECs, and/or periportal hepatocytes and BECs already in an altered state. The data from the Slack Lab and recent work evidencing resident Sox9-expressing cells in the adult mouse liver suggest that the origin of stably reprogrammed cells is a ductal bipotent progenitor (Banga et al., 2012; Furuyama et al., 2009).

From the previous studies, this thesis work aims to address: First, can Sox9+ ductal cells be isolated from mouse liver and cultured? Second, can they be reprogrammed in vitro into stable beta-like cells that produce insulin? The answers to these questions intend to provide proof of principle evidence that a liver-based, autologous biopsy-transplant ex vivo reprogramming regimen is a possible Type I diabetes cure.
Methods

1. Liver Ductal Cell Harvest

C57BL/6 background, Sox9-CreERT2;mTmG mice and CD1 mice were used in experiments. They were housed in the McGuire Translational Research Facility at the University of Minnesota and cared for by the Research Animal Resources (RAR) staff under The University of Minnesota Animal Care and Use Committee guidelines.

Sox9-CreERT2;mTmG mice were labelled with tamoxifen in utero at E18.5 by intraperitoneal injection of mothers with 3mg tamoxifen dissolved in sunflower seed oil. Labelled mice were sacrificed at 6-8 weeks of age. To preserve the health of cells in tissues, particularly in highly perfused organs like the liver, mice were sacrificed by cervical dislocation according to IACUC guidelines. Liver removal shortly followed.

2. Liver digestion, ductal cell isolation

Livers were removed within 10 minutes of sacrifice and transferred to a BSL2 hood in 10cm petri dishes. Within 20 minutes of sacrifice removed livers were minced with scalpels until tissue was smooth and uniform. Each adult liver was placed in 50mL of dissociation buffer (10mM HEPES with pH 7.4, 5mM KCl, 0.5mM EGTA, 5mM Glucose, 1X penicillin/streptomycin (from 100X stock), 1X anti/anti (from 100X stock), 250mM gentamicin in Earle’s Buffered Salt Solution (EBSS)) and placed in a 37°C water bath for 50 minutes, with shaking agitation every 5-10 minutes. Tissue was then centrifuged for 6 minutes at 900 rpm. Pellets were resuspended in 25mL of digestion buffer (1.67mg/mL collagenase type II (Invitrogen), 1.33mg/mL bovine hyaluronidase (Sigma Aldrich), 5% FBS,1X anti/anti, 1X penicillin/streptomycin, 250mM gentamicin in DMEM:F12). Tissue was incubated again in a 37°C water bath and inverted as well as mixed with a pipet every 10 minutes. After 45-50 minutes 1.34units/mL of dispase (BD Biosciences) were added and liver contents incubated for 30-40 minutes longer in a 37°C water bath. Pellets were washed 4 times in 40X volumes of washing media (5% FBS, 1X anti/anti, 1X penicillin/streptomycin, 250mM gentamicin in DMEM) with centrifuging settings at 1000 rpm for 5 minutes. Pellets were then treated in one of two ways:

1) Resuspension in complete Keratinocyte-Serum Free Medium (Life Technologies; 250mM gentamicin, 1X anti/anti, 1X penicillin/streptomycin) for 1-2 days suspension
culture in 2 wells of ultra-low attachment plate (Corning), collected and resuspended in 15% FBS Williams E medium (supplemented with 20ng/mL hEGF, ITS-X, 250mM gentamicin, 1X anti/anti, 1X penicillin/streptomycin, 1X NEAA) for an additional 1-2 days in suspension culture.

2) Resuspension in 15% FBS Williams E medium for 1-2 days followed by suspension culture in complete KSFM for 1-2 days suspension culture.

The two methods of suspension culture were used to assess whether one mitigated mesenchymal cell co-culture better than the other. No difference was observed. Ductal cell aggregates were formed in suspension culture 3-4 days after isolation. After one of the two suspension culturing methods, contents of wells were collected and filtered through 37µm reversible PES filters (StemCell Technologies) with sterile PBS. The large fraction, >37µm, was centrifuged at ~1000 rpm for 5 minutes, resuspended and plated.

3. Ductal cell culture

For infection, immunostaining or in suspension for recollection, cells were plated in 15% FBS Williams E medium and were kept in a 37°C, 5% CO₂ incubator. Media was changed every 2 days unless necessary to change daily in wells with larger cell numbers.

For plating cells in collagen sandwiches, 2-3mg/mL rat tail Type I collagen (Millipore) stock was used to make the 3D matrix. Ice cold Type I collagen stock was diluted into a tube of 10X Williams E and 0.34N NaOH with a ratio of 8:1:1 respectively. Approximately 20% of well volume was initially plated; after the matrix solidified a cell suspension was added. Cells adhered overnight and the next day media was removed and the second half of ~20% well volume was added. The homogenous mixture was aliquoted to 6-well plates so that once a sandwich was complete, the volume of each well was 40% full with matrix. After solidification 1.5-2mL of media were added. Purified cells from one liver were plated in 1 or 2 collagen sandwiches of a 6-well plate to allow space for population expansion.

For passaging cells out from collagen, serum-containing media was removed and 2mL of 20mg/mL Type I collagenase (Worthington) was added. Cells were incubated for 15-20 minutes with pipet agitation until the collagen matrix was disassembled. Cells were washed 5 times with 40x media volume in 10% FBS DMEM washing media. The final pellet was resuspended in plating media, gently mixed with a pipet and plated onto first layers of
collagen I matrix in new wells at a 1:2 dilution.

4. Ad-PNM infection

Dutton Lab Batch A4 virus was amplified and titered by Caitlin Hill, MS. Batch A4 led to the highest levels of Pdx1 and Insulin expression in treated AR42J-B13 cells, a rat exocrine cell line (Longnecker et al., 1979), with 90% of cells coexpressing Pdx1 and Insulin (Hill, 2014). The titer was determined to be 1.2 x 10^11 infectious units/mL (ifu/mL).

Aggregated cells were treated with 0.25% Trypsin/EDTA for 5 minutes before being transferred to wells of 6-well type I collagen-coated BioCoat plate (Corning BioCoat Cellware) before infection. They were then incubated with 1µg/mL polybrene (PB) for 5 minutes. As the optimum titer of Ad-PNM virus for primary mouse liver cells was unknown, virus was added in one of three stock dilutions: 10^{-3}, 10^{-5}, 10^{-6}. ‘No PB, no virus’ and ‘no virus’ controls were used. ‘No PB, no virus’ controls were used in addition to ‘no virus’ controls to evaluate any toxicity of polybrene. No toxicity was observed. No virus controls were used to examine whether Ad-PNM was the cause of any Pdx1 and Insulin expression. All controls showed no Pdx1 or insulin by immunostaining. Cells were incubated for 2 days after which cells adhered and the media was changed.

5. Immunostaining

Aggregated cells were plated on BioCoat plates for immunostaining post isolation and post infection. Cells were washed twice with PBS and fixed with 10% formalin for 10 minutes. 10% formalin was removed and cells were washed three times with PBS-T. Cells were either covered with PBS and placed in 4°C for storage or permeabilized with 0.2% Triton-X100 PBS for 20 minutes at room temperature (RT). Cells were blocked with 5% donkey serum, 0.5% BSA, PBS-T for 30-40 minutes at RT. Antibodies were added at respective dilutions to 1% BSA PBS-T (Table 1). Incubation with primary antibodies occurred overnight at 4°C, and with secondary antibodies at RT covered on the benchtop for 1 hour. Cells were washed three times with PBS-T between antibody incubations and finally covered in PBS. DAPI (10mg/mL stock) was added to each well at a 1:1000 dilution. Cells were imaged using inverted microscope Leica DMI 6000B.
Table 1. Primary antibodies and dilutions

<table>
<thead>
<tr>
<th>Primary</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Millipore rabbit Anti-Sox9</td>
<td>1:1000, 1:5000</td>
</tr>
<tr>
<td>Santa Cruz goat Anti-Cytokeratin 19</td>
<td>1:75-1:50</td>
</tr>
<tr>
<td>chicken Anti-Vimentin</td>
<td>1:1000</td>
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<tr>
<td>SCBT rat E-cadherin</td>
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<td>Millipore rabbit Anti-Pdx1</td>
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<tr>
<td>Abcam guinea pig Anti-Insulin</td>
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Table 2. Secondary antibodies and dilutions

<table>
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<th>Secondary</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Alexa Fluor 488 donkey anti-rabbit</td>
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</tr>
<tr>
<td>Alexa Fluor 555 donkey anti-goat</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 633 goat anti-chicken</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-guinea pig</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 488 donkey anti-rat</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Results

1. Use of Sox9-CreERT2:mTmG mouse

Several studies using chronic injury models and viral infection for lineage-tracing have revealed a proliferative, ductal cell-like population that expresses Sox9 near the portal triad. The interpretation was that the observed cells are a reactivated Sox9-expressing resident progenitor population in adult liver of at least rats and mice that is bipotent and able to give rise to hepatocytes and BECs. Based on this idea of a Sox9+ liver stem/progenitor cell (LSPC), embryonic lineage tracing was used by Banga et al. (2012) to investigate whether the successfully reprogrammed cells were progeny of a once Sox9-expressing parent population. In uninfected reporter mice labelled at E15.5, mGFP+Sox9+ ductal cells were present. Subsequently, in labelled mice treated with Ad-PNM ectopic ductal cells were mGFP+insulin+ (Banga et al., 2012). Based on these data, possible target cells for stable reprogramming in adult mice are ductal progeny of embryonic Sox9-expressing liver cells, specifically BECs and, if present, LSPCs. Hence, embryonic labelling of Sox9-reporter mice was continued for in vitro infection with Ad-PNM to examine reprogramming in ductal cells of Sox9-lineage. If Sox9-expressing LSPCs with ductal morphology are present in adult mouse livers, these cells would be isolated along with BECs.

At E15.5, ductal plate cells (a late-stage hepatoblast population) begin their final stages of committing to the ductal lineage. At the time of the initial experiments with Ad-PNM, it was commonly thought that between E15 and E18 hepatocytes and BECs are committed, and BECs as well as LSPCs are established from ductal plate cells. To label Sox9-expressing cells at late gestation, pregnant mothers were injected with tamoxifen at E18.5 for convenience. Labelling with tamoxifen injection at E18.5 will not label all bile duct cells as some Sox9-expressing progenitors presumably have committed to non-Sox9-expressing bile duct cells by this time.

2. Ductal cell isolation and initial cell culture

After the isolation procedure, initial suspension cultures showed that not all cells in aggregates were labelled (mGFP+) indicating that they were not progeny of cells labelled by Sox9 driven CreER expression at E18.5 (Figure 6). Additionally, not all aggregates were free from ‘contaminating’ co-culturing mesenchymal cells after 1-2 days of suspension culture in
serum-free media.

Aggregates were purified after filtering with a 37µm strainer, however batch-to-batch variation did not allow a consistent understanding of co-culturing cell levels (*Figure 7*).

*Figure 6. Labelled and unlabelled ductal aggregate cells in suspension. Lineage-labelling was done by tamoxifen injection at E18.5. Adult livers were digested, aggregated cells filtered and cultured in suspension. (A) Pre-filtration, not all ductal aggregates contain labelled (mGFP⁺) cells. (B) A typical aggregate is shown. Cells are 3 days post isolation. Red= mTomato, Green= mGFP. Scale bars= 50µm*
Figure 7. Aggregates before and after filtration. Aggregates 3 days (A,B) and 4 days (C,D) post isolation, pre (A,C) and post (B,D) filtration with 37µm strainer. Magnified views of aggregates pre- (E) and post (F) filtration. Scale bars= (A-D) 250µm, (E,F) 50µm

3. Short-term culture on collagen I coated surface

As enzymatic digestion is crude and no FACS or MACS system was used to select for Sox9-
expressing and/or ductal cells, immunostaining after cell enrichment (suspension culture followed by filtration) was used to survey the identity of cells. For immunostaining the cells were plated on Corning BioCoat plates which are coated with a thin layer of type I collagen.

Characterization of the populations described the relative proportion of putative LSPCs based on commonly used markers: Ck19 and Sox9. Fixation for immunostaining was performed 5-7 post isolation.

Despite batch-to-batch variation, plated aggregates contained many Sox9⁺ cells, however the relative proportion of Sox9⁺ cells per isolation was not quantified (Figures 8,9,10). The morphology of Sox9⁺ cells was mostly ductal with some mesenchymal cell co-culturing (Figure 8). Interestingly, some cells with tightly defined cell borders exhibited more discriminate Sox9 immunostaining. An incidence of more discriminate Sox9 immunostaining in tight epithelial cell clusters could indicate that widespread Sox9 detection in less tightly packed epithelial cells is evidence of stress from the isolation procedure and dedifferentaion upon culturing (Figure 9B,D). E-cadherin (Ecad) and Sox9 immunostaining was done as well, Ecad expression was weak however present, this may be due to an old secondary antibody (Figure 9). Ck19 co-stained with Sox9 in cells with epithelial morphology (Figure 10). No Ck19⁺ cells were found to be Sox9⁻.

Based on the morphology of the flat, extended Sox9⁻Ck19⁺ cells the question arises whether culturing induces dedifferentiation and if so, do plated Ad-PNM infected cells experience dedifferentiation that significantly alters the ability of cells to reprogram compared to in vivo Ad-PNM infection? Immunostaining for other BEC-specific antibodies might be used and qRT-PCR done with lysates of isolated aggregates to look for stress response and biliary gene expression, and verify Sox9 and Ck19 expression.

Though less specific, suspension culture is possibly a less stressful isolation method than MACS or FACS. A less stressful procedure encourages the preservation of cell identity and may better serve in vitro reprogramming. However, the methods used here are inconclusive regarding the viability and purity of cells post-isolation. The isolated cells in the aggregates are mostly Sox9⁻Ck19⁺ with ductal morphology, however variable morphology would seem to indicate differences in the identity/health of the early culture populations.
Figure 8. Characterization of adherent aggregate cells with immunostaining. (A,C,E) Phase images of aggregates show ductal morphology and compact groups of cells. (B,D,F) Sox9+ cells are surrounded by co-culturing fibroblasts. Cells analyzed 5 days post isolation. Red= Sox9, Green= Vimentin. Scale bars= (A,B)250µm, (C-F)50µm
Figure 9. Epithelial characterization of adherent aggregate cell with immunostaining.

(A,C,E,G) Phase images of adherent aggregate cells. (B,D,F,H) Weak E-cadherin detection in Sox9\(^+\) cells. Cells analyzed 7 days post isolation. Red = Sox9, Green = E-cadherin. Scale bars = 50\(\mu\)m
Figure 10. Ductal characterization of adherent aggregate cells with immnostaining. (B,D) Phase images of adherent aggregate cells. (A,C,E) Cytokeratin 19$^+$ ductal cells are Sox9$^+$. Cells analyzed 6 days post isolation. Red= Cytokeratin 19, Green= Sox9. Scale bars=50µm
4. Infection of short-term cultured cells

Treatment of cells with Ad-PNM was performed with aggregates 3-4 days post isolation. Short-term infection was defined as culturing cells for 7 days or less after exposure to the virus (11 days or less post isolation). The outcome determining successful infection was Pdx1 protein detection and for successful reprogramming detection of insulin protein by immunostaining. Corning BioCoat plates were used for plating infected cells.

Upon examining mGFP (embryonically labelled using Sox9 Cre^{ER}) and mTomato (unlabelled)-expressing cells for any bias in infection rates, there was no observable difference in Pdx1 expression 5-7 days after infection (Figure 11 A,B,D). This observation was seen in several rounds of infection. There was no observable toxicity from polybrene administration, and no Pdx1 expression was detected in ‘no virus’ controls.

The \(10^{-5}\) virus dilution was found to maximize infection with minimal cell loss (by visual observation). Reflective of the variable sizes of ductal cell aggregates, Pdx1 detection varied from 0.7% to 18.1% of counted cells, with higher percentages correlating with smaller patches of cells (Table 3). The groups of cultured cells used for counting contained at least 100 cells. A lack of consistently sized and dissociated aggregates inherently led to an inconsistent MOI among cells within the same aggregate and among all the cells in a well. Inconsistent cell numbers and inconsistent MOI are significant barriers to assessing infection rates.
Table 3. Counts of Pdx1-expressing cells

<table>
<thead>
<tr>
<th>Percent Pdx1+</th>
<th>Cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5%</td>
<td>n= 106</td>
</tr>
<tr>
<td>18%</td>
<td>n= 155</td>
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<tr>
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</tr>
<tr>
<td>0.7%</td>
<td>n= 429</td>
</tr>
<tr>
<td>1.8%</td>
<td>n= 446</td>
</tr>
<tr>
<td>5.3%</td>
<td>n= 469</td>
</tr>
</tbody>
</table>

Table 3. Counts of Pdx1-expressing cells after infection with $10^{-5}$ dilution of Batch A4 Ad-PNM virus. Mean average = 5.4%, Range = 0.7-18%. Aggregate cells were infected in suspension, cells were analyzed by immunostaining 5 days after cells adhered to plates. Data from cells isolated from two mice.

Despite detection of Pdx1 expression at 5 and 7 days after infection, no insulin was detected in cells from 3 different mice infected with Ad-PNM. It is possible that the culture conditions inhibited efficient reprogramming and/or insulin expression would not occur until more than 7 days after infection. Additionally, dissociation of the aggregates would allow increased access of virus to all cells (to increase MOI), however dissociation disrupts the cell-to-cell contacts normally experienced by epithelial cells. This disruption may affect the reprogramming of ductal cells.
Figure 11. Infection of adherent aggregate cells. Upon infection aggregates were 3-4 days post isolation, infected cells adhered to the plate surface by 6 days post isolation, and fixation occurred 11 days post isolation. (B, C, D) Pdx1 is expressed in lineage-labelled (mGFP⁺) and unlabelled (mTomato⁺) cells. (A, E, F) Phase images show morphology of infected cells. Red=mTomato, Green= mGFP, Magenta= Pdx1, Blue = DAPI.

Scale bars= (C, F) 50µm, (A, B, D, E) 100µm

5. Long-term culture: duct-forming cells

Expansion of target cells would likely be necessary for a clinically relevant ex vivo
reprogramming procedure. This could be done before reprogramming or after. To gauge the
difficulty of expanding before reprogramming, long-term culturing of isolated liver cells was
attempted. After ductal cell collection, a Type I collagen matrix was used for 3D culture of
aggregates.

A simple Type I collagen matrix and a previously used epithelial cell culture medium
formulation led to expansion of cells in a collagen “sandwich”. Passaging was done
infrequently, once a month, and culturing of populations was done for up to 6 months.

As no expression levels were measured nor proteins labeled with immunostaining, the
identity of the cells after 5 or 6 months is unknown. 3D culturing encouraged tubular
extensions (Figure 12). Morphology of \textit{mGFP}-expressing (\textit{Sox9} lineage-labelled) cells was
not distinctly different from that of \textit{mTomato}-expressing (unlabelled) cells. Passaging
encouraged growth and tubular structures that are consistent with duct-forming epithelial cell
cultures (Sirica, A. E., & Gainey, T. W., 1997).

Cells from one isolation procedure experienced a great deal of stress in their first
month of 3D culturing (Figure 12A,B,C,E; Figure 13). Less cellular debris and mesenchymal
cells were removed and cells were mixed with the ‘wet’ collagen mixture before the matrix
was formed. These cells also experienced less frequent media changes in their first month of
culture, approximately once a week. However, they were able to be passaged four times into
type I collagen sandwiches and continued to proliferate. Cultures appeared to decrease their
doubling time with each passage. Small hollow structures were observed that contained
unlabelled (\textit{mTomato}⁺) cells after 6 months of culture (Figure 13). It is possible that cells
from the original aggregates were enriched for a robust, proliferative ductal cell population.

The culturing techniques used allowed expansion and formation of ductal structures
within a Type I collagen matrix. Nonetheless culturing the cells in a less dynamic and
complex system is likely to lead to gene expression changes that through many passages may
induce larger phenotypic changes. To encourage conservation of starting cell identity, more
physiologically accurate culture conditions should include choosing a matrix with more liver-
specific ECM proteins, e.g. Type IV collagen and laminin, as well as a matrix that is matched
to liver ECM mechanics/mechanical forces and additional soluble factors added to the
medium.
Figure 12. Long-term 3D culture of ductal aggregate cells. (A,B,C) Branched structures with hollow ductal extensions formed after 2 months of 3D culture in Type I collagen matrix. (D) Cellular extensions can be seen 2 weeks after passaging (3 months of 3D culture). (E) Passaged cells from the culture shown in (A-C) continue to form branched structures after 6 months of culture. No morphological differences were observed in labelled (mGFP⁺) and unlabelled cells (mTomato⁺). Red=mTomato, Green=mGFP. Scale bars= (C,D) 50µm, (E) 250µm
Figure 13. Ductal structures reform after multiple passages. (A,C,E) Unlabelled (mTomato-expressing) ductal cells proliferate and form hollow structures after fourth passage, 6.5 months of 3D culture in Type I collagen matrix. (B,D,F) Phase. Scale bars= 50µm
Discussion

1. Efficacy of ductal cell isolation, in vitro short-term reprogramming

The crude methods described allow predominantly Ck19^+Sox9^+ ductal cells to be collected as aggregates 3 days after isolation from whole livers. The relative proportion of Ck19^+Sox9^+ ductal cells was inconclusive and multicellular aggregates contained both Sox9 lineage-labelled and unlabelled ductal cells. Labelled and unlabelled cells maintained the capacity to form ductal structures and proliferate after months of culturing in a 3D Type I Collagen matrix. The identity of cells after long-term culturing is unknown, however it is possibly altered due to lack of a physiologically matched matrix and soluble factors in the medium.

Ad-PNM infection of ductal aggregate cells occurred with a range of 0.7-18.1% Pdx1^+ cells. Variability in infection rates among ductal aggregates was most likely due to variability in aggregate size. The vitality of these cells is uncertain in vitro as well as the degree of reprogramming. Insulin expression was not observed in cells that were treated with Ad-PNM and cultured for 7 days. In vitro reprogramming efficiency may be improved by changing the matrix on and in which cells are cultured, as well as by culturing cells longer, and examining them for insulin expression 10 and 14 days after infection.

2. Identity of reprogrammable population, resident Sox9^+ cells?

It was clear from the work of the Slack Lab (Banga et al., 2012; Banga, et al., 2014) that Ad-PNM can reprogram some cell type(s) in adult mouse livers yielding sufficient insulin secretion to reverse diabetes in NOD-SCID and immunocompetent mice. However, the evidence is unclear what starting cell types are most permissive to reprogramming toward a beta-like state. Knowing these cell types would be useful for establishing populations to be infected for ex vivo reprogramming as well as would contribute to the understanding of liver biology.

Coexpression of Sox9 and Insulin and lack of Albumin expression from histological data was shown in mice 10 days after Ad-PNM delivery in vivo; gene expression data was analyzed from samples taken 12 weeks after Ad-PNM delivery (Banga et al. 2012). It has been documented that virally-infected cells can change expressional programs in timespans less than one week (Dorn et al., 2005) and replication-deficient adenovirus vector infection alters cytoskeletal dynamics (Luo et al., 2006; Warren, J. C., Rutkowski, A., & Cassimeris,
L., 2006; Zamir et al., 2007) which over time can affect intracellular signalling in pathways that influence transcriptional regulation (Kumar et al., 2007). Hence data regarding cell identity at even 1 week post infection may be uninformative of the cell’s original phenotype.

Additionally, in Banga et al. (2014) an immune response was observed to lower reprogramming rates in CD1 mice compared to NOD-SCID mice. Given that immune cells and non-hematopoietic liver cells were encountering the same transgenic virus, and that viral infections in vivo induce immune cell and infected cell antiviral responses, then an immune cell response observed by the group likely indicates that infected liver cells also reacted to Ad-PNM virus itself. Intracellular virus detection alone alters cellular transcriptional programs in addition to the influence of the introduced genes Pdx1, Ngn3, and Mafa, further influencing the transcriptional changes from the starting cell type.

Recently, the existence and prevalence of a resident Sox9+, bipotent LSPC has been questioned (Grompe, 2014). Work by Carpentier et al. (2011) demonstrated heterogeneity in the progeny of Sox9+ labelled ductal plate cells. The group showed that Sox9+ cells labelled at E15.5 contributed to intrahepatic and extrahepatic BECs as well as periportal hepatocytes. Demonstration of labelling by Banga et al. (2012) also shows that not all lineage-labelled cells are still expressing Sox9 nor appear to be in a bile duct in the uninfected adult control Sox9-CreERT2;mTmG mice. Additionally, histological data from Banga et al. (2012) and (2014) lacks clear tissue architecture, e.g. the portal vein and hepatic artery of the portal triad, for anatomical context of ductal structures and progeny of labelled cells.

Work by Yanger et al. (2014), Tarlow et al. (2014), and O’Neill et al. (2014) support the possibility that stably reprogrammed cells could be hepatocytes. Yanger et al. (2014) used Cre-based lineage-tracing methods to determine the major cell type that repopulates the liver after injury from four different methods: CDE diet, DDC diet, CCl₄ injection, and ANIT treatment. They labelled cytokeratin 19 (Krt19)-expressing bile duct cells in Krt19-CreER;R26R-YFP transgenic mice. In separate experiments hepatocytes were labelled by infecting R26R-lsl-YFP mice with an adeno-associated virus serotype 8 (AAV8) encoding a thyroid-binding globulin (TBG) promoter sequence upstream of that encoding Cre recombinase, specifying hepatocyte expression of Cre recombinase (Yanger et al., 2014). Examining recovered livers revealed little to no contribution of Krt19 lineage-labelled cells to the hepatocyte population. Complementary to that result, recovered livers showed no
decrease in labelled hepatocytes, suggesting little to no contribution from or ‘crowding out’ by an unlabelled, non-hepatocyte cell type. This group provided significant evidence to discredit the existence of a LSPC in adult mouse livers.

Tarlow et al. (2014) generated liver chimeras such that mTomato-labelled *FAH*+/+ hepatocytes repopulated the livers of unlabelled *FAH*−/− mice. The group showed that 6 weeks after 0.1% DDC diet-induced injury, EdU-incorporating, OPN+ biliary-like cells were found that also expressed mTomato, demonstrating that donor hepatocytes entered the cell cycle and adopted a biliary identity. Sorting of mTomato+ cells also expressing “oval cell” marker, *MIC1-1C3*, revealed that the biliary-like cells derived from donor-hepatocytes also had much higher levels of Sox9, CK19, EpCam, Spp1 expression and much lower levels of HNF4α and albumin expression compared to hepatocytes. Additionally, they generated chimeras with *Sox9-CreERT2; mTomG FAH*+/+ donor hepatocytes in unlabelled *FAH*−/− mouse livers. DDC diet-induced injury occurred over 6 weeks with one low dose of tamoxifen for *Sox9*-specific labelling. Livers examined at the end of 6 weeks demonstrated mGFP+, oval cell marker A6+, FAH cells. Because these cells were mGFP-expressing they were known to be hepatocytes at the time of injury and by 6 weeks after the start of injury they expressed Sox9 and A6. The group demonstrated the capacity of hepatocytes to adopt a Ck19, Sox9-expressing pseudobiliary identity after chronic injury/stress.

O’Neill et al. (2014) examined the process of hepatocyte dedifferentiation in culture. The group labelled hepatocytes in *Alb-Cre; R26R LacZ* mice. X-gal was used for hepatocyte identification to demonstrate the purity of their population after perfusion isolation. After 3 days of culture in DMEM medium, a medium known to induce dedifferentiation of hepatocytes, Sox9 was one of the genes distinctly upregulated. Additionally, Sox9 protein levels were increased when compared to cells cultured in keratinocyte serum-free medium (KSFM), a medium known to retain hepatocyte identity. Overexpression of Sox9 led to modest increases in Ck19 and OPN expression compared with freshly isolated hepatocytes. 5 days of culture in DMEM lead to observational increases in Sox9 and OPN immunostaining. The group provided evidence that environmental changes such as changes in culture media can induce Sox9 expression in cultured hepatocytes, and that Sox9 expression was an early event in the transdifferentiation of hepatocytes toward a OV6, Ck19, OPN, Sox9-expressing biliary-like cell.
Hence there is evidence to support the possibility that the target cell type reprogrammed by Ad-PNM and observed in Banga et al., 2012, 2014, could result be an injured/stressed hepatocyte. As there are metabolic differences among hepatocytes (Kang et al., 2012), it is possible that one type versus the other is more susceptible to Ad-PNM reprogramming.

Also to consider when re-examining the work of Carpentier et al. (2011) as Sox9+ ductal plate cells give rise to extrahepatic BECs there remains the possibility that these are another reprogrammable cell type of liver origin. Recent work has demonstrated that a small fraction of extrahepatic BECs express insulin in neonatal and adult mice (Dutton et al., 2007). Histology from Banga et al. (2012) and Banga et al. (2014) demonstrate that some of the insulin+ ducts are large and could represent reprogrammed extrahepatic ducts of the hilar region.

*In vivo* data have not excluded the possibility that stressed hepatocytes and extrahepatic BECs contribute to stably reprogrammed, insulin+ ductal cells in livers after exposure to Ad-PNM. Hence, *in vitro* reprogramming should include treating primary hepatocytes and extrahepatic BECs with Ad-PNM.

Experiments in this thesis work showed that the protocol used here could reliably isolate ductal cells of the mouse liver and that these cells can be infected *in vitro* in suspension culture upon exposure to Ad-PNM. They were shown to be infected and expressing Ad-PNM genes after 5 days, however at this time were not observed to be producing insulin protein. Further experiments specifically isolating intrahepatic BECs and extrahepatic BECs of the hilar region could continue to explore reprogramming efficiency and timelines *in vitro* for the two liver ductal cell populations. With more physiologically accurate culturing surfaces and 3D matrices, the infection efficiency and insulin production may be improved, as well as expansion of the cells. Excitingly, Ad-PNM virus remains a potential means to develop a system of *in vivo* or *in vitro* reprogramming toward a stable, insulin-producing state using cells of healthy tissue in T1D patients and non-patients.
Bibliography


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