Novel cell therapies for liver disease and type 1 diabetes

by

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CERTIFICATE OF APPROVAL

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AAV	Adeno-associated virus
BSA	Bovine serum albumin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
DBZ	Dibenzazepine
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
eGFP	Enhanced green fluorescent protein
ELSIA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cell
FAA	4-fumarylacetoacetate
FACS	Fluorescence activated cell sorting
FAH	Fumarylacetoacetate hydrolase
FAR	Fumarylacetoacetate reductase
FSC	Forward scatter
GBC	Gall bladder cell
GSI	Gamma secretase inhibitor
HCC	Hepatocellular carcinoma
HSC	Hematopoietic stem cell
HGD	Homogentisic acid dioxygenase
HPD	4-hydroxyphenylpyruvate dioxygenase

HT1	Hereditary tyrosinemia type 1
iPSC	Induced pluripotent stem cell
ITR	Inverted terminal repeat
KO	Knockout
KRB	Krebs Ringer Buffer
MAI	Maleylacetoacetate isomerase
MIP	Mouse insulin promotor
MODY	Maturity-onset diabetes of the young
MOI	Multiplicity of infection
NEO	Neomycin phosphotransferase
NRG	NOD-Rag1^{null} IL2rγ^{null}
NSG	NOD-SCID IL2rγ^{null}
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
PCR	Polymerase chain reaction
PERV	Porcine endogenous retrovirus
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
qRT	Quantitative reverse transcription
RA	Retinoic acid
RAR	Retinoic acid receptor
RIP	Rat insulin promoter
RXR	Retinoid X receptor
SCID	Severe combined immune deficiency

SCNT	Somatic cell nuclear transfer
TAT	Tyrosine amino transferase
T1D	Diabetes mellitus type 1
uPA	Urokinase-type plasminogen activator
WT	Wild-type
ZFN	Zinc finger nuclease

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completed the IHC, and Maria Grompe performed the antibody screening of primary gall bladder cells. I am grateful to all of them for their collaboration.

ABSTRACT

Liver disease and type 1 diabetes combined affect over 28 million people in the USA alone, with the incidence of both diseases increasing significantly. Medically refractory liver failure is currently only successfully treated by orthotopic liver transplantation. Similarly, whole pancreas transplantation is the only definitive intervention for type 1 diabetes. An alternative to whole organ transplantation is a cell therapy approach to restore the cellular deficit caused by hepatocyte loss or beta cell destruction in the respective disease. Importantly, initial hepatocyte and islet transplantations in humans have demonstrated that these procedures can be performed safely and effectively, but their widespread use has been limited by a number of factors, with one of the major restrictions being the paucity of transplantable material. Therefore, new methods to generate transplantable, functional cells are needed urgently.

My thesis research focused on two different approaches to address this problem. Firstly, we created fumarylacetoacetate hydrolase (*Fah)*-null heterozygote pigs by gene targeting and somatic cell nuclear transfer (SCNT) in order to eventually generate *Fah*-null homozygote pigs to expand human hepatocytes in. In a novel approach we used the chimeric adeno-associated virus DJ serotype (AAV-DJ) and homologous recombination to target and disrupt the porcine *Fah* gene. The AAV-DJ vector was used to deliver the *Fah* knockout construct to fetal pig fibroblasts with an average knockout targeting frequency of 5.4%. Targeted *Fah*-null heterozygote

fibroblasts were used as nuclear donors for SCNT to porcine oocytes, and multiple viable *Fah*-null heterozygote pigs were generated. *Fah*-null heterozygotes were phenotypically normal, but had decreased *Fah* transcriptional and enzymatic activity compared to wild-type animals.

The second focus of my research was to generate pancreatic beta cells from mouse gall bladder cells (GBCs) using a direct reprogramming approach that could eventually be used to treat patients with type 1 diabetes. Murine GBCs were robustly expanded *in vitro*, allowing the generation of billions of cells from a single gall bladder. We determined that expression of *Neurog3*, *Pdx1* and *MafA* to be the minimal required transcription factors for reprogramming to the beta cell fate. Reprogramming towards the beta-cell fate occurred rapidly within 48 hours, and was augmented by retinoic acid and by inhibition of notch signaling. Using flow cytometry to isolate reprogrammed cells, we confirmed reprogrammed GBCs were differentiating towards a beta-like cell fate by both gene and protein analyses, including whole genome RNA-Sequencing analysis. In order to determine if these cells could be used to reverse hyperglycemia *in vivo*, we transplanted these cells into diabetic mice. Although the transplanted cells were unable to consistently reverse the hyperglycemia, the transplanted cells were able to engraft long term in these mice and were insulin-positive for at least 12 weeks post-transplantation.

In summary, both these studies explore different approaches to generate new cells for the purpose of cell therapy. There is an urgent need to generate transplantable

cells for treating myriad liver diseases and type 1 diabetes. Chapter one of this thesis outlines the background to the problems of liver disease and type 1 diabetes, and the different approaches currently being attempted to treat these disorders by cell therapy. In chapters two and three, I describe in detail my efforts in generating *Fah*-null heterozygote pigs and mouse pancreatic beta cells, respectively. Finally, in chapter four I summarize the significance of my research, the current obstacles with both approaches and the future directions I see this research going, as well as speculating on the general challenges associated with all cell therapies currently focused on treating liver disease and type 1 diabetes.

CHAPTER 1

Introduction

A historical perspective on cell therapy

The American Society of Gene and Cell Therapy defines cell therapy as the administration of live whole cells or maturation of a specific cell population in a patient for the treatment of a disease. Blood transfusions were the first type of cell therapy, with the earliest reports of such a procedure dating as far back as 1492 (1). In that year, Pope Innocent VIII was in a coma after suffering a stroke. In an effort to save the dying pontiff, his physician infused the blood of three boys through the pontiff's mouth. Although this may have been the burgeoning of the field of cell therapy, the initial results were not encouraging with the deaths of the three donors and the eventual death of the Pope. Although it took nearly five centuries to eventually perfect the technique, blood transfusions are now routine with millions of patients having undergone successful therapy (2, 3).

Bone marrow transplantation is another common and well-established type of cell therapy and is considered the treatment of choice for many blood disorders, including anemias, leukemias and lymphomas (4-7). Thomas and colleagues were the first to report the radical new approach to cancer treatment that involved chemotherapy followed by infusion of bone marrow (8). Good and colleagues at the University of Minnesota performed the first successful allogeneic bone marrow transplant in 1968 for a non-cancer patient, in which a five month-old boy received cells from his eight year-old sister to treat his severe combined immune deficiency disorder (9). A proper conditioning regimen prior to transplantation is an obligatory factor for successful transplantation (10). However, another key element to the

success of this transplantation is the existence of the hematopoietic stem cell (HSC), a cell that is able to give rise to the entire spectrum of differentiated mature blood cells (11). Firstly, the HSC can be isolated safely and routinely (12). Secondly, the HSC has the ability to home to the bone marrow of the recipient, where it can survive, undergo replication and give rise to all the blood lineages (11). None of these facts are true for the liver or pancreas, as no cell in the mouse, let alone the human, has been identified that can replicate indefinitely and give rise to all the lineages of that specific organ (13, 14).

Further complications for all cell therapies are the problems of immune rejection and graft-versus-host disease following allogenic transplantation (15, 16). In the former, the immune system of the recipient recognizes the transplanted cells or tissues as foreign, eliciting two types of responses. The first is that of acute rejection, and occurs rapidly following transplantation. The second is termed chronic rejection and can occur even in the absence of acute rejection. Graft-versus-host disease can also occur if immunologically competent T cells are contained in the graft and recognize recipient tissue antigens as foreign, leading to an alloimmune attack on the host tissue. Despite advances in administration of immune suppression drugs, a large proportion of transplanted grafts are lost due to immunological events (17). In addition, despite advances in immunosuppressive drug therapy, complications from long-term immunosuppression in solid organ transplantations continue to have a significant impact on post-transplant morbidity and mortality (18). The “holy grail” of cell and organ transplant, therefore, is the use of autologous cells to obviate the need for long-term immunosuppression.

Liver disease

According to the annual report of the American Liver Foundation in 2000, liver diseases affect 25 million Americans. Liver failure is the 8th most frequent cause of death in the USA, accounting for roughly 43,000 deaths each year. Liver transplantation is currently the only effective treatment for medically refractory liver failure (19). This transplantation involves major surgery and is a complex technique: the native liver is first removed prior to implantation of the new liver and reconstruction of the vascular and biliary architecture. However, liver transplantation has been successfully performed for a number of different liver disorders. Acute liver failure is defined as the clinical manifestation of sudden and severe hepatic injury and has a number of causes, most commonly viral infection and drug-induced injury (20). Chronic liver disease most commonly manifests from hepatitis C infection and alcoholic liver disease, although the term encompasses a number of conditions that lead to cirrhosis or fibrosis of the liver (21). Another class of liver diseases that can be ameliorated by liver transplantation is the metabolic liver disorders that are mostly caused by a single gene defect that affects a specific enzyme that causes the disease (22). There are many examples of metabolic disorders that have been treated by transplantation, including Wilson disease, hemochromatosis, hereditary tyrosinemia type 1, urea cycle defects and familial hypercholesterolemia (23). These single gene disorders are most often caused by inherited mutations that produce defective enzymes that cause accumulation of specific substrates or deficiency of certain proteins. Although in some cases

biochemical intervention or proper nutritional management can ameliorate the condition, most often liver transplantation is the sole curative option.

Cell therapy for liver disease

An alternative strategy to orthotopic liver transplantation is hepatocyte transplantation (22, 24). The initial preclinical experiments occurred in the Gunn rat, a rodent model for Crigler-Najjar syndrome type 1 (25). Healthy hepatocytes from heterozygote Gunn rats were infused into the hyperbilirubinemic rats and resulted in a sustained decrease in plasma bilirubin concentrations. Further experiments followed in which chemically or surgically induced liver failure was performed in animals and treated with hepatocyte transplantation to improve survival (26, 27). Since then, a number of other model metabolic liver diseases in animals have been treated by hepatocyte transplantation, including hereditary tyrosinemia type 1 and familial hypercholesterolemia (28, 29). These animal experiments have paved the way for a number of human hepatocyte transplantations to occur. Initial human hepatocyte transplantations were performed in patients with liver failure with some success (30). The first metabolic disorder to be treated by hepatocyte transplantation was for ornithine transcarbamylase deficiency, however the first published partial correction was performed by Fox and colleagues in a patient with Crigler–Najjar syndrome type 1 (31). Since then a number of patients with liver-based metabolic disorders have undergone hepatocyte transfusions with encouraging, albeit modest, success (24).

Cell therapy for liver disease – the challenges

Liver transplantation has several shortfalls, the most prominent being a shortage of donor organs. According to the American Liver Foundation, 1,848 candidates for liver transplantation died on the waiting list in 2005 alone. A potential solution to the shortage of organs is direct transplantation of hepatocytes, a method that could serve as a bridge to transplantation or until spontaneous recovery of the native liver occurs (24, 32). However, a major barrier to successful liver cell therapy is the availability of primary human hepatocytes. Human hepatocytes are available from liver biopsies, surgical hepatic resections, and discarded cadaveric organs. However, these sources are highly variable in quantity and quality, and hepatocytes obtained from these tissues cannot be expanded *in vitro*. Alternatives to primary human hepatocytes have been examined, including using hepatocytes from other mammalian sources such as mice and pigs, but obstacles exist to their use. For example, rodent and porcine hepatocytes possess different metabolic enzymes, which respond differently than human hepatocytes in drug metabolism studies (33). Some researchers have examined the use of immortalized human hepatocytes but these cells fail to express specific genes involved in various hepatocyte functions, such as ammonia detoxification and normal drug metabolism (34). Transformed cells also possess a potential risk for malignant spread if these cells were able to metastasize into the circulation of an immunosuppressed patient. Therefore, an abundant supply of primary human hepatocytes would be the ideal source for direct transplantation into the patient.

Expanding human hepatocytes in mice

To overcome the inability of *in vitro* propagation of human hepatocytes, researchers have begun to engraft and expand human hepatocytes in rodents. Two main strategies have been used. The first system uses immunodeficient urokinase-type plasminogen activator-transgenic SCID mice (uPA/SCID mice). These transgenic mice express urokinase-type plasminogen activator (uPA) under the transcriptional control of the albumin promoter, making it hepatotoxic to the mouse. Human hepatocytes can be engrafted in these immunodeficient mice without rejection (35, 36). As the murine hepatocytes die, healthy non-affected human hepatocytes are able to expand within the mouse liver yielding chimeric human/mouse livers with engraftment levels as high as 92% achieved. An alternative approach to expand human hepatocytes in genetically modified mice uses immunodeficient fumarylacetoacetate hydrolase (*Fah*)-knockout mice that causes an essential deficit in hepatocytes of these mice leading to a selective pressure for the stable engraftment and expansion of human hepatocytes in the mouse liver (37). FAH is an essential enzyme in tyrosine catabolism (see Figure 2-1). Mice deficient for FAH develop tyrosinemia and liver disease in the absence of the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (29, 38). Using these mice, it was demonstrated that transplanted FAH-positive human hepatocytes have a selective growth advantage upon NTBC withdrawal, allowing for near complete repopulation of the liver with human cells.

Importantly, the expanded human hepatocytes were indistinguishable from normal human adult hepatocytes in standard drug metabolism assays (37).

Type 1 diabetes

Diabetes mellitus type 1 is an autoimmune disorder affecting millions of people. In the USA, according to the Juvenile Diabetes Research Foundation, the number of type 1 diabetes patients is estimated at three million people, with approximately eighty new cases being diagnosed daily. The problem is so profound, in fact, that the global worldwide prevalence of diabetes (type 1 and 2) is estimated to be 7.7% by the year 2030 (39). The disease is caused by the destruction of the insulin-producing beta cells in the islets of Langerhans within the pancreas. The beta cells function to secrete insulin in response to increased glucose concentration within the blood, leading to increased glucose uptake into tissues, thereby maintaining physiological glucose levels within a narrow range. Upon destruction of the beta cells, patients lose blood glucose control, leading to both acute and chronic complications (40, 41). Acute conditions can manifest in multiple forms, including ketoacidosis and severe hypoglycemia. Chronic illness, due to prolonged inability to achieve normoglycemia, can result in secondary complications such as blindness and kidney failure. These complications can and do arise even with current insulin therapies (42). The pathophysiology of the disease has been intensely studied, and is thought to develop as a consequence of genetic predisposition and multiple environmental factors (43).

Cell therapy for type 1 diabetes

Whole pancreas transplantation has been performed since 1966, however it is a major surgical procedure that is commonly associated with post-transplant morbidity (44). Pancreatic islet transplantation, though, is associated with decreased morbidity due to it being a less invasive procedure. In this procedure, islets, which constitute 1-2% of the pancreas mass, are enzymatically and mechanically isolated from the organ of the cadaveric donor, and infused percutaneously via a catheter into the hepatic portal vein. Due to their size, islets become entrapped in the portal vein. Two to three donor pancreata are usually required for each diabetic patient (44, 45). The results of the first islet transplantation were reported in the year 2000 and demonstrated rapid insulin independence in all seven transplanted patients after one year (45). Since then, there have been about 1000 recipients of islet transplantations worldwide. Unfortunately, results have been mixed, with only 7.5% of patients remaining insulin-independent after five years (44, 46). In the same study though, 82% of patients had detectable c-peptide levels in the blood, indicating persistent but insufficient graft function. The cause of the loss of graft function is unclear, however numerous reasons have been postulated including insufficient vascularization of the islets, the toxic environment of the liver caused by the high concentration of immunosuppressive drugs and low oxygen concentration, graft rejection, or recurrence of autoimmunity to transplanted islets (47, 48). Overall, though, other factors must also be considered that indicate beneficial effects of islet

transplantation. These include decreases in vascular complications caused by inappropriate blood glucose control, increased quality of life, and, importantly, increased patient survival (44).

Cell therapy for type 1 diabetes – the challenges

The primary problem for islet transplantation is the paucity of transplantable material. Two to three cadaveric donors are required for each islet transplantation and often the diabetic patient will have to undergo further rounds of islet infusions to maintain insulin independence. While pancreatic islets can be maintained in culture for extended periods, they cells that constitute them, particularly the insulin-producing beta cells, do not undergo replication (49). Neither can dissociated beta cells themselves be expanded in culture. Therefore, researchers are investigating novel ways to generate new beta cells. There have been many approaches attempted to generate new beta cells by nuclear reprogramming. Initially, *in vivo* reprogramming of hepatic cells by expressing different pancreatic transcription factors, including *Neurog3* and *Pdx1*, was able to restore euglycemia in hyperglycemic mice (50, 51). More recently, *in vivo* reprogramming of exocrine acinar cells into insulin-positive cells by expression of *Neurog3*, *Pdx1* and *MafA* was able to reverse hyperglycemia in mice. Another *in vivo* approach that was successful in mice involved ectopic expression of *Pax4* which converted pancreatic progenitor cells into alpha cells and subsequently beta cells (52). In addition, there have been several approaches *in vitro* to reprogramming pluripotent cells, including both

embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), by directing differentiation of these cells along the pancreatic lineage to generate insulin-producing cells (53-56). These strategies are inherently risky, however. *In vivo* approaches using viral vectors can result in transduction of the virus into non-targeted cells, despite advances in targeted design of these vectors (57). This approach can also result in random integration into the genome of the transduced cell, potentially disrupting an important gene (58, 59). *In vitro* approaches are intrinsically safer, as the targeted cell population can be exclusively manipulated, with additional genetic interrogation of the cell possible prior to transplantation. However, the use of pluripotent stem cell-derived cells is associated with particular risks also, most notably generation of teratomas by the transplanted cells (55, 60).

Nuclear reprogramming

Development, differentiation and cell fate are represented well by Waddington's epigenetic landscape (61). In this model, cell differentiation is compared to a ball rolling down a hill, with the cell fate determined by which path the ball takes (Figure 1-1). This differentiation is unidirectional: in this model the ball cannot roll up the hill, nor can it traverse across the valleys. In normal development, this model is essentially correct. For example, the fully differentiated beta cell arises following a series of fate choices through which it differentiates through the endoderm lineage, becoming a pancreatic progenitor, then a pancreatic endocrine progenitor before eventually becoming a fully functional, terminally

differentiated, beta cell (62). However, under specific conditions, there are now numerous examples of a terminally differentiated cell being reprogrammed to an entirely different cell type by nuclear reprogramming (63). A modified landscape is depicted in Figure 1-2, demonstrating both proven and hypothetical avenues for a differentiated pancreatic beta cell to be generated.

The definitive example of reprogramming is nuclear transfer from a differentiated cell to an enucleated egg to generate an entire organism, first demonstrated in *Xenopus laevis* eggs, but most famously validated in sheep with the creation of Dolly (64, 65). The initial study in 1962 demonstrated that the fully differentiated cells of the intestinal epithelium of *Xenopus* could be transferred into enucleated eggs to generate fully viable male and female frogs (64). Many years later in 1997, Wilmut and colleagues reported the generation of viable lambs following transfer of fully differentiated cells from the mammary gland of an adult sheep into enucleated eggs (65). An important example of the ability of primate cells to be reprogrammed completely was reported in 2007 with the generation of ESCs from adult rhesus macaque skin fibroblasts (66). The exact mechanism by which reprogramming of a fully differentiated cell can occur in the egg is unknown. However, it is known that this process can take place in the absence of cell division and without protein synthesis, indicating that the entire complement of reprogramming factors is present within the cytoplasm and mitochondria of the egg (63, 67).

An alternative method, however, is forced expression of particular transcription factors. This was elegantly demonstrated with the generation of iPSCs

from adult fibroblasts by the expression of four genes – *Oct3/4*, *Sox2*, *c-Myc* and *Klf4* (68). Both this method and somatic cell nuclear transfer (SCNT) require the cell to undergo a dedifferentiation to a pluripotent cell state. However, this pluripotent intermediary has been demonstrated to be unnecessary for the generation of particular differentiated cell types. Weintraub and colleagues first demonstrated this with the identification of *MyoD*, overexpression of which was sufficient to turn a number of non-muscle cell types into cells expressing a range of muscle-specific genes (69). Recently, there have been a number of publications showing that expression of specific genes can directly differentiate one cell type directly into another functional cell, without undergoing a pluripotent intermediary. This was elegantly first demonstrated by Graf and colleagues by reprogramming B cells directly into macrophages by forced expression of C/EBPα and C/EBPβ (70). More recently, Wernig and colleagues who were able to directly convert mouse fibroblasts into functional neurons by expressing the three transcription factors *Ascl1*, *Brn2* and *Myt1l* (71). This strategy has now been replicated to generate functional cardiomyocytes, blood cells, and hepatocytes from fibroblasts (72-74).

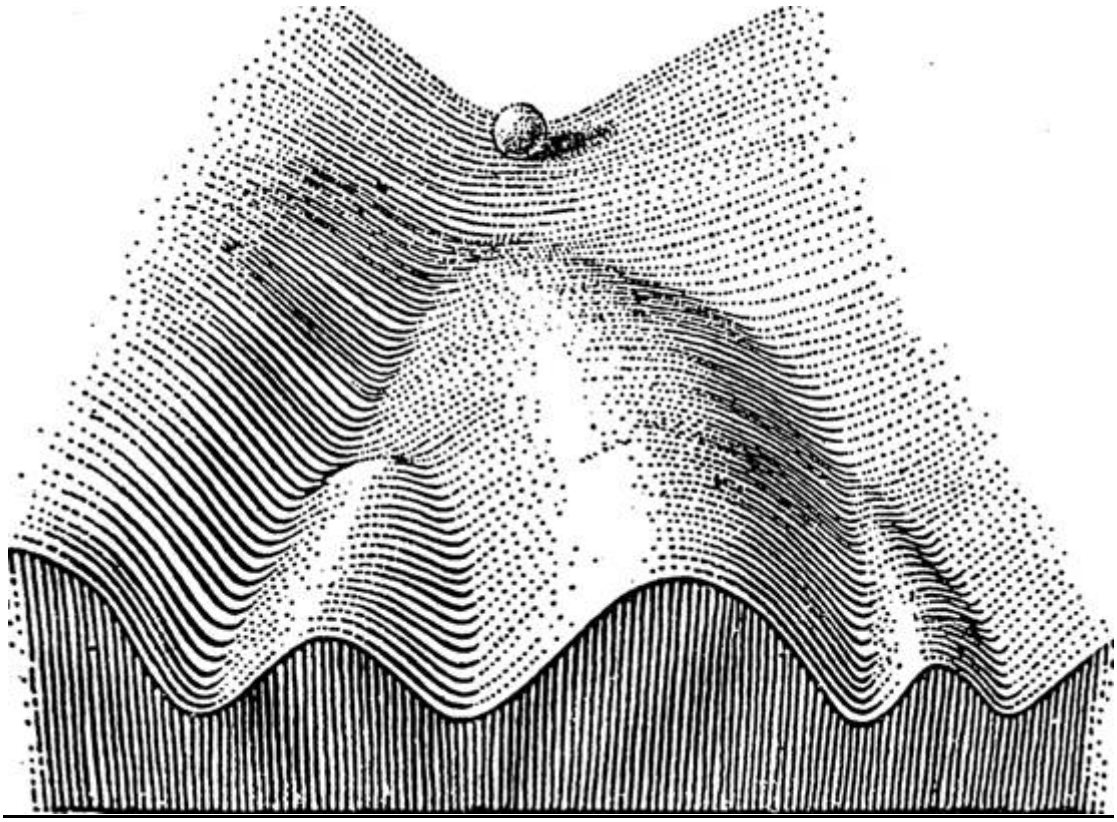

Figure 1-1. Waddington's original landscape.

Replicated from Waddington, 1957 (75).

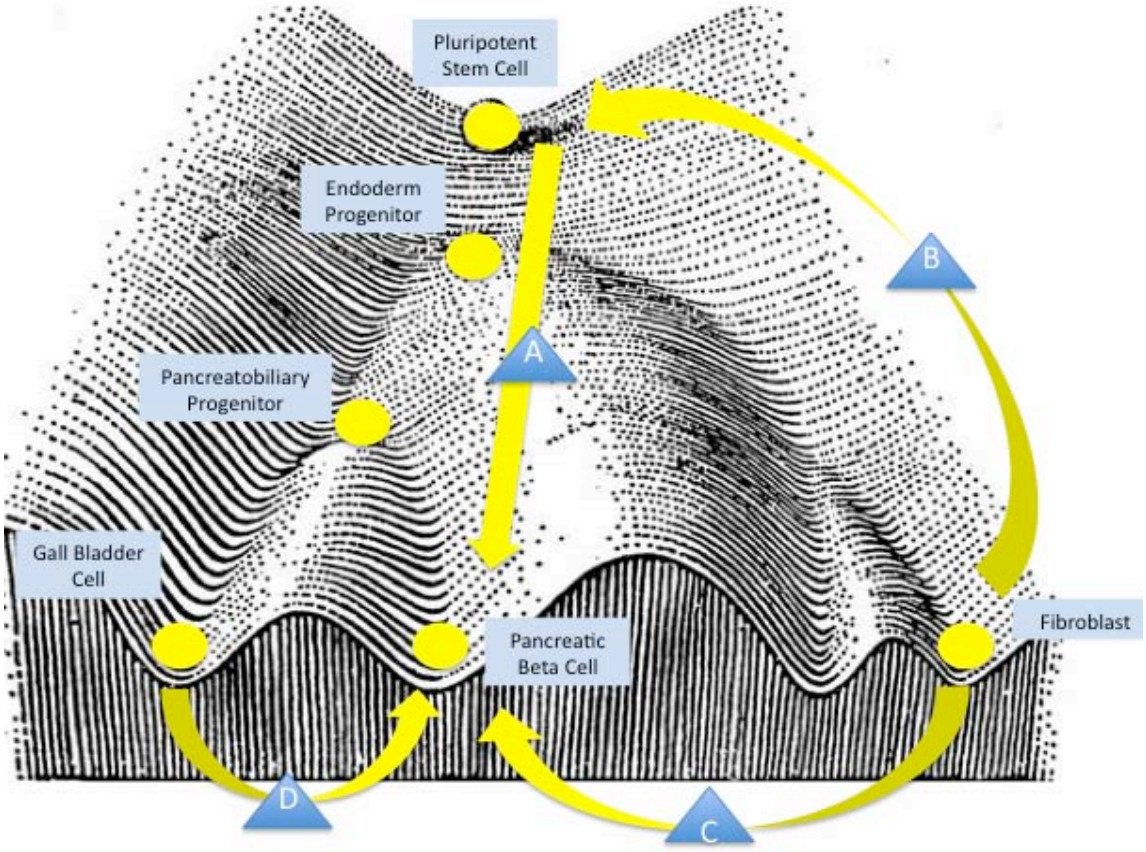

Figure 1-2. A modified nuclear reprogramming landscape.

A pancreatic beta cell can be generated by several methods. **(A)** In development, and recapitulated by differentiation of an ESC or iPSC, a pluripotent stem cell can be differentiated along the normal development pathway to give rise to different intermediate progenitors prior to "terminal" differentiation to a beta cell. **(B)** However, it has now been demonstrated that a "terminally" differentiated cell can be reprogrammed to a pluripotent cell by either forced expression of different transcription factors, or by SCNT into an enucleated egg. **(C)** Even more recently, the malleability of one cell type to be genetically reprogrammed directly into another cell type has been demonstrated for various cells such as neurons and cardiomyocytes, but not beta cells. **(D)** Hypothetically, a developmentally related cell, for example a gall bladder cell, could be genetically reprogrammed to its developmental cousin, in this case, a pancreatic beta cell, without undergoing a pluripotent intermediate step.

CHAPTER 2

Towards a novel resource for expanding human hepatocytes: Generation of *Fah*-null heterozygote pigs by chimeric adeno-associated virus-mediated gene knockout and somatic cell nuclear transfer.

ABSTRACT

Hereditary tyrosinemia type 1 results in hepatic failure, cirrhosis, and hepatocellular carcinoma (HCC) early in childhood and is caused by deficiency in the enzyme fumarylacetoacetate hydrolase (FAH). In a novel approach we used the chimeric adeno-associated virus DJ serotype (AAV-DJ) and homologous recombination to target and disrupt the porcine *Fah* gene. AAV-DJ is an artificial chimeric AAV vector containing hybrid capsid sequences from three naturally occurring serotypes (AAV2, 8 and 9). The AAV-DJ vector was used to deliver the knockout construct to fetal pig fibroblasts with an average knockout targeting frequency of 5.4%. Targeted *Fah*-null heterozygote fibroblasts were used as nuclear donors for somatic cell nuclear transfer to porcine oocytes, and multiple viable *Fah*-null heterozygote pigs were generated. *Fah*-null heterozygotes were phenotypically normal, but had decreased *Fah* transcriptional and enzymatic activity compared to wild-type animals. This study is the first to use a recombinant chimeric AAV vector to knockout a gene in porcine fibroblasts for the purpose of SCNT. In using the AAV-DJ vector we observed targeting frequencies that were higher than previously reported with other naturally occurring serotypes. We expect that the subsequent generation of FAH-null homozygote pigs will serve as a significant advancement for translational research and cell therapy in the areas of metabolic liver disease, cirrhosis and HCC.

INTRODUCTION

In humans, hereditary tyrosinemia type 1 (HT1) is a severe, autosomal recessive inborn error of metabolism caused by deficiency of fumarylacetoacetate hydrolase (FAH), a metabolic enzyme that catalyzes the last step of tyrosine metabolism (Figure 2-1) (76). Affected children develop micronodular cirrhosis and 36% develop HCC by age five if untreated (77). We have previously generated a small animal model of HT1 by generating *Fah* mutant mice by gene targeting in mouse embryonic stem cells (78). The phenotype of these mice is analogous to many of the key features of the human disorder, including the formation of liver cancer, and has proven to be an important research model for both HT1 and HCC (38, 79-81). However, this mouse model fails to recapitulate all of the aspects of the human disorder, most importantly cirrhosis.

Similar deficits in small animal models of other human disease have been reported as well. In cystic fibrosis (CF), multiple mice and rabbit models were engineered to contain several of the common genetic mutations seen in humans. However, these models failed to fully reproduce the disease phenotype observed in humans (82, 83). The pig is an appropriate research model because of its similarity in size, anatomy and biology to the human (84). This led researchers to create a porcine model of CF by using adeno-associated virus (AAV)-mediated gene targeting in combination with SCNT to create their pig model of CF, which has now been shown to display the characteristic manifestations of CF seen in humans (85, 86).

The CF pig was the first time AAV had been used to generate a porcine gene-knockout model. Up to this point, the generation of large animal models of disease had been hindered by the inability to apply mouse embryonic stem cell targeting approaches to non-murine models. However, AAV-mediated gene targeting and SCNT provide an alternative method to generate gene-knockout animals. AAV vectors have been shown to be able to introduce specific mutations, including insertions, into homologous chromosomal sequences of many cell types and species (87, 88). In addition, AAV-mediated gene targeting has been shown to be more efficient than conventional techniques based on transfection or electroporation of plasmid constructs (89, 90). However, the highest published targeting efficiency of AAV in various systems remains close to 1% (91).

The goal of this study was to develop a more efficient method to create pig knock-out models of human diseases and to create *Fah*-null heterozygote pigs to be used to generate homozygote *Fah*-null animals for future studies related to metabolic liver disease, cirrhosis, HCC, cell and gene therapy. We used for the first time the novel chimeric AAV-DJ serotype to disrupt the porcine *Fah* gene by targeted gene knockout by homologous recombination. We report here on the successful and efficient generation of targeted *Fah*-null heterozygote fibroblasts and their use by SCNT to generate *Fah*-null heterozygote pigs.

EXPERIMENTAL PROCEDURES

Genomic clone construction.

Genomic DNA was isolated and purified (Qiamp; Qiagen) from pig fetal liver. Two fragments of DNA, adjacent to exon 5 of the *Fah* locus of chromosome 7, were amplified using primers MG2616 and MG2678 (left homologous recombination arm; 1479bp) and MG2619 and MG2680 (right homologous recombination arm; 1523bp) and a high fidelity polymerase (Phusion; Finnzymes). Primers were designed based on the domestic pig working draft genomic sequence (Genbank accessions CU468492 and CU467891). These PCR products were subcloned into pCR-Blunt II-TOPO (Invitrogen) and confirmed by restriction digest and sequencing.

Fah-null targeting vector construction.

The overall strategy to knock out the *Fah* gene was to insert an in-frame stop codon and a neomycin-resistance cassette (PGK-neo) into exon 5 of the porcine *Fah* gene. To generate a PGK-neo expression cassette, with an additional in-frame TGA stop codon at the 5' end, a 1681bp fragment was amplified using primers MG2622 and MG2679, subcloned into pCR-Blunt II-TOPO, and confirmed by restriction digest and sequencing. To generate the complete targeting vector, each fragment was sequentially subcloned into pcDNA3.1- (Invitrogen) and orientation confirmed by restriction digest and sequencing. Once the full-sized 4683bp-targeting construct was generated, it was cloned into an AAV2 plasmid backbone, thus providing it with the inverted terminal repeat (ITR) sequences required for viral packaging. Plasmids

containing the AAV-DJ shuffle capsid sequences were generously provided to us by Dr. Mark Kay at Stanford. AAV-DJ virus containing the *Fah*-null construct was produced using a standard triple plasmid transfection protocol, as described previously (92).

Culture of fetal fibroblasts.

Porcine fetal fibroblasts were isolated from day 35 fetuses as previously described (93). Fetal gender was determined by PCR amplification of the *Sry* gene on the Y chromosome (94). Cells were initially grown in a 37°C, 5% CO_2 humidified incubator in high glucose DMEM media (HyClone), containing 15% fetal bovine serum (HyClone) and antimicrobials (100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml Amphotericin B; Cellgro).

Infection and selection of targeted fibroblasts.

Fetal fibroblasts (1.0×10^6) were thawed and plated on a 100 mm collagen I-coated culture dish (Biocoat; BD BioSciences). 24 hours later, cells were infected with virus (200μl, $3x10^{11}$ viral particles/ml). 22 hours later, cells were trypsinized (0.05% Trypsin; HyClone) and 500-2000 cells were transferred to 96-well collagen I-coated plates in media supplemented with 150μg/ml G418. Ten to twelve days later, cells were again trypsinized and split three different ways. For future cell freezing, 20% of the cells were transferred to a 96-well collagen I-coated plate. For cell expansion and further molecular analyses, 20% of the cells were transferred to an additional

96-well collagen I-coated plate. For PCR screening, 60% of the cells were transferred to a 96-well PCR plate.

PCR screening to identify targeted clones.

Cells in the 96-well PCR plates were spun down and washed in 250μl of PBS. The plates were spun again, and the cell pellets resuspended in 5μl lysis buffer (stock lysis solution = 660μl 0.01% SDS; 60μl 10mg/ml proteinase K; 30μl 0.5M EDTA). Following a 90-min incubation at 50°C and 30-min denaturation at 95°C, 1μl of the lysed cells were used for each 25μl PCR reaction. Primer pairs MG2844/2821 and MG2824/2851 were used to detect targeted integration at the 5' and 3' ends, respectively. PCR conditions were as follows: 3 min at 98°C, 40 cycles of 98°C for 10 s, 68°C for 20 s, and 72°C for 75 s, and then 72°C for 5 min. PCR generated products of 1622bp and 1774bp at the 5' and 3' ends, respectively, which were electrophoresed on a 2.0% TAE agarose gel and visualized with ethidium bromide staining.

Processing of positive clones.

Following identification of double positive (5' and 3' PCR products) PCR clones, cells in the freezing-down 96-well plate were grown to 90% confluency and trypsinized with 30μl 0.05% Trypsin. 15μl of detached cells were placed into each of two cryovials (Nalgene) and 300μl of freezing media (90% FBS; 10% DMSO) was added to each cryovial. These vials were then transferred to an isopropanol cryofreezing container at -80°C. 16 hours later, the vials were transferred to liquid nitrogen for

storage. In order to increase the cell number to allow for sufficient DNA isolation for additional molecular analyses, clones in the 96-well expansion plate were grown to confluency and transferred to 24-well plates, and subsequently expanded in 6-well and 100mm collagen-coated dishes. DNA was purified using a standard salting out procedure, as previously described (95).

Southern probe design and labeling.

The *Fah* specific probe was generated by using primers MG2840 and MG2841 to amplify a 601bp fragment of genomic *Fah* sequence located outside of the homologous recombination region. The Neo specific probe was generated by using primers MG3159 and MG3160 to amplify a 542bp fragment of Neo. PCR products were purified (Qiaquick; Qiagen) and 25ng of template was labeled with ^{32}P-dCTP using random oligonucleotides (Roche). Labeled probes were purified (Qiaquick; Qiagen) prior to hybridization.

Southern blotting.

4μg of isolated genomic DNA was digested overnight with *AflII* (NEB) at 37°C and electrophoresed on a 0.7% TAE agarose gel. Following acid depurination, the genomic digests were then transferred to a positively charged nylon membrane (Zeta-Probe; BioRad) by alkaline transfer in 0.4M NaOH. The membrane was prehybridized at 63°C in Church buffer with 150μg/ml fish sperm sodium salt (Amresco) for 60 min. For both *Fah* and Neo blots, the membranes were hybridized overnight in Church buffer with the ^{32}P-labeled probe at 63°C. Membranes were

subsequently washed in successive baths of 2x, 1x, and 0.1x SSC with 0.1% SDS at 63°C for 10 minutes each. Membranes were developed by autoradiography for 2-7 days (BioMax MS; Kodak).

Preparation of fibroblasts and oocytes for SCNT.

Porcine fetal fibroblast were seeded in a 4-well plate and grown until contact inhibited. The cells were trypsinized until cells started to become detached and resuspended in salt-buffered NCSU-23 containing 10% FCS. Oocytes were matured in Earle's TC199-Hepes supplemented with 5 mg/mL insulin, 10 ng/mL EGF, 0.6 mM cysteine, 0.2 mM sodium pyruvate, 25 mg/mL gentamicin, 5 mg/mL FSH, and 10% porcine follicular fluid for 40 hours prior to manipulation.

SCNT, surrogate preparation and embryo transfer.

All SCNT and embryo transfers were performed by Viagen (Austin, TX) and Exemplar Genetics (Sioux Center, IA) following standard protocols previously described (96, 97). All reconstructed oocytes were transferred into naturally cycling gilts on the first day of standing estrus. A midline laparotomy was performed exposing the uterus following which the reconstructed embryos were transferred into the oviduct at the ampullary-isthums junction. Four gilts underwent embryo transfer with each gilt receiving 136 embryos.

Phenotype characterization of *Fah*-null heterozygote pigs.

Histological analyses and FAH immunostaining were performed as previously described (98). For western blot analysis, liver samples were homogenized in cell lysis buffer (Cell Signaling) and 30μg of isolated total protein were analyzed by SDS-PAGE followed by immunoblotting onto a polyvinylidene fluoride microporous membrane (Immobilon-P, Millipore). The primary antibodies against FAH and beta-Actin (Cell Signaling) were detected with a secondary HRP anti-rabbit antibody (BioRad), and imaged using a chemiluminescent substrate for detection of HRP (Thermo Scientific). FAH enzyme assays were carried out on a cytosolic fraction of homogenized liver as described previously (78). Protein concentrations were measured by fluorometric quantification according to the manufacturer's instructions (Qubit, Invitrogen). Concentrations of succinyl acetone, tyrosine and phenylalanine were measured from dried blood spots as previously described (99). Quantitative RT-PCR gene expression was performed as described previously (100). The primers for the transcripts *Fah* and *Actb* are detailed in Table 1-1.

RESULTS

Creation of a targeting vector to disrupt the pig Fah gene.

Unlike other mammalian organisms, including the human and mouse, the genome of the domestic pig, *Sus scrofa*, has not been completed or annotated at the time of this study. Using nucleotide search algorithms available online, we were able to determine the location and structure of the porcine *Fah* gene. In addition, preliminary cDNA sequences determined from pig tissue aligned almost perfectly with the human and mouse *Fah* orthologs, hinting that this sequence was indeed the correct locus for *Fah* in the pig genome (Figure 2-2). Additionally, no other genomic sequence was identified by nucleotide alignment programs to be of similar sequence identity, indicating that the *Fah* locus had probably not undergone duplication. However, to confirm this a southern blot was performed using three distinct restriction enzymes to digest the DNA and hybridized with a probe generated from around the *Fah* exon 5 locus (Figure 2-2). Only a singe band was detected in each digest, confirming no other similar sequence was located in the pig genome.

Using a similar approach as was used to disrupt the mouse *Fah* gene, we created an *Fah* knockout targeting construct to disrupt exon 5 of the porcine *Fah* gene with a neomycin resistance cassette (Neo^R) and an in-frame stop codon (Figure 2-4). The in-frame stop codon will lead to nonsense-mediated mRNA decay and prematurely interrupt any translation of FAH (101). In addition, exon 5 of the porcine *Fah* gene is 92 bp long and the 1.5 kb neo insertion should lead to a significant frameshift and subsequent null allele, even if the TGA-stop codon is

bypassed during transcription. The Neo^R inserted in the middle of exon 5 also served as a method to select for integration of the targeting vector within the genome during fibroblast expansion using G418 selection (Figure 2-3B).

To improve the process of gene targeting in pigs we chose to use the chimeric AAV-DJ vector to deliver our knockout construct. AAV-DJ has been shown to have high tissue tropism for fibroblasts, an essential cell type used in the pig cloning process (102-104). In a preliminary experiment, pig fetal fibroblasts were infected with AAV-DJ containing the GFP transgene. The rAAV-DJ infected 93% of cells with the relatively low MOI of 185 (Figure 2-5A). This result helped support the decision to use the chimeric AAV-DJ for specific gene targeting of the *Fah* locus.

rAAV-DJ vector delivered the Fah disruption cassette.

In pigs, cloning is performed through the process of SCNT followed by embryo transfer (96, 105, 106). Therefore, all gene-targeting steps occurred using fetal fibroblasts and after selection and confirmation these targeted fibroblasts were used as nuclear donors in the SCNT step. Fetal fibroblasts were obtained from 35-day-old male and female pig fetuses. Primary cultures of pig fetal fibroblasts were infected with the rAAV-DJ targeting vector containing the *Fah* disruption cassette. Twenty-two hours after infection, fibroblasts were transferred to a number of 96-well plates and cultured under G418 selection. All wells in all plates were screened by PCR to identify wells containing *Fah* exon 5-targeted clones. A sensitive PCR screening strategy was created to identify targeted events using two different sets of PCR primers (Figure 2-5B). The 5' PCR screen was designed using a forward primer

outside the targeting region and a reverse primer for unique sequences inside our targeting construct. Similarly the 3' PCR screen utilized a forward primer for unique sequences inside our targeting construct and a reverse primer for sequences outside the targeting regions. In order to optimize the PCR screening, we initially designed a genotyping construct that allowed troubleshooting of the PCR reaction prior to actual infection, ensuring a robust and sensitive PCR analysis was available at the time of actual screening for positive targeted clones (Figure 2-3A).

Targeted fibroblast clones generating PCR products obtained from the 5' and 3' PCR screen were considered double positive and were subsequently confirmed by repeat PCR, sequencing and Southern blot. Southern blot was conducted using a *Fah*-specific probe located outside of the homologous targeting region (Figure 2-5C). The targeting frequencies obtained using the rAAV-DJ were extremely high, with an average targeting frequency of 5.4% (range = 2.29-8.50%) (Table 2-2). Confirmed *Fah*-null heterozygote fibroblasts that had been in culture for 15-19 days were frozen down for SCNT. Southern blot using a neo-specific probe was used to identify clones with targeted *Fah* alleles that were free of other random integration events (data not shown). All double positive PCR clones were also positive by Southern blot and no additional random integration events or sequence anomalies were observed.

SCNT followed by embryo transfer produced Fah-null heterozygote piglets.

To produce heterozygote pigs, *Fah*-null targeted fetal fibroblasts were used as nuclear donors for transfer to enucleated oocytes. Then to each of 4 surrogate

females, 134 embryos were transferred with only one surrogate reaching full term and delivering five viable female offspring by natural vaginal birth**.** PCR and Southern blot revealed that all five of the offspring were *Fah*-null heterozygotes (Figure 2-6). One of the newborn *Fah*-null heterozygote piglets was euthanized 24 hours after birth because of failure to thrive. Figure 2-7 shows pictures of the surviving 4 *Fah*-null heterozygote piglets 12 and 24 hours after their birth.

Phenotypic characterization of Fah-null heterozygote piglets.

Upon reaching reproductive maturity, female *Fah*-null heterozygote pigs were bred to male wild-type pigs. The *Fah*-knockout allele was inherited by newborn piglets with the expected Mendelian result: 50% of males and 55% of females carried the knockout allele. *Fah*-null heterozygotes were then compared to wild-type littermates. *Fah*-null heterozygote piglets were phenotypically normal and had normal levels of the amino acids phenylalanine and tyrosine, as well as the tyrosinemia type 1 marker succinylacetone, which indicated normal tyrosine metabolism in these animals compared to wild-type sibling controls (Table 2-3). In addition, H&E staining of livers of *Fah*-null heterozygotes appeared histologically normal and were positive for FAH by immunostaining within the hepatocytes (Figure 2-8A&B). However, qPCR analysis revealed a 55% reduction of the *Fah* transcript, in addition to a reduction of the overall FAH protein seen by western blot analysis from livers of *Fah*-null heterozygotes when compared to wild-type animals (Figure 2-8C&D). Finally, FAH enzyme activity can be measured by fluorometric quantification. FAH converts 4-fumarlacetoacetate (FAA) to acetoacetate and

fumerate. The loss of FAA is detected as decreased absorbance at 330nm. In accordance with FAH protein levels, the *Fah*-null heterozygotes showed reduced FAH enzyme activity when compared to their wild-type littermates (Figure 2-8E).

FIGURES

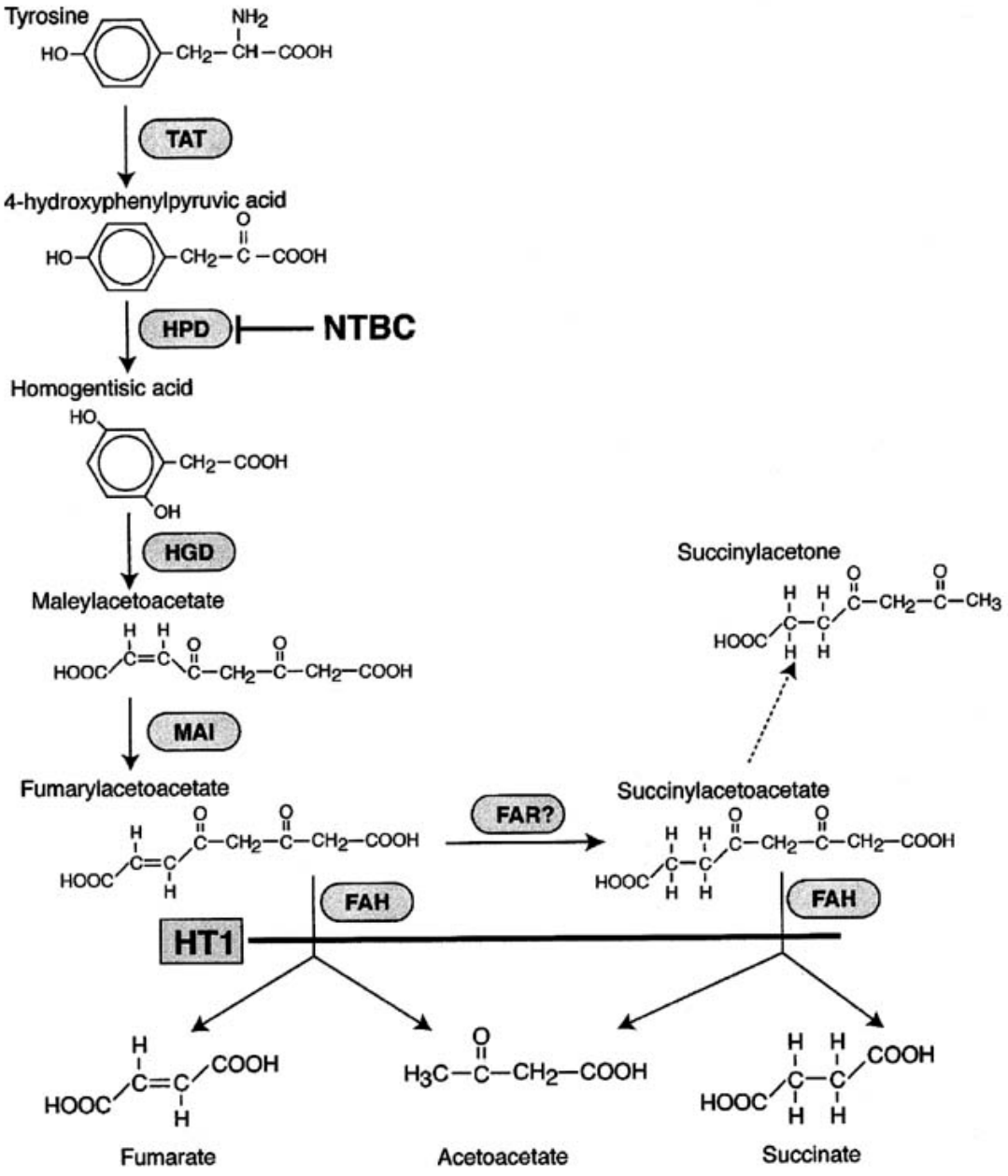

Figure 2-1. The tyrosine catabolic pathway.

TAT, tyrosine amino transferase; HPD, 4-hydroxyphenylpyruvate dioxygenase; HGD, homogentisic acid dioxygenase; MAI, maleylacetoacetate isomerase; FAR, fumarylacetoacetate reductase. Reproduced from Grompe, M 2001 (107).

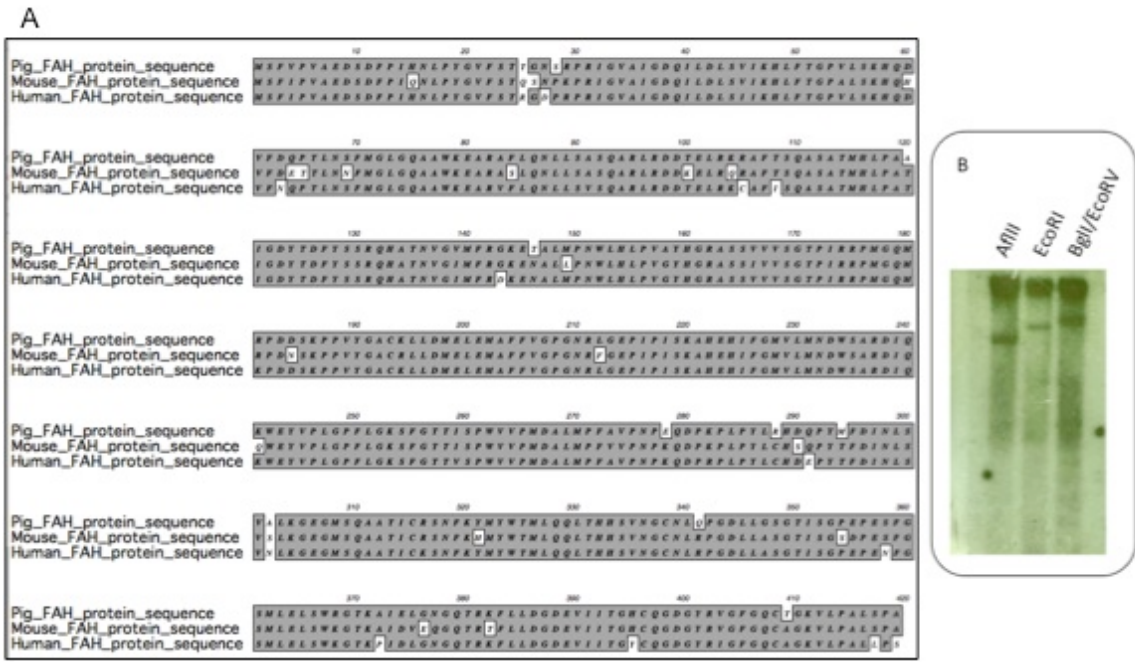

Figure 2-2: Determining the structure of the pig *Fah* locus.

(A) Based on the cDNA sequences, the predicted FAH peptides were aligned by computer algorithms, showing almost perfect homology between the pig (top line), mouse (middle) and human (bottom) amino acid sequences of the predicted FAH protein. **(B)** Pig genomic DNA isolated from liver tissue was isolated and digested with the restriction enzymes AflII, EcoRI or Bgl1 & EcoRV and hybridized with a probe generated from the *Fah* gene. Only a single band was detected in each of the lanes.

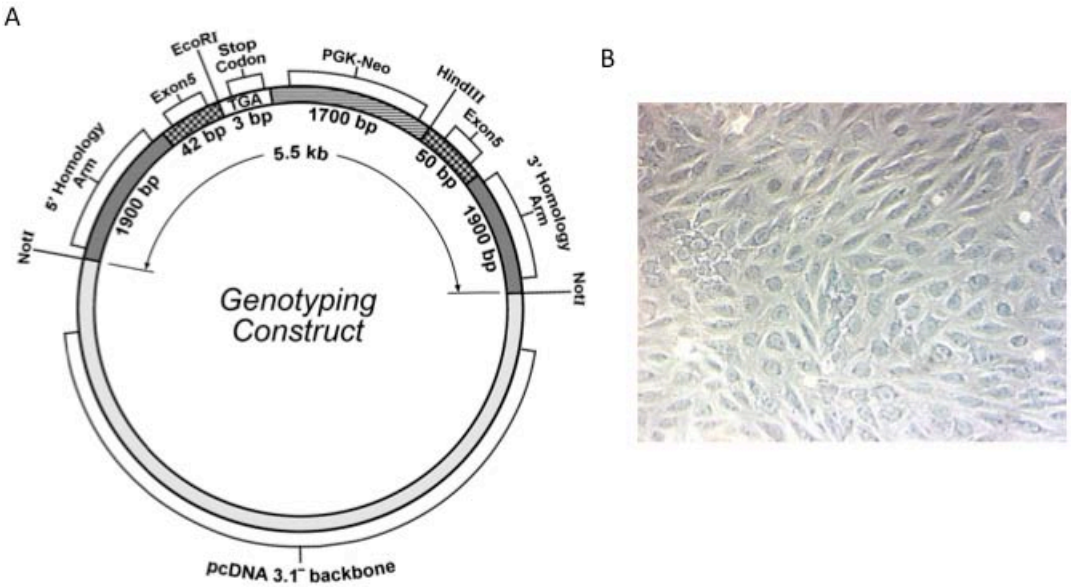

Figure 2-3: Generating a genotyping construct for preliminary experiments.

(A) In order to test the ability of a PCR to detect targeted fibroblasts with only small amounts of DNA, a larger construct was generated that included an additional 400 nucleotides on each end of the homology arms. This construct was assembled in the pcDNA3.1- backbone, and was not subcloned into the AAV2 backbone. This construct was linearized and transfected into fetal fibroblasts. Using limiting amounts of cells, optimal PCR primers and conditions could be deduced for use in the actual screening of cells. **(B)** In addition, this construct allowed for the testing of the functional capability of the PGK-Neo construct to confer antibiotic resistance on targeted cells. This picture shows a typical confluent fibroblast colony after 14 days of selection in G418-containing media.

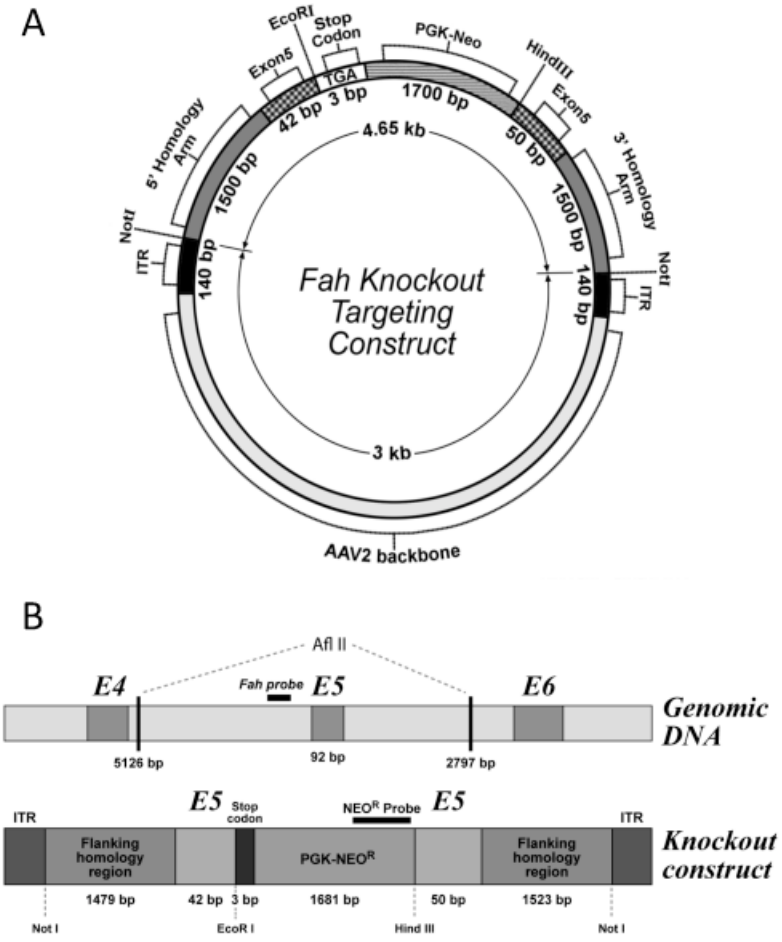

Figure 2-4. Generation of the AAV-DJ targeting vector.

(A, B) The strategy used to generate the targeting vector is schematically depicted here. The PCR primers located within exon 5 were designed to contain tails that introduced the stop codon as well as restriction sites that facilitated facile ligation into the PGK-Neo expression vector. Unique restriction sites were then used for the 5' and 3' homology arms. Once the full-sized 4683bp targeting construct had been generated, it was cloned into the backbone of an AAV2 plasmid. The approximate location of Southern blot probes is also depicted here.

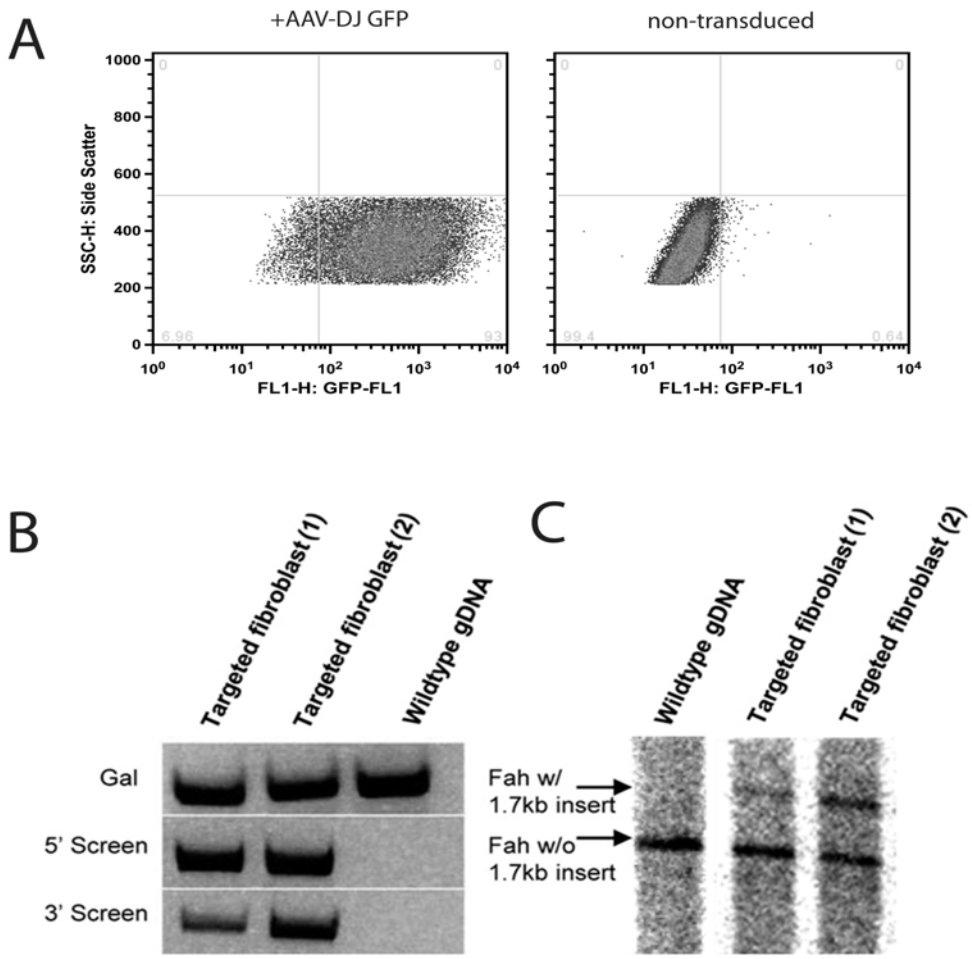

Figure 2-5. AAV-mediated *Fah* gene knockout.

(A) The AAV-DJ GFP construct can transduce pig fetal fibroblasts. 24 hours post transduction, 93% of the pig fetal fibroblast were GFP-positive as detected by flow cytometry compared to non-transduced cells. **(B)** PCR gel image showing two targeted fibroblast lines (1) and (2). Wild-type genomic DNA (gDNA) was isolated from the liver tissue of an adult pig. PCR primers designed to amplify the α1,3-galactosyltransferase (Gal) gene served as an internal control for the lysis procedure and PCR. Two separate sets of primers were used to amplify either the 5' or 3' end of the construct. One primer is placed in the PGK-neo cassette and the other outside the 1.5 kb targeting arm so that only properly targeted fibroblasts would yield a PCR product. **(C)** Southern Blot with *Fah* probe. Two representative targeted lines are shown in addition to wild-type gDNA. The lower bands represent the native wild-type *Fah* locus and the upper bands are the result of a 1.7 kb *stop-neo* cassette insertion into exon 5 of the genomic *Fah* gene. The Southern probe was generated from unique gDNA sequence located in intron 4 of the *Fah* gene outside of the homologous targeting region.

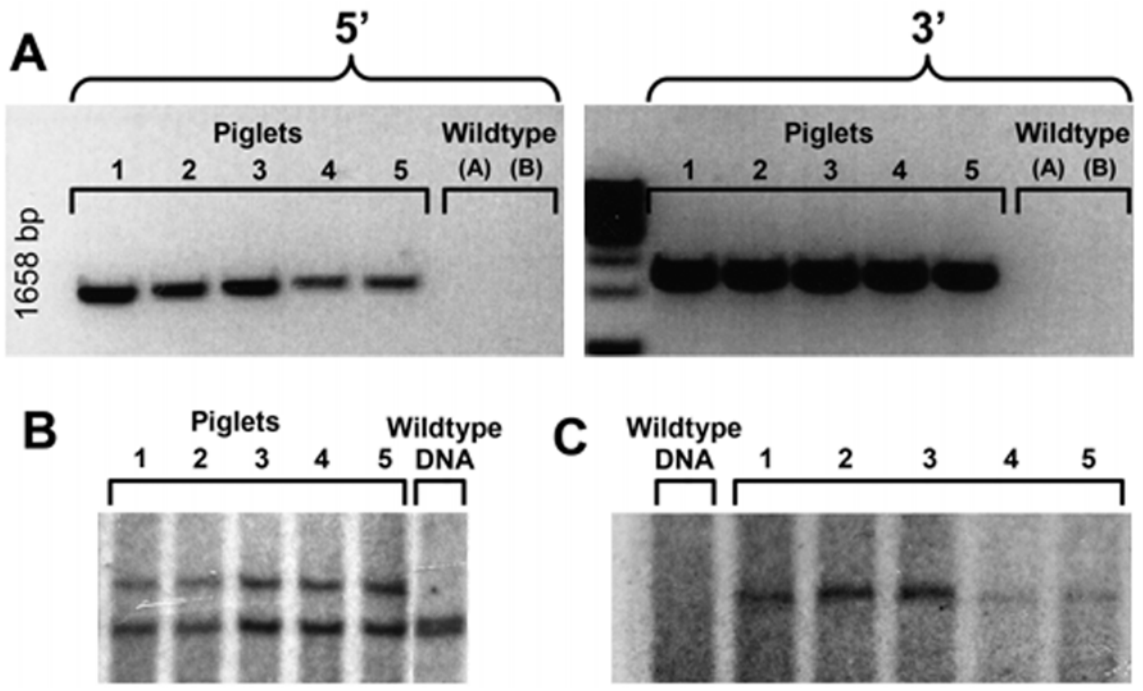

Figure 2-6. Genotype confirmation of *Fah*-null heterozygote pigs.

(A) PCR gel image showing the genotyping confirmation of the newborn piglets. Five piglets are represented (numbers 1-5). Wild-type DNA (WTA and WTB) was isolated from the livers of adult pigs and served as a negative control. Two separate sets of primers were used to amplify either the 5' or 3' end of the disruption cassette. One primer is placed in the PGK-neo cassette and the other outside the 1.5 kb targeting homology arm but within the *Fah* gene so that only properly targeted fibroblast would yield a PCR product. **(B)** Southern Blot with *Fah* probe. Lower bands represent the native wild-type *Fah* locus and the upper bands are the result of the insertion of the 1.7 kb disruption cassette into exon 5 of the genomic *Fah* gene. The Southern probe was generated from unique gDNA sequence located in intron 4 of the *Fah* gene outside of the homologous targeting region. **(C)** Southern Blot with neo probe. A unique probe homologous to the neo cassette was generated and hybridized to detect integrated targeting constructs within the pig genome. Only a single band was ever detected, indicating a single integration within the genome.

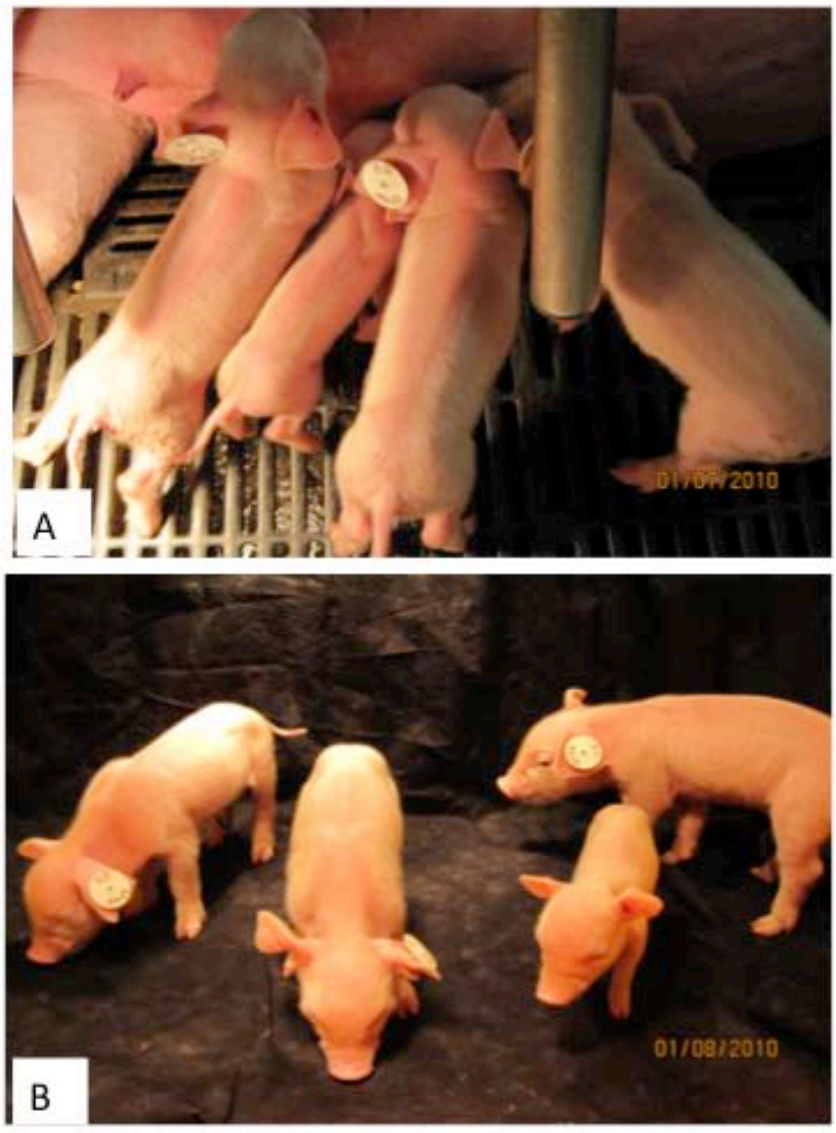

Figure 2-7. *Fah*-null heterozygote piglets.

(A) Piglets 12 hours after being born. **(B)** Piglets 24 hours after being born.

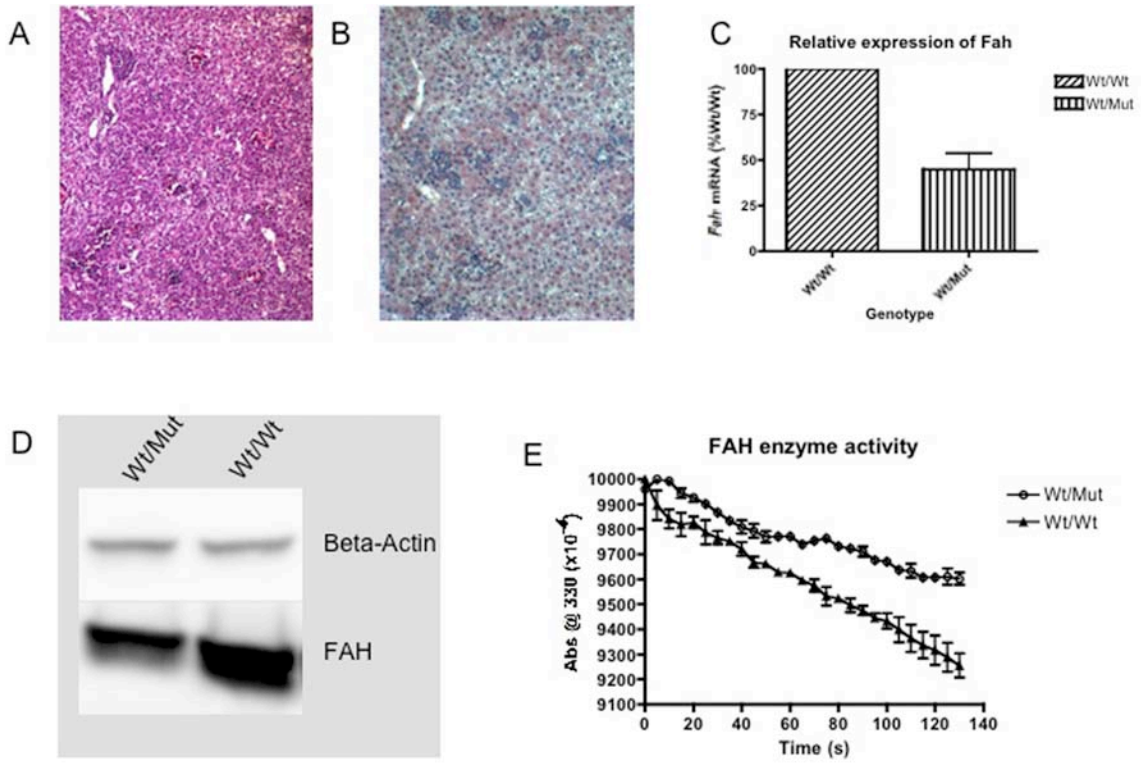

Figure 2-8. Phenotypic characterization of *Fah*-null heterozygote piglets.

(A) H&E stained section of liver from a *Fah*-null heterozygote piglet (magnification x125). The liver histology is normal. **(B)** FAH protein was detected by IHC using a rabbit anti-FAH polyclonal antibody in a formalin-fixed liver section from a *Fah*-null heterozygote piglet (magnification x200). There is characteristic FAH staining in the hepatocytes. **(C)** There is a 55% reduction (average = 55.3%, standard error = 0.09) of *Fah* gene expression in heterozygotes compared to wild-type siblings by qPCR analysis. *Fah* gene expression is compared to expression of the housekeeping gene beta-actin (*Actb*). Error bars represent standard error, n = 2. **(D)** Western blot using a polyclonal rabbit anti-FAH antibody and imaged using a chemiluminescent substrate for detection of the HRP-conjugated secondary antibody. 30μg of protein lysate was added for each sample. There is a decrease of FAH protein in the liver lysates from *Fah*-null heterozygote piglets when compared to wild-type littermates. **(E)** FAH enzyme activity is reduced in *Fah*-null heterozygote piglets. The absorbance of FAA at 330nm is plotted on the Y-axis and time in seconds is plotted on the X-axis. Error bars represent standard error, n = 2.

TABLES

Table 2-1. List of primers used for generating *Fah*-null heterozygote pigs.

Primer Name	Sequence (5'–3')
MG2616	GTAGCGAATTCGCGGCCGCGCAATGTTTTGCTAATTTCTGC
MG2678	GGATAGAATTCCTGCCGGGAGGAATAGAAGT
MG2619	GTAGCAAGCTTCACGCCACAAACGTCGGAGT
MG2680	GGATAAAGCTTGCGGCCGCACTCTTCCACCAGCAAGCAT
MG2622	GTAGCGAATTCTGATCTACCGGGTAGGGGAGGCG
MG2679	GGATAAAGCTTTAGAACTAGTGGATCTCGAG
MG2844	GAACCCAAATTCTCATGGATACC
MG2821	CTAAAGCGCATGCTCCAGAC
MG2824	ATTGCATCGCATTGTCTGAG
MG2851	TATGCCTCCTGATCCTAAATCTTCC
MG2840	CCGTTGTGTAGGCATCACATT
MG2841	TAACAATTTCTGCCCCCTTG
MG3159	TGCTCCTGCCGAGAAAGTAT
MG3160	CAACAGATGGCTGGCAACTA
Fah qPCR F	ACGACCAGCCCTACATGTTC
Fah qPCR R	GAGTGGTGAGTGAGCTGCTG
Actb qPCR F	CACGCCATCCTGCGTCTGGA
Actb qPCR R	AGCACCGTGTTGGCGTAGAG

Table 2-2. Gene targeting frequency in donor fibroblasts. Two cell lines are shown here, 1 and 2. The percentage of G418-resistent clones was obtained by diving the number of G418-resistent clones by the number of infected cells and then multiplied by 100. *Fah*-null heterozygote targeted clones were those that were PCR-positive for targeted homologous recombination.

Donor (Cell Line)	**Sex**	**G418-resistant clones (%)**	**Targeted/ G418-resistant clones (%)**
1	F	0.15	8.50
2	M	0.29	2.29

Table 2-3. Biochemical parameters in *Fah*-null heterozygote pigs. Measurements were made from dried blood spot samples from 7 and 8-day old piglets for either *Fah*-null heterozygotes (Wt/Mut) or their wild-type siblings (Wt/Wt). Values in parentheses represent the standard error for each reading. N is the number of pigs for each group.

	Units	**Wt/Wt Female n = 5**	**Wt/Wt Male n = 6**	**Wt/Mut Female n = 4**	**Wt/Mut Male n = 5**	**Normal Range**
Succinylacetone	μmol/L	0.96 (.05)	0.94 (0.09)	0.92 (0.12)	0.91 (0.04)	< 3
Tyrosine	μmol/L	105 (23)	53 (10)	86 (40)	93 (19)	55-147
Phenylalanine	μmol/L	97 (5)	74 (17)	77 (21)	73 (13)	38-137

DISCUSSION

As limitations in murine models have become more apparent, a substantial need for the creation of improved models of human diseases is required. The advent of SCNT and improved gene targeting strategies has made the pig a preferred choice for generating large animal models of human diseases (86, 108). In this report we were able to reproduce and improve on initial efforts of gene targeting in pigs by using for the first time a chimeric AAV vector (AAV-DJ) to target and disrupt the porcine *Fah* gene. After nuclear and embryo transfer steps, multiple *Fah*-null heterozygote females were generated. These animals were reproductively healthy and were able to give rise to viable, healthy offspring that also inherited the mutant allele. *Fah*-null heterozygotes do not suffer from any abnormal liver pathology and are healthy, able to reproduce and are phenotypically normal when compared to their wild-type littermates. However, *Fah*-null heterozygote animals have reduced FAH levels and a substantially reduced ability to hydrolyze FAA, thereby confirming that the targeted disruption does produce a defective *Fah* allele.

The use of AAV vectors allows for the targeting of genes that are transcriptionally inactive, as is the case for the *Fah* gene in fetal fibroblasts (92, 109). In addition, the single stranded genome of the AAV particle is ideal for homologous recombination and can efficiently target sequences in the genome when large areas of flanking homology are inserted around the disruption cassette as was used in this study. Rogers et al. noted variability in targeting frequencies between sibling fibroblast clones (85). Controlling for experimental variations, targeting events between different fibroblast clones differed by as much as 100-fold

using AAV1 in their studies. This high degree of clone-to-clone variability was not seen in our experiments (Table 2-2). Additionally, using the chimeric AAV-DJ, the targeting frequencies were observed to be higher than those found using AAV1 in the CF model, a result that leads to substantial time and cost savings when producing these porcine knockout models. Although locus heterogeneity may explain the discordance between the variability in the results of Rogers et al. and our findings, we believe three important factors may have contributed to the high targeting efficiency seen this report.

Firstly, in our preliminary work, we determined there to be as many as two base pair differences in the 5' region and 15 base pair differences in the 3' region of the targeting homology between the two alleles of the same animal's genome. Previous work done by others have shown that as few as one base pair difference is enough to disrupt homologous recombination between the AAV vector and target genomic sequence and so we ensured complete sequence homology between the targeting arms and a single allele of *Fah* (110, 111). Secondly, we designed the neo insertion to be centrally located between the homologous arms of the vector, which others have shown to give the greatest targeting efficiency (112). Thirdly, we contend that the use of AAV-DJ resulted in a more efficient targeting. AAV-DJ is an artificial chimeric AAV vector containing hybrid capsid sequences from three naturally occurring AAV serotypes (AAV2, 8 and 9) (102). This and other chimeric AAV vectors are currently being used because of their improved tissue tropism and transduction frequencies (102, 109, 113). However, understanding of the factors

that influence AAV gene targeting are still incomplete and more work in this area will likely improve our ability in the future to modify genes in primary somatic cells.

According to the annual report of the American Liver Foundation, hepatitis, cirrhosis and HCC affect 25 million Americans. However, research in the area of liver disease lags behind other well studied prominent disorders because of the lack of appropriate animal models. The HT1 pig will potentially address several significant needs, including serving as the first large animal model of HCC arising spontaneously in the background of cirrhosis. In addition, the *Fah*-null mouse has proven an invaluable model for cell and gene therapy work, including its use for hepatocyte and bone marrow transplantation studies, as well as both viral and non-viral mediated gene therapy approaches (29, 114-116). We anticipate that the pig model will also be extensively used for similar gene and cell therapy studies. Finally, we recently developed a method whereby primary human hepatocytes were efficiently expanded in immune-deficient mice mutant for *Fah* (37). In these mice, transplanted $Fah^{+/+}$ primary human hepatocytes were able to engraft and expand to greater than 90% repopulation of the mouse liver. These hepatocytes were fully functioning adult primary hepatocytes capable of performing all the necessary metabolic and synthetic functions that are required in the normal liver. However, a limitation in the repopulated FAH deficient mouse is related to its small size. The absolute number of primary human hepatocytes that can be obtained from these animals is low, making a large animal model of FAH deficiency highly desirable.

CHAPTER 3

Towards a novel source of pancreatic beta cells: Direct reprogramming of mouse gall bladder cells into insulin-positive beta-like cells.

ABSTRACT

Current cell therapies for type 1 diabetes are restricted to transplantation of donor islets, but success has been hindered by both lack of donor islets available and by immune rejection of transplanted cells. We hypothesized that reprogrammed gall bladder cells (GBCs) could be used as an autologous cell therapy. Here, we show that murine GBCs can be robustly expanded *in vitro*, allowing the generations of billions of cells from a single gall bladder. These GBCs can be transduced by Adenovirus, although optimal transduction of GBCs requires complexing of the viral particles with DEAE-Dextran. We determined the combination of *Neurog3*, *Pdx1* and *MafA* to be the minimal required transcription factors for expression of both *Ins1* and *Ins2* gene transcripts. Interestingly, reprogramming to the beta-cell fate occurs rapidly within 48 hours, with maximum *in vitro* reprogramming occurring by 72 hours. We also determined that inhibition of notch signaling as well as activation of retinoic acid-effector genes increased the reprogramming efficiency of GBCs into beta-like cells. Using flow cytometry to isolate reprogrammed cells, we confirmed reprogrammed GBCs were differentiating towards a beta-like cell fate by both gene and protein analyses. Additionally, these reprogrammed cells also begin to lose their gall bladder phenotype. Next, in order to determine the global expression profiles of these cells, we performed RNA-Sequencing analysis to compare reprogrammed GBCs to control GBCs, as well as true pancreatic beta cells. Importantly, this analysis confirmed the qPCR results of previously analyzed genes, however a number of other beta-cell specific genes were also upregulated. However, reprogrammed GBCs

also had significant gene expression differences compared to beta cells, indicating these cells were not fully differentiated towards the beta cell fate. Finally, to determine if these cells could be used to reverse hyperglycemia *in vivo*, we transplanted these cells into diabetic recipients. Although the transplanted cells were unable to consistently reverse the hyperglycemia, the cells were able to engraft long term in these mice and were insulin-positive for at least 12 weeks post-transplantation.

INTRODUCTION

Diabetes mellitus type 1 is caused by destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. According to the Juvenile Diabetes Research Foundation, the number of patients in the United States is estimated at three million people, with approximately eighty new cases being diagnosed daily. Type 1 diabetes is treatable by regular injection or infusion of insulin, however patients are at increased risk of hypoglycemic or hyperglycemic episodes, both of which can be fatal (40). Cell therapy to cure this form of diabetes is severely limited by the lack of transplantable pancreatic beta cells. Success of the Edmonton protocol, which uses islets from up to three cadaveric donors to treat a single patient, has also been limited by the need for long-term immune suppression in the patient (45, 117). Ideally, therefore, the source of these transplantable cells would be autologous and abundant.

There have been many approaches attempted to generate new beta cells by reprogramming. Initially, *in vivo* reprogramming of hepatic cells using expression of different pancreatic transcription factors, including *Neurog3* and *Pdx1*, was able to restore euglycemia in hyperglycemic mice (50, 51). More recently, *in vivo* reprogramming of exocrine acinar cells into insulin-positive cells by expression of *Neurog3*, *Pdx1* and *MafA* was also able to reverse hyperglycemia in mice (118). In addition, there have been several approaches *in vitro* to reprogramming pluripotent cells, including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), by directing differentiation of these cells along the pancreatic lineage to

generate insulin-producing cells (53-56). Although there have been challenges with using pluripotent cells, such as teratoma formation by transplanted cells, there has also been success in restoring normal blood glucose control in diabetic mice (60).

It has long been known that the pancreas, biliary system and liver arise from the posterior ventral foregut endoderm (62). However, recently it was demonstrated that the extrahepatobiliary system shares a common development origin with the ventral pancreas, from a cell termed the pancreatobiliary progenitor (119). These progenitors coexpress Pdx1 and Sox17, prior to differentiating into Pdx1+ ventral pancreas and Sox17+ biliary tissue. Segregation of these distinct lineages was partly regulated by the notch effector *Hes1*. Furthermore, loss of *Hes1* results in generation of ectopic pancreatic tissue in the common duct with concomitant agenesis of the gall bladder (120). Additionally, it was demonstrated that inhibition of Hes1 in cultured gall bladder cells (GBCs) is sufficient to induce some insulin expression (121).

In this current study, we investigated if cultured mouse GBCs could be directly reprogrammed to a beta cell fate, without undergoing a pluripotent intermediate step. We transduced GBCs with adenoviruses expressing the transcription factors *Neurog3*, *Pdx1* and *MafA* and showed that a proportion of these cells could be differentiated into a beta-like cell. Importantly, while the transplanted cells were unable to reverse hyperglycemia in diabetic mice, these cells had the ability to engraft, survive and remain insulin-positive up to 12 weeks post-transplantation. Therefore, our findings indicate that the gall bladder could be used a source of reprogrammable cells for the treatment of type 1 diabetes.

EXPERIMENTAL PROCEDURES

Mouse gall bladder cell isolation and culture.

Gall bladders (GBs) were removed by a surgical incision and bile released by making a single cut in the wall. GBs were rinsed twice in DPBS (Life Technologies, Ca) and then cut into several pieces. This material was then incubated at 37°C with 0.25% Trypsin/EDTA (Life Technologies, Ca) for 45 minutes to obtain a cell suspension. Cells were cultured using a modified protocol to that previously described (122). Briefly, cells were plated on a 70-80% confluent irradiated LA7 rat epithelial feeder layer that had been previously γ-irradiated at 60 Gy. Cells were cultured in DMEM/F12 (Life Technologies, Ca) supplemented with 0.5% FBS (Hyclone), 1% insulin-transferrin-selenium (Roche, IN), 15mM HEPES (Fisher Scientific, MA) and antimicrobials (100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml Amphotericin B; Cellgro, VA) in a 37°C incubator with 5% CO_2. Media was changed every two to three days. When GBCs were 70-90% confluent, they were passaged by incubation with 0.05% trypsin/EDTA (Life Technologies, Ca) at 37°C, followed by incubation with DNaseI at 37°C for 10 minutes to obtain a single cell suspension.

Fibroblast culture.

For initiating fibroblast cultures, mouse ears from euthanized animals were washed with DPBS, cut into several pieces, and digested with 0.25% Trypsin/EDTA for 60 minutes at 37°C with regular mixing. Upon inactivation of trypsin, the tissue mix was spun at 1000 rpm for 5 mins, resuspended in DMEM supplemented with 15%

FBS and antimicrobials, followed by plating in a 37°C incubator with 5% CO_2. Media was changed every two to three days.

Adenovirus transduction of GBCs.

Each adenovirus consisted of the full-length cDNA (human *Neurog3*, rat *Pdx1*, mouse *MafA*; *Neurog3* and *Pdx1* provided by Michael German, University of California at San Francisco; *MafA* provided by Roland Stein, Vanderbilt University Medical Center) driven by the CMV promoter. For additional amplification, each virus was expanded in HEK293 cells and purified using the FastTrap Purification Kit (Millipore, MA) as per the manufacturer's protocol. *Neurog3* (MOI 1000), *Pdx1* (MOI 500), and *MafA* (MOI 500) were incubated with DEAE-Dextran for 30 minutes at room temperature with regular mixing prior to addition to the cell media. Media was changed 16-20 hours post transduction.

GBC reprogramming.

GBC cultures were grown to ~70% confluency. On this day, day 0, cells were transduced with the adenovirus/DEAE-Dextran mix. 16-20 hours later, the media was changed to include 2 μM retinoic acid (Sigma-Aldrich, MO) and 1% DMSO (Fisher Scientific, MA). 24 hours later, the media was changed to include 250 nM of the γ-secretase inhibitor dibenzazepine (DBZ; EMD Chemicals, PA) and 1% DMSO. 48 hours later, the media was changed to DMEM, supplemented with 10% FBS and antimicrobials. For time course studies, the media was changed every other day. All

cells, both attached and in suspension, were kept by spinning the media to pellet all cells prior to addition of new media.

Flow cytometry and FACS.

Dissociated cells were resuspended in DMEM, supplemented with 2% FBS and 0.25 mg/ml DNaseI (Sigma-Aldrich, MO). Propidium iodide staining was used to label dead cells for exclusion. The forward scatter (FSC):pulse width gating excluded cell doublets from sorts, as previously described (100). Cells were analyzed with a FACScalibur or sorted by an inFluxV-GS (BD Biosciences, San Jose, CA, USA for both) at 15 psi using a 100 μm nozzle. Data were analyzed using FlowJo (Treestar, Ashland, OR).

RNA isolation and qRT-PCR.

For RNA isolation, cells were either directly FACS-sorted into Trizol Liquid Sample (Invitrogen, CA) or trypsinized and pelleted by centrifugation prior to cell lysis with Trizol. RNA was purified using RNeasy (Qiagen, CA) per the manufacturer's protocol. First strand cDNA synthesis was completed using MMLV reverse transcriptase and random oligonucleotide primers (Invitrogen, CA). Relative mRNA expression levels were determined by qRT-PCR using a BioRad iCycler with a single color MyiQ detection system. All reactions were performed with Platinum Taq DNA Polymerase (Invitrogen, CA) and SYBR Green using 45 cycles of 95°C for15s, 68°C for 20s, and 72°C for 20s. The full list of primers is given in Table 3-1.

Immunohistochemistry and immunofluoresent imaging.

For cytospin imaging, either reprogrammed GFP+ cells or non-adenovirus transduced cells were spun at 1000rpm for 5 mins onto Superfrost Plus slides (Thermo Fisher Scientific, PA). Cells were fixed in either 4% paraformaldehyde or 90% methanol at 4°C for 10 mins. Prior to labeling, cells were blocked in 5% BSA for 60 mins at 23°C. Primary labeling was performed overnight at 4°C in PBS supplemented with 2% BSA and 0.05% Triton-X using rabbit polyclonal antibodies against Insulin (H-86; Santa Cruz Biotechnology, CA), C-peptide (BCBC collection #1042), Neurod1 (16508; Abcam, MA) and Nkx6-1 (BCBC collection #1069). Secondary labeling was performed for 60 mins at 23°C in PBS supplemented with 2% BSA and 0.05% Triton-X with a 1:200 dilution of Alexa 555-conjugated goat anti-rabbit IgG (Cell Signaling, MA). Nuclei were stained using Hoechst 33342 (Sigma-Aldrich, MO). For IHC analysis, formalin-fixed paraffin-embedded kidneys were sectioned and labeled with a primary antibody against insulin (H-86; Santa Cruz Biotechnology, CA) and detected using previously described methods (29).

Enzyme-linked immunosorbent assay.

Control and reprogrammed GBCs were harvested at the indicated time points. Cells were washed twice in Krebs Ringer Buffer supplemented with 2.8 mM glucose (KRB-2.8) and incubated at 37°C for 90 minutes in KRB. Following two further washes in KRB-2.8, cells were incubated in either KRB-2.8 or Krebs Ringer Buffer supplemented with 16.7 mM glucose (KRB-16.7) for 30 minutes at 37°C. Cells were spun at 1200 rpm for 5 mins and the media collected and stored at -80°C. Insulin

quantitative analyses were performed per the manufacturer's instructions (Insulin Ultrasensitive ELISA, Alpco, NH).

Cell transplantation.

GBCs were expanded to 70% confluency prior to reprogramming at the indicated passage number (Table 3-2). Cells were transduced with the three adenoviruses expressing *Neurog3*, *Pdx1* and *MafA* with or without retinoic acid. One to three days later, cells were harvested and shipped on ice to the University of Massachusetts for transplantation on the following day. 10-20x10^6 cells were transplanted into the renal subcapsular space of spontaneously diabetic NRG-Akita or NSG-Akita mice, as previously described (123). Non-fasting blood glucose levels were monitored following transplantation by blood glucose measurements with an ACCUCHEK active glucometer (Hoffman-LaRoche, Basel, Switzerland).

RNA-SEQ analysis.

Islets from MIP-GFP mice were isolated by standard collagenase digestion, as previously described (124). Islets were trypsinized into a single cell suspension and FACS-sorted based on GFP expression. Control and GPF+ GBCs were also FACS-sorted for RNA isolation. RNA library preparation, oligonucleotide sequencing and bioinformatic analysis was performed at the University of Pennsylvania Functional Genomics Core Laboratory.

RESULTS

Expansion of mouse gall bladder cells.

We modified a protocol first reported by Mahohar et al. to isolate and expand mouse GBCs *in vitro* (122). Using a simple trypsinization of the entire gall bladder, we were able to isolate 200,000-400,000 cells from a single mouse. These cells were cultured on a feeder layer of irradiated LA7 rat epithelial cells that allowed their robust expansion (Figure 3-1A&B&C). The predominant population of cells, and the only cell type to exhibit the ability to be passaged indefinitely, was flat colonies of gall bladder cuboidal epithelial cells, consisting of cells with a large nuclear-cytoplasmic population, as described by Mahohar et al. These cells were cultured to 70-80% confluency, at which time the cells were passaged onto a new irradiated feeder layer. We were able to culture these GBCs to at least fourteen passages, with no detectable decrease in morphology or growth rate (data not shown). In addition to this type of culture system, other systems were explored to expand GBCs, including growth on collagen-coated dishes, fibronectin-coated dishes, and in 3D culture using geltrex (a Matrigel-equivalent) droplets (Figure 3-2). However, it was determined that the optimal system for expanding mouse GBCs was a LA7-based feeder culture and this system was used for all further reprogramming experiments described in this thesis.

Determining the cellular makeup of the mouse gall bladder.

The mouse gall bladder is not an extensively studied organ, and little or no information is known about the different cell populations present. Additionally, at the time of this study no gall bladder stem cell has been identified and it is not know if such a cell even exists. This finding would have important implications for the biology of gall bladder development, but it could also play an important role in delineating the cell responsible for causing gall bladder cancer. In addition, it would be fair to hypothesize that a stem cell would be more readily reprogrammable to another cell fate than a more differentiated cell. We initially set out to determine if an *Lgr5*-positive cell was present in the gall bladder, as this has shown to be an principal marker for stem cells in other tissues, including the intestine and stomach (125, 126). We were able to identify a population of *Lgr5*-positive cells in the gall bladder (Figure 3-3A). However, these cells were not more reprogrammable to a beta cell fate than other subsets of cells in the gall bladder nor did they show preferential growth and expansion in the conditions tested (Figure 3-3C&D). Additionally, we performed a flow cytometry analysis of GBCs using a panel of antibodies that had previously been shown to identify specific cell types in the liver and pancreas (Figure 3-4) (100, 127). Once again, although different populations exist in the gall bladder, no single cell type was identified that was more readily reprogrammable to the beta cell fate than another, nor was any cell more clonogenic than another (data not shown). Therefore, for all the following experiments, unfractionated gall bladder cells were used for expansion and subsequent reprogramming.

Mouse GBCs can be transduced by adenovirus.

In order to express the relevant transcription factors needed for reprogramming, we chose to use adenovirus to transduce and express specific genes. We first tested the ability of adenovirus to express GFP in these cells, however less than 10% of the cells were positive by flow cytometry using standard transduction protocols (Figure 3-1D). Previous groups have shown that DEAE-Dextran can complex with adenovirus to increase the ability of this vector to be adsorbed to the cell membrane of lung epithelia (128, 129). We tested several concentrations of DEAE-Dextran and found that concentrations greater than 20 μg/ml allowed transduction of greater than 50% of the GBCs (Figure 3-1D&E&F). Although the higher concentrations of DEAE-Dextran gave even greater transduction efficiencies, these concentrations were also associated with increased cell morbidity (data not shown). Therefore, for all the reprogramming experiments, we chose a final DEAE-Dextran concentration of 20 μg/ml for adenovirus transduction of GBCs.

MafA is required for expression of both *Ins1* and *Ins2* in reprogrammed GBCs.

We next determined what transcription factors were required to reprogram mouse GBCs into insulin-positive cells. We utilized a mouse reporter strain in which the mouse *Ins1* promoter drives expression of GFP (130). In control cultured GBCs, there was never any GFP expression detected, indicating no transcriptional activity from the *Ins1* promoter in these cells. In contrast, under specific reprogramming conditions, the *Ins1* promoter is activated, causing detection of GFP expression by

flow cytometry (Figure 3-5A). We also created another mouse reporter strain by breeding a mouse expressing Cre recombinase under control of the rat *Ins2* promoter with a dual reporter Tomato-RFP/GFP mouse in which expression of GFP is only detected in cells in which a Cre-mediated recombination event has occurred (131, 132). Again, control cultured GBCs were always GFP negative, except under defined reprogramming conditions (Figure 3-5B).

Previous groups have shown that induced expression of *Neurog3* or *Pdx1* is sufficient to differentiate liver cells towards a pancreatic fate *in vivo* (50, 51). Although expression of these two factors induced GFP expression in some cells, we determined that expression of *MafA*, together with *Neurog3* and *Pdx1*, was required for optimal GFP expression in GBCs *in vitro* (Figure 3-5C).

Expression of *Neurog3*, *Pdx1* and *MafA* causes polyhormonal pancreatic gene expression.

As *Neurog3* and *Pdx1* are associated with differentiation of both endocrine and exocrine lineages during pancreatic development, we hypothesized that expression of these factors may also induce expression of other pancreatic subtypes (133). By quantitative PCR analysis of mRNA expression in non FACS-sorted cells, we determined that other endocrine hormones were also being produced, based on detection of *Sst*, *Ppy*, and *Ghrl* gene transcripts (Figure 3-5D). Interestingly, we never detected any mRNA expression of the alpha cell hormone *Gcg*. We also analyzed expression of the exocrine genes *Cela2a* (chymotrypsin-like elastase family, member 2A), *Cpa2* (pancreatic carboxypeptidase A2) and *Prss1* (trypsin 1) (Figure 3-5E).

While no *Cela2a* mRNA was ever detected in reprogrammed GBCs, and no significant increase in *Prss1* between control and reprogrammed cells, there was an increase in expression of *Cpa2* in reprogrammed GBCs. However, while expression of the endocrine genes was comparable to that of the pancreas, the overall expression of the analyzed exocrine genes in reprogrammed cells was less than 0.001% of that detected in normal pancreas.

Retinoic acid and inhibition of Notch signaling augment GBC reprogramming.

In addition to genetic reprogramming, we determined if inhibition or activation of particular signaling pathways would augment reprogramming towards the beta cell fate. Retinoic acid (RA) signaling is essential for proper pancreatic development and acts by interacting with the nuclear receptor families RARs and RXRs which control transcription of genes containing RA responsive elements (134, 135). By including RA in the reprogramming media at a concentration of 2 μM, there was an approximately 2.5 fold increase in GFP+ GBCs reprogrammed with NPM, compared to GBCs only reprogrammed with NPM alone, or NPM and vehicle (DMSO) only (Figure 3-7A). We also examined the effect of inhibiting Notch signaling by using the gamma secretase inhibitor DBZ. Inhibition of Notch signaling has been has been shown to be play an important role in lineage commitment in pancreatic and extrahepatic biliary tissues (136, 137). Concomitant with these findings, inhibition of notch signaling at day 2 of reprogramming caused an approximate two-fold increase in GFP+ reprogrammed cells (Figure 3-7B).

Reprogrammed GBCs rapidly lose their gall bladder phenotype.

Using the optimized reprogramming strategy, we next ascertained the timeline of reprogramming of GBCs to the beta cell fate. We chose two different gall bladder samples and analyzed the expression of GFP over a period of 12 days (Figure 3-7C). The first GFP+ cells appear within 48 hours after transduction with NPM with the maximum number of GFP+ cells detected 24 hours later. After this time, the percentage of GFP+ cells decreases. Although we could not determine that these specific cells were undergoing either apoptosis or silencing of the *Ins1* promoter driving GFP, based on the decreased cell viability over time in these cultures, we deemed this was due to the reprogrammed cells undergoing cell death (data not shown).

We next wanted to confirm that in addition to reprogrammed GBCs turning on certain pancreatic genes, these GFP+ cells were also losing their gall bladder identity. Normal GBCs in culture express high levels of *Muc1*, *Aqp1* and *Krt19*. However, within 3 days post reprogramming, GFP+ GBCs show significant decreases in expression of these three genes by qPCR (Figure 3-7D). In addition, the pancreatobiliary modifiers *Sox17* and *Hes1*, required for normal gall bladder development and homeostasis, have reduced mRNA expression (Figure 3-7D).

Reprogrammed GBCs differentiate into insulin-secreting beta-like cells.

After 4 days of reprogramming, GFP+ GBCs were analyzed by flow cytometry and FACS-sorted for RNA and protein analyses. By qPCR analysis of mRNA expression levels, reprogrammed GBCs expressed both insulin genes, *Ins1* and *Ins2*,

as well as the transcription factors *Neurod1*, *Nkx2-2* and *Nkx6-1*. None of these transcripts were detected in normal cultured GBCs (Figure 3-8A). Additionally, two of the proprotein convertases required for processing proinsulin into insulin and c-peptide, *Pcsk1* and *Pcsk2*, were highly upregulated in reprogrammed cells (Figure 3-8B).

We next confirmed that the cells were also expressing these genes at the protein level. By cytospinning GFP+ cells onto glass slides, we were able to detect insulin, c-peptide, Nkx6-1 and Neurod1 in reprogrammed, but not control, GBCs by immunocytochemistry (Figure 3-9). Finally, we tested if reprogrammed cells were able to secrete insulin in response to glucose stimulation. By using an ELISA specific for insulin, we detected insulin in the incubation media of reprogrammed cells, but never in that of control cells (Figure 3-8C). However, the amount of secreted insulin detected was not increased in response to high glucose stimulation.

GBCs are more readily reprogrammed to the beta cell fate than fibroblasts.

Although other groups have demonstrated the ability of fibroblasts to be differentiated into other cell types such as neurons, we hypothesized that the developmental lineage sharing between the gall bladder and the pancreas would mean GBCs would be more readily reprogrammed to the beta cell fate than a non-developmentally related cell like a fibroblast. Tail tip mouse fibroblasts were expanded and transduced with *Neurog3*, *Pdx1* and *MafA*. Three days later, these cells were FACS-sorted based on GFP expression and gene expression analyzed by qPCR. Interestingly, expression of *Neurog3*, *Pdx1* and *MafA* was able to induce promoter

activity at the *Ins1* locus, as determined by GFP expression and detection of *Ins1* mRNA (Figure 3-10A&B). However, other beta cell transcripts such as *Ins2*, *Neurod1* and *Nkx6-1* are significantly upregulated in GFP+ GBCs compared to GFP+ fibroblasts (Figure 3-10B). Moreover, other transcripts upregulated in reprogrammed GBCs, *Nkx2-2*, *Pax4* and *Pcsk2*, are not even detected in GFP+ fibroblasts (Figure 3-10C). Therefore, although the expression of *Neurog3*, *Pdx1* and *MafA* were able to instigate expression of insulin from the *Ins1* promoter, the degree of reprogramming to the beta cell fate was only minimal in fibroblasts.

RNA-SEQ analysis confirmed reprogramming of GBCs to the beta cell fate.

In order to obtain a global representation of gene expression in reprogrammed cells, GBCs transduced with *Neurog3*, *Pdx1* and *MafA* were FACS-sorted after 3 days based on GFP expression and RNA isolated for RNA-Sequencing analysis. In order to compare these samples, control, non-transduced GBCs were also FACS-sorted. Additionally, GFP+ cells from pancreatic islets of MIP-GFP mice were analyzed as the optimal true beta cell population. This analysis confirmed initial qPCR results that GFP+ GBCs were being reprogrammed to the beta cell fate with concomitant loss of gall bladder phenotype (Figure 3-11). The list of upregulated genes compared to control cells was in the hundreds but, importantly, included the pancreatic transcription factors *Isl1*, *Neurod1*, *Nkx2-2*, *Pax6*, *Pax4*, *Rfx6* and mouse *Pdx1*. Additionally, genes involved in insulin processing and packaging into secretory granules were also upregulated and included mRNA for *Pcsk1*, *Pcsk2*,

Cbe, Scgn, *Scgn2*, *Scgn3*, *Chg1*, and *Chgb*. Two other genes involved in beta cell function are the potassium and calcium channels and mRNA for *Kcnj11* (Kir6.2 potassium channel) and *Itpr1* (inositol 1,4,5-trisphosphate receptor, type 1 calcium channel) were upregulated compared to control GBCs. Interestingly, there were several components of glucose sensing, insulin packaging and insulin secretion that had significantly decreased gene expression relative to true beta cells, including *Slc2a2* (glucose sensing), *Slc30a8* (vesicle maturation), *Tmem27* and *Nnat* (insulin secretion). Finally, concomitant with differentiation to the pancreatic fate, GFP+ GBCs had significantly decreased mRNA expression of a number of gall bladder associated genes, including *Muc1* (epithelial mucin), *Cftr* (ion channel), *Aqp1* (water channel), *Slc12a2* (ion transporter), *Slc10a1* (bile acid transporter), and *Clca4* (chloride channel).

Transplanted reprogrammed cells can engraft, survive and produce insulin in diabetic mice.

We next tested whether *in vitro* reprogrammed cells could reverse hyperglycemia in diabetic mice. We used the Akita diabetic model in which a mutation in the *Ins2* gene causes spontaneous hyperglycemia in immunodeficient animals (123, 138). GBCs reprogrammed with *Neurog3*, *Pdx1* and *MafA* and incubated with or without RA were harvested from culture after two to three days and transplanted under the kidney capsule of recipient mice (Table 3-2). Blood glucose levels were monitored weekly. Of the 21 transplanted mice, only one mouse showed a temporary reversal of hyperglycemia that was not sustained (data not

shown). Between 8-12 weeks after transplantation, all mice were euthanized and the kidneys collected for immunohistochemistry. Interestingly, although the transplanted cells were unable to permanently reverse the hyperglycemia in any of the recipients, 8/19 of the mice had insulin-positive cells in the graft region of the kidney (Figure 3-12) compared with no positive cells detected in any of the control transplanted GBCs (n = 6).

FIGURES

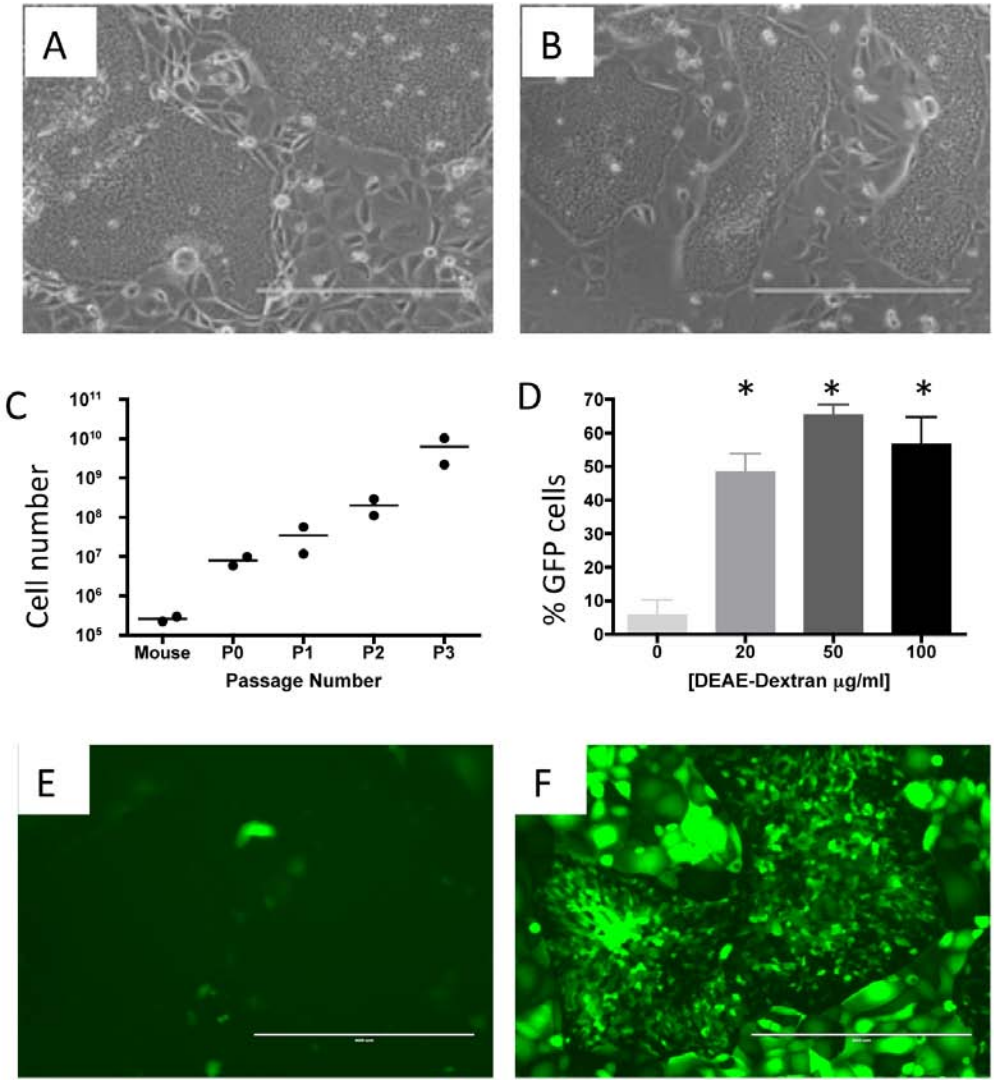

Figure 3-1. Expansion and adenovirus transduction of GBCs.

(A) Passage 0 and **(B)** passage 3 typical GBC colonies grown on a LA7 feeder monolayer. **(C)** Murine GBCs can be robustly expanded. A typical mouse gall bladder yields approximately 200-400,000 cells that show unlimited ability to replicate and expand, normally generating hundreds of millions of cells by passage 3. **(D)** Adenovirus does not transduce GBCs efficiently, without DEAE-Dextran. At 0 μg/ml DEAE-Dextran there are approximately 5% cells transduced by Adenovirus expressing GFP. However, under increasing concentrations of DEAE-Dextran, there is a significant increase in the number of GBCs transduced (* $P<0.05$). **(E)** Few GBCs are GFP+ with 0 μg/ml DEAE-Dextran. **(F)** At 20 μg/ml concentration, over half the cells are GFP+.

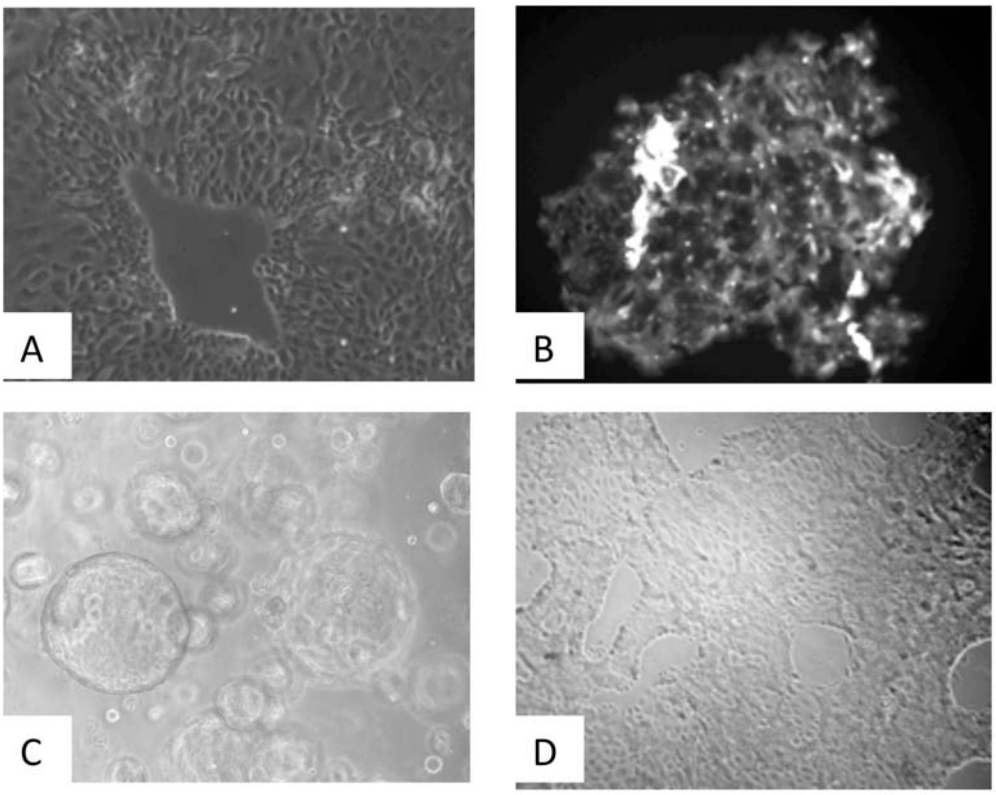

Figure 3-2. Alternative methods to expand GBCs.

(A) Cells can be expanded in feeder-free conditions on either collagen-coated or **(B)** fibronectin-coated plastic tissue culture plates. The cells expanded on fibronectin-coated plastic were from a constitutively active GFP-expressing mouse and this figure was taken using a GFP-detecting microscope. **(C)** Alternatively, mouse GBCs can be resuspended in Geltrex, a Matrigel-alternative. This format permits cells to grow rapidly and form spheroid-type structures in a 3D culture. **(D)** GBCs can also be expanded on a thin-layer of Geltrex in a monolayer culture.

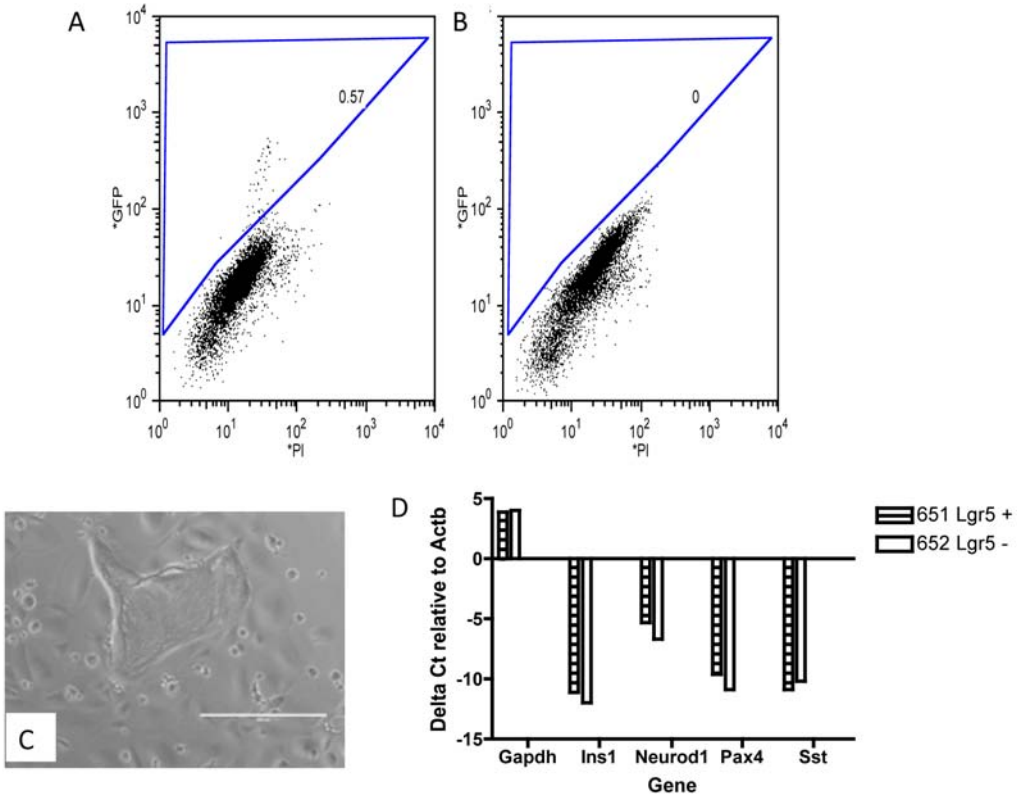

Figure 3-3. *Lgr5* expression in the gall bladder.

(A) 0.57% of GBCs directly isolated from the mouse are *Lgr5*+ based on GFP expression from a mouse in which the *Lgr5* promoter drives expression of GFP. **(B)** There are never any GFP+ cells from control mice, in this case a wild-type C57Bl6 age and sex-matched mouse. **(C)** *Lgr5*+ cells can form colonies on a LA7 feeder layer, however the colonies are smaller and have a reduced colony forming efficiency than non FACS-sorted cells. **(D)** *Lgr5*+ cells are not more reprogrammable to the beta cell fate than *Lgr5*- cells based on mRNA expression levels of *Ins1*, *Neurod1*, *Pax4* and *Sst*. The y-axis represents the change in Ct values relative to Actb and demonstrates that there is no difference in gene expression between the different *Lgr5* populations.

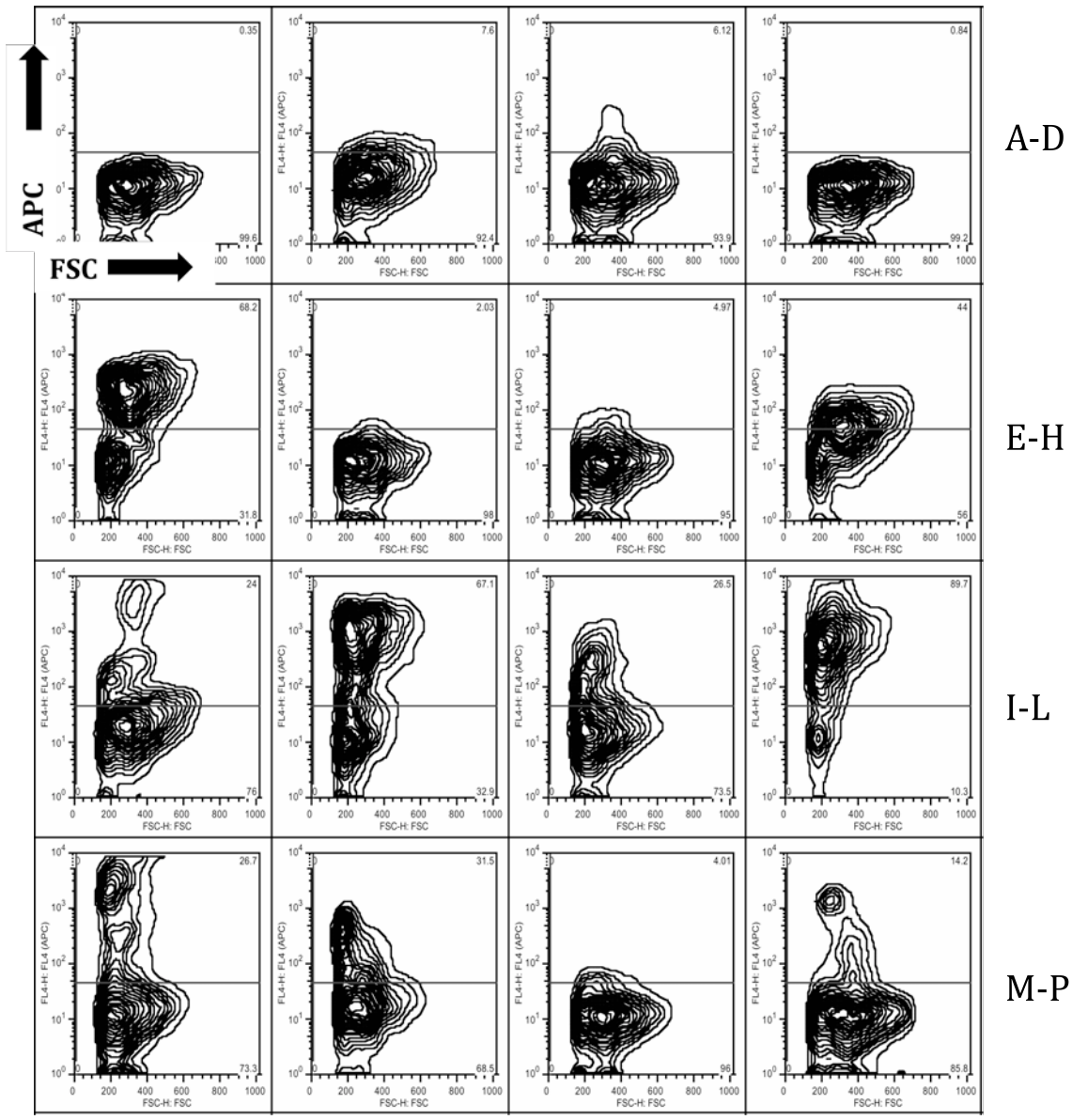

Figure 3-4. Antibody screening on primary mouse GBCs.

(A) 2° antibody APC only control (B) MIC0-2A6 (C) MIC1-6A2 (D) OC2-1F3 (E) OC2-1D11 (F) OC2-1C6 (G) OC2-1C8 (H) OC2-2A6 (I) OC2-2B9 (J) OC2-2F3 (K) OC2-2F8 (L) OC2-2G9 (M) OC2-3C5 (N) OC2-3C7 (O) OC2-4E8(P) OC2-5F6.

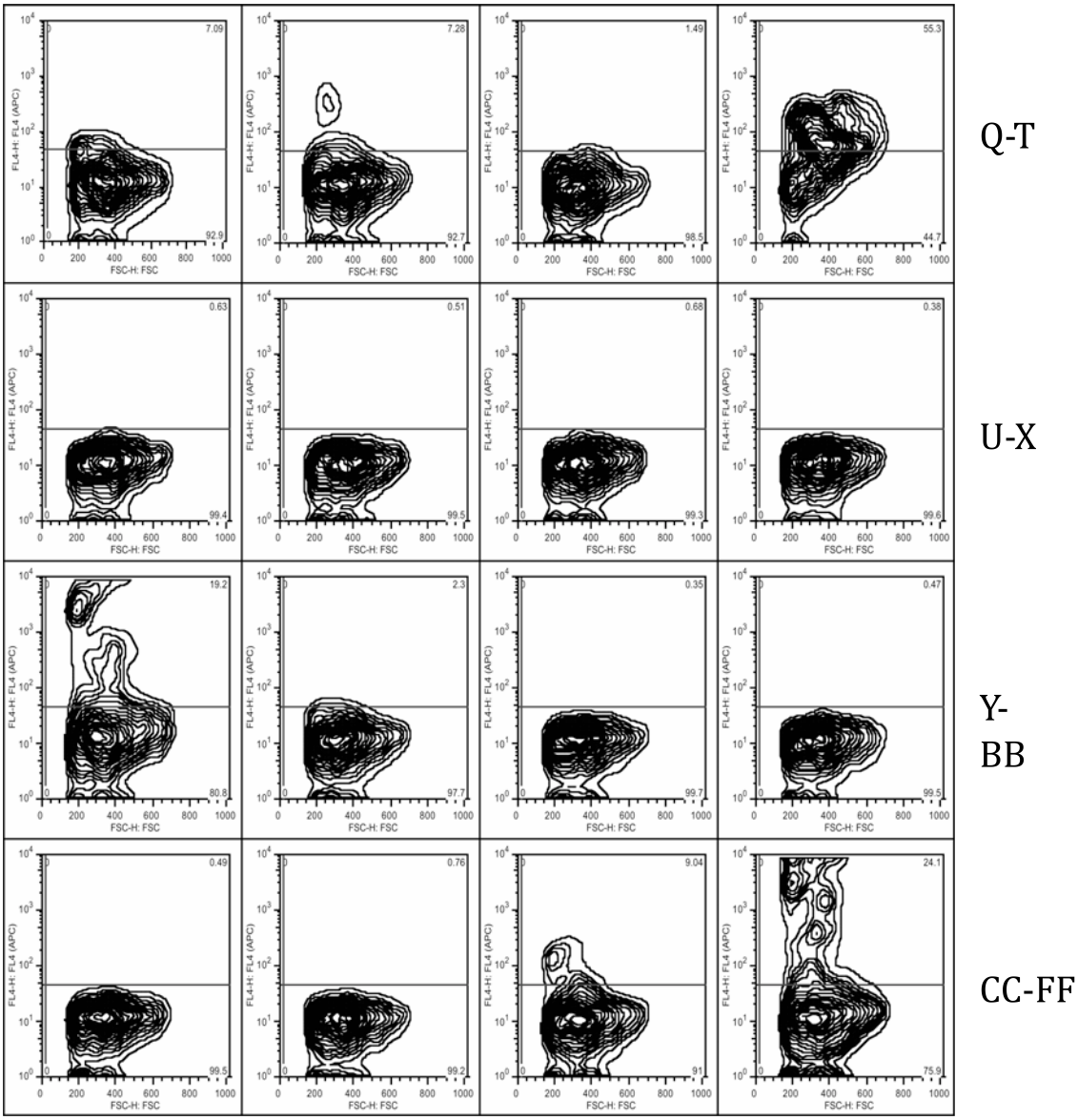

Figure 3-4 (continued). Antibody screening on primary mouse GBCs.

(Q) OC2-5H9 (R) OC2-6E10 (S) OC2-2F9 (T) MKC-5C4 (U) MKC-4A7 (V) MKC-1F9 (W) MKC-4H7 (X) MKC-6A2 (Y) OC2-6E7 (Z) MLPC3-3F7 (AA) MLPC3-1F10 (BB) MLPC3-2A7 (CC) MLPC3-2D2 (DD) MLPC3-3F7 (EE) MLPC3-5A9 (FF) MLPC3-5F3.

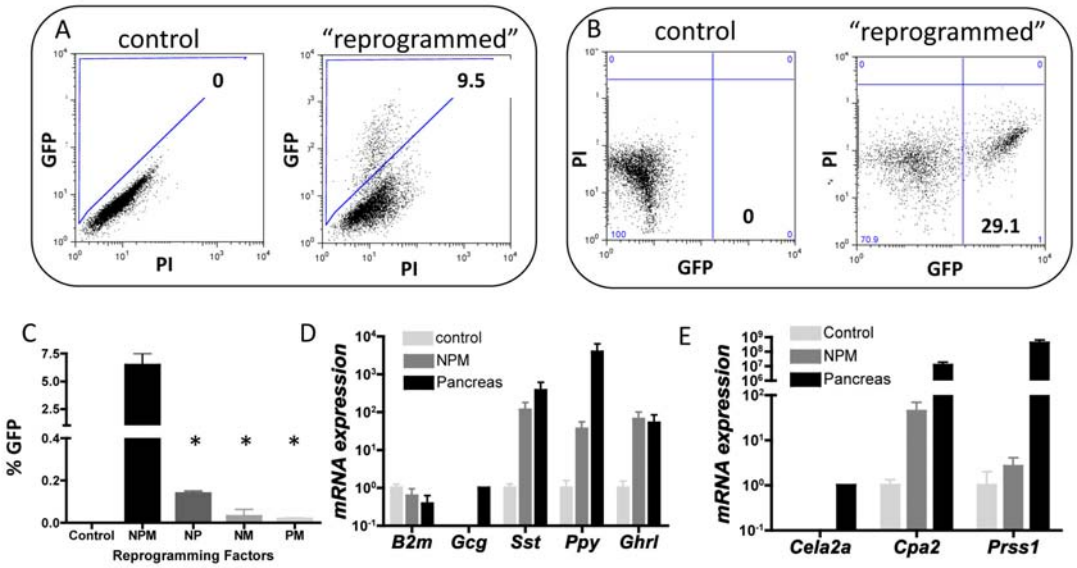

Figure 3-5. GBCs can be reprogrammed to the pancreatic fate.

(A) GBCs from MIP-GFP mice do not have any GFP+ cells, indicating no promoter activity at the *Ins1* locus. However, under appropriate reprogramming conditions, a proportion of GBCs become GFP+. **(B)** Ins2-Cre/^{fl}Tom^{fl}GFP GBCs are uniformly GFP negative under normal conditions. However, under optimal reprogramming conditions, a certain percentage of GBCs express Cre recombinase that leads to GFP expression. **(C)** Optimal GBC reprogramming requires all three transcription factors - *Neurog3*, *Pdx1* and *MafA* (NPM). There is a significant difference in the number of GFP+ cells transduced with NPM compared to with only two factors together (* $P<0.05$). **(D)** NPM-transduced GBCs express increased levels of the endocrine hormones *Sst*, *Ppy*, and *Ghrl*. However, no expression of *Gcg* is detected in these reprogrammed cells. **(E)** In addition to endocrine gene activation, reprogrammed GBCs express increased levels of the exocrine genes *Cpa2* and *Prss1*, but do not express any *Cela2a* mRNA.

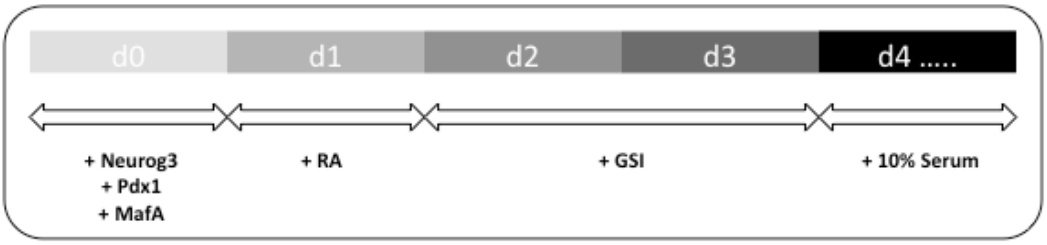

Figure 3-6. Schematic outline of reprogramming timeline.

GBCs are expanded to 60-80% confluency prior to d0. On this day, cells are transduced with *Neurog3*, *Pdx1* and *MafA*. 24 hours later, RA is added to the cell media. On d2, the gamma secretase inhibitor DBZ is added for 48 hours. At this point, cells are FACS-sorted for RNA and protein analysis. For longer-term experiments, cells are maintained in DMEM supplemented with 10% FBS with no additional factors. Full details are provided in the experimental procedures.

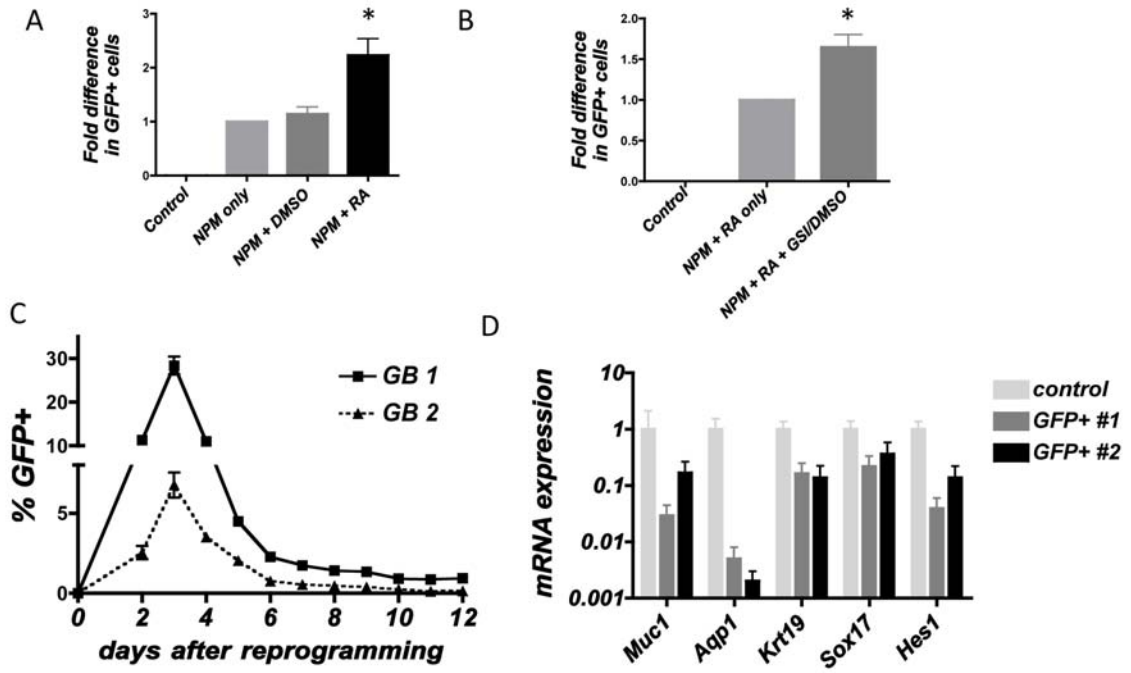

Figure 3-7. Reprogramming to the beta-cell fate is augmented by addition of RA and inhibition of Notch signaling.

(A) RA increases the % of GFP+ GBCs in cultures by approximately two fold (* P<0.05). The addition of the vehicle DMSO alone has no significant effect. **(B)** Adding the gamma secretase inhibitor DBZ and DMSO to the reprogramming mix on d2 significantly increases the number of GFP+ GBCs in the culture (* P<0.05). **(C)** Timeline of reprogramming GBCs from day 0 through day 12. The peak of GFP+ cells occurs on day 3, after which the number of GFP+ cells decreases rapidly. **(D)** Gene expression data of the GFP+ cells compared to control GBCs after three days. The gall bladder markers *Muc1*, *Aqp1* and *Krt19* show decreased mRNA expression in reprogrammed GBCs. The developmental markers *Sox17* and *Hes1* also show decreased expression in reprogrammed GFP+ cells.

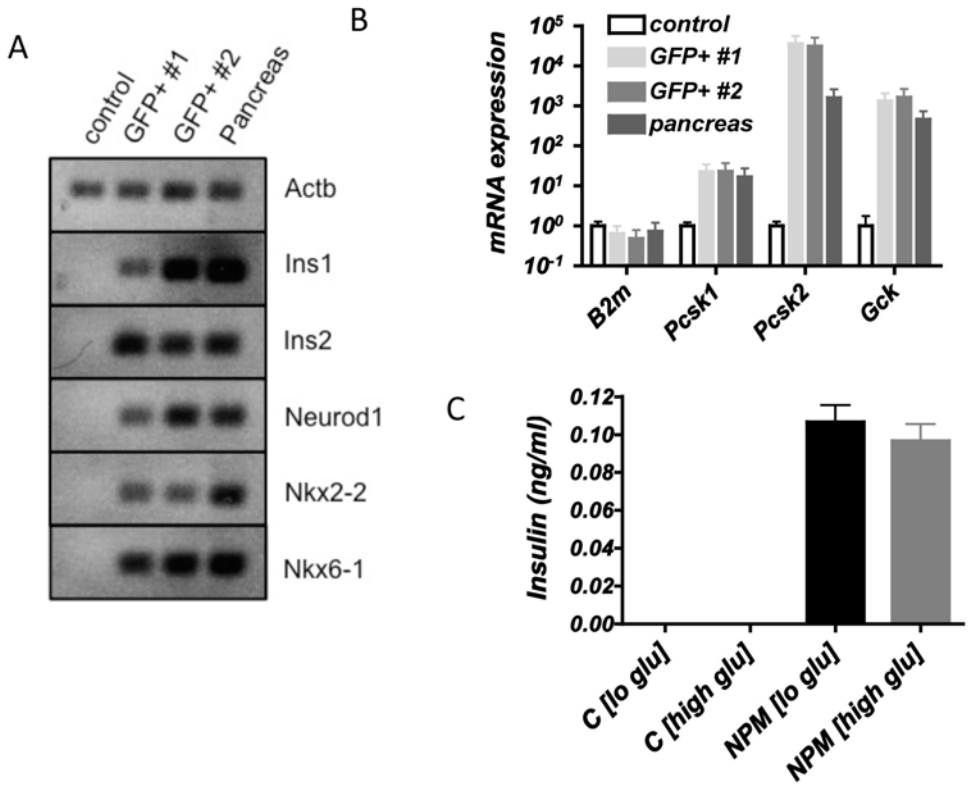

Figure 3-8. Characterization of reprogrammed GBCs.

(A) Under optimal reprogramming conditions GFP+ GBCs turn on the insulin genes *Ins1* and *Ins2*, as well as the transcription factors *Neurod1*, *Nkx2-2* and *Nkx6-1*. Control GBCs do not normally express these genes. **(B)** Similarly, the proinsulin processing genes *Pcsk1* and *Pcsk2* are upregulated compared to controls by a factor of approximately 10 and 10,000 fold. The glucose metabolism enzyme *Gck* mRNA is also significantly increased compared to control GBCs. mRNA levels are at comparable or higher levels than that of the whole mouse pancreas. **(C)** Control (C) GBCs do not secrete insulin under either low or high glucose stimulation. In contrast, reprogrammed GBCs (NPM) are able to secrete insulin into the media. However, insulin secretion is not increased in response to glucose stimulation.

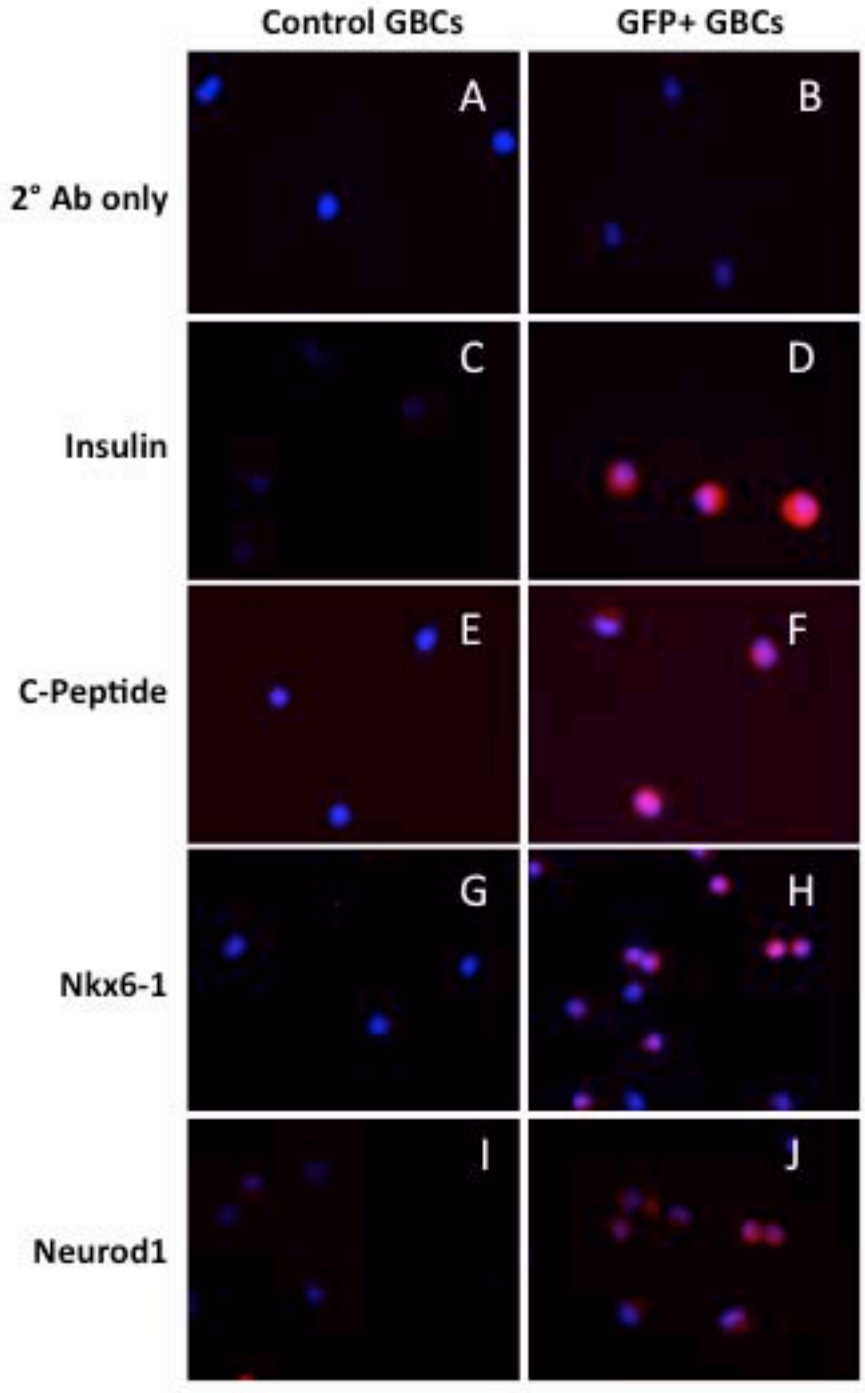

Figure 3-9. Immunocytochemistry of reprogrammed GBCs.

Control or GFP+ GBCs were cytospun onto glass slides, fixed in 4% PFA and stained with antibodies against Insulin (C&D), C-peptide (E&F), Nkx6-1 (G&H) or Neurod1 (I&J). Secondary labeling was performed with an Alexa 555-conjugated antibody, and nuclei counterstained with Hoechst 33342.

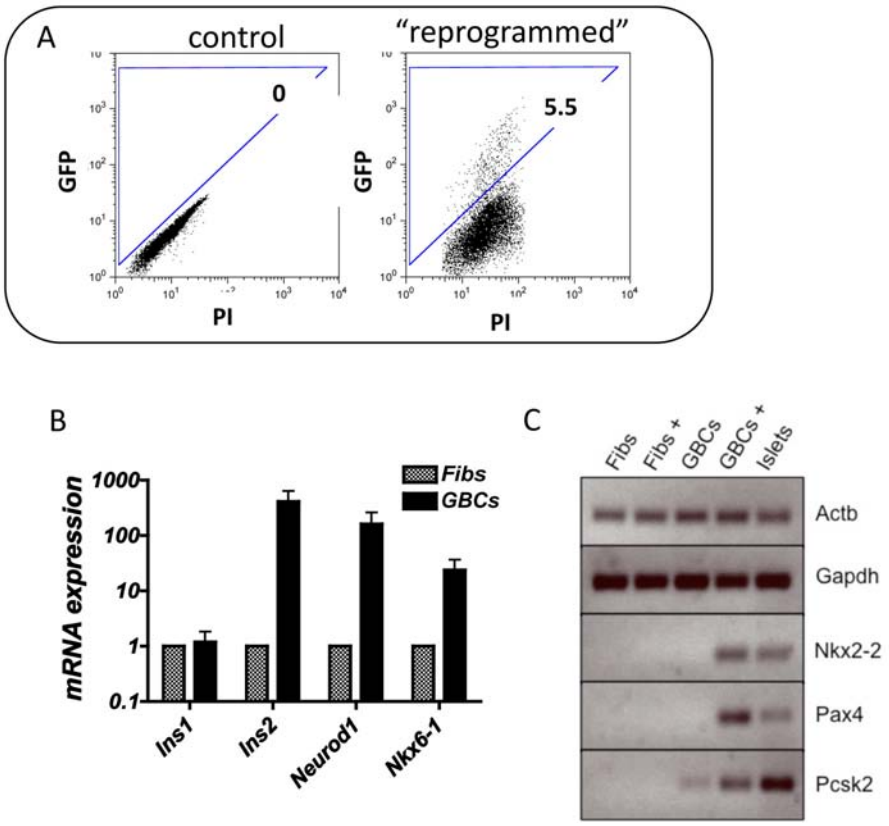

Figure 3-10. GBCs are more readily reprogrammed to the beta cell fate than fibroblasts.

(A) Tail-tip fibroblasts from MIP-GFP mice were expanded and transduced with adenoviruses expressing *Neurog3*, *Pdx1* and *MafA*. Control fibroblasts did not contain any GFP+ cells, however adenovirus-infected cells contained 5.5% GFP+ cells after three days. **(B)** These GFP+ cells were FACS sorted for RNA analysis. While these cells do indeed express *Ins1* mRNA, the levels of mRNA for other beta cell-specific transcriptions factors are not increased to the same degree as reprogrammed GFP+ GBCs. **(C)** Concomitant with the qPCR results, GFP+ fibroblasts do not express *Nkx2-2*, *Pax4* or *Pcsk2*.

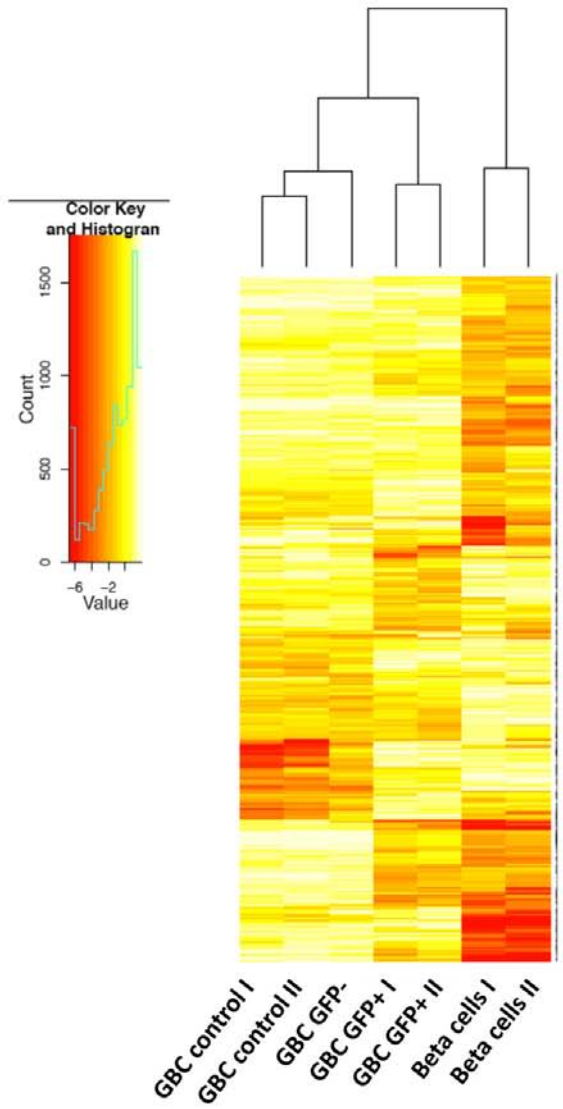

Figure 3-11. RNA-Seq analysis of reprogrammed GBCs.

Depicted is a heat map of a subset of analyzed genes. There are two control samples, two reprogrammed GFP+ samples, and two true beta cell samples. All replicates for each group were collected on different days using different cells. Included is also a GFP- reprogrammed GBC sample. The cladogram on top summarizes the data: reprogrammed GBCs form an intermediate cell population that is not entirely gall bladder, but is not entirely beta cell either.

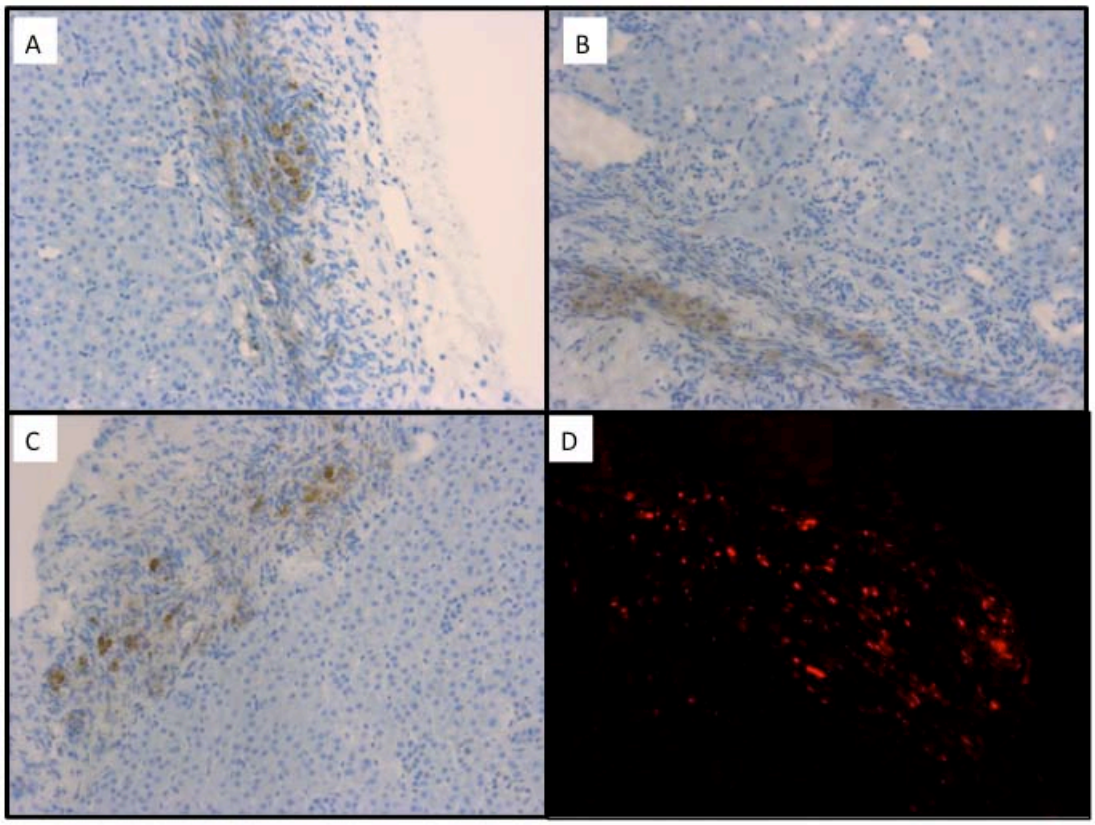

Figure 3-12. Immunohistochemistry of transplanted reprogrammed GBCs.

(A,B,C) Kidney grafts were harvested and stained with an anti-insulin antibody. Insulin-positive cells were detected in several of the grafts and representative images are shown here. Insulin-positive cells were only detected in the grafts, and never in the adjacent kidney. **(D)** Insulin-positive cells were also detected using a primary anti-insulin antibody and a fluorescent Texas Red-conjugated secondary antibody.

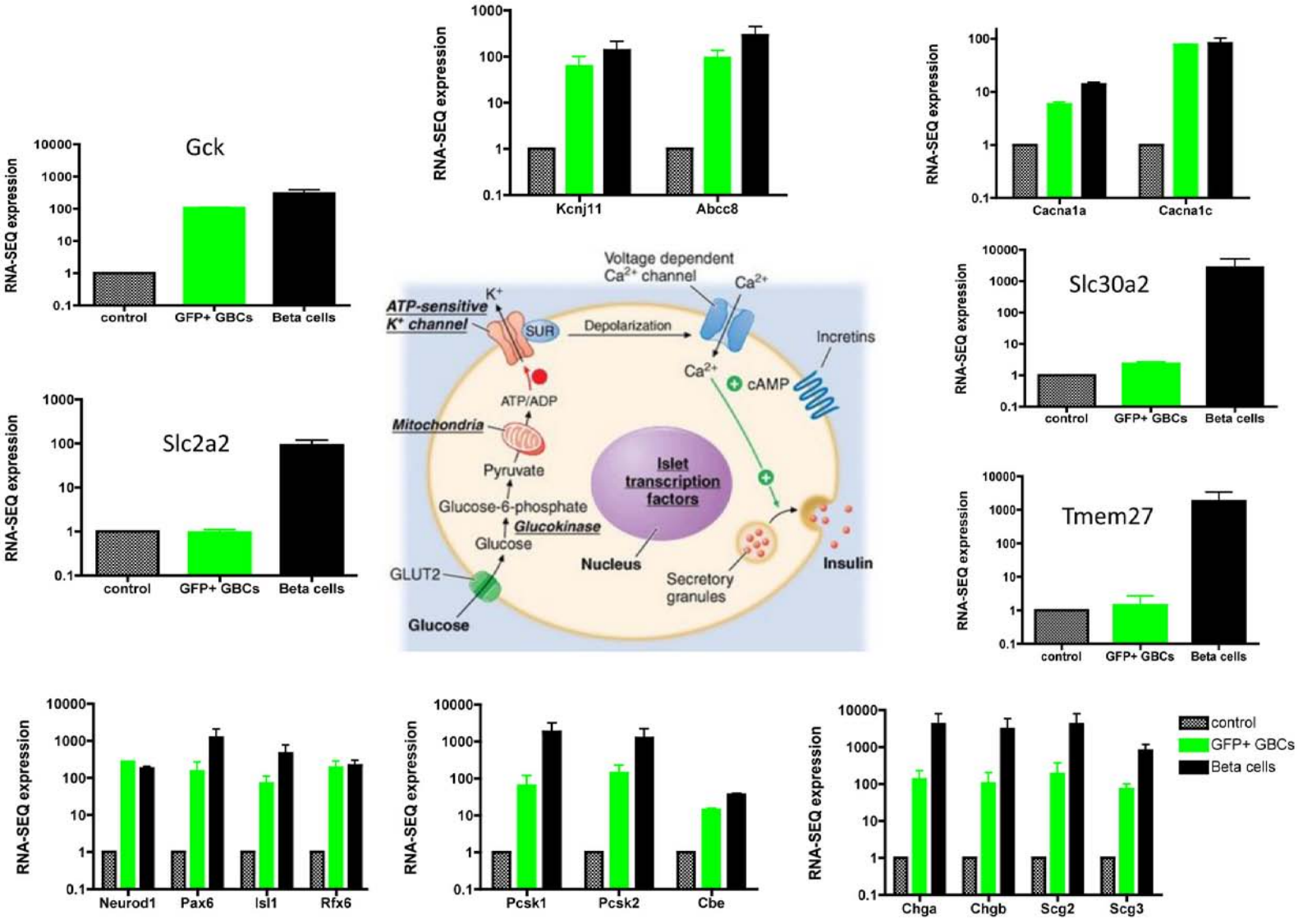
Gck
RNA-SEQ expression
control
GFP+ GBCs
Beta cells
Kcnj11
Abcc8
Cacna1a
Cacna1c
Slc2a2
Slc30a2
Tmem27
Voltage dependent
Ca2+ channel
ATP-sensitive
K+ channel
SUR
Depolarization
Incretins
cAMP
ATP/ADP
Mitochondria
Pyruvate
Glucose-6-phosphate
Glucokinase
Glucose
GLUT2
Islet
transcription
factors
Nucleus
Secretory
granules
Insulin
Neurod1
Pax6
Isl1
Rfx6
Pcsk1
Pcsk2
Cbe
Chga
Chgb
Scg2
Scg3

Figure 3-13. Overview of beta cell function in reprogrammed GBCs.

The middle schematic is the hypothetical overview of how a mature beta cell functions to secrete insulin in response to glucose stimulation. The beta cell consists of a unique combination of transcription factors and reprogrammed GBCs (rGBCs) show increased expression of many of these, including *Neurod1*, *Pax6*, *Isl1* and *Rfx6*. Insulin is translated as a larger preproinsulin prior to processing into a mature insulin protein by the enzymes *Pcsk1*, *Pcsk2* and *Cpe*, all of which are upregulated in rGBCs. Insulin is then packaged into secretory granules prior to release which involve many genes, including *Chga* and *Chgb* (chromogranins) and *Scg2* and *Scg3* (secretogranins), and their expression is also increased in rGBCs. However, proper insulin packaging involves crystallization of insulin with zinc and calcium and a main zinc transporter (*Slc30a2*) is not upregulated in rGBCs.
Release of insulin in response to glucose is the result of a complex series of events. Firstly, glucose enters the cell through the glucose transporter Glut2, encoded by *Slc2a2* that is not increased in rGBCs compared to beta cells. Once inside the cell, glucose in phosphorylated by *Gck*, expression of which is increased in rGBCs. After a series of reactions, there is an increase in the amount of ATP in the cell that closes the potassium channel. Two genes that make up this channel in beta cells are *Kcnj11* and *Abcc8*, but of which have increased expression in rGBCs compared to control GBCs. This then results in depolarization of the cell that leads to an influx of calcium through certain voltage-dependant calcium channels. Two genes that encode for components of this channel are *Cacna1a* and *Cacna1c*, which have increased mRNA detection in rGBCs. Finally, insulin is secreted from the cell via excytosis through interaction with the SNARE complex. Collectrin, encoded by *Tmem27*, is a key factor in exocytosis and its expression is not increased in rGBCs.

TABLES

Table 3-1. Primers used for quantitative PCR analysis of gene expression.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Gapdh	CGTTGAATTTGCCGTGAGTGGAG	AAGGTCGGTGTGAACGGATTTGG
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC
Alas1	CTGTCCACATCAGCTGTCCA	GTCTGTGCCATCTGGGACTC
B2m	TGGTGCTTGTCTCACTGACC	TTCAGTATGTTCGGCTTCCC
Ins1	CCAGCTATAATCAGAGACCATCA	GTTTGACAAAAGCCTGGGTG
Ins2	GCTTCTTCTACACACCCATGTC	AGCACTGATCTACAATGCCAC
Neurod1	AATTTGGTCGCCGGCTGCCT	GGCAGATGCGGGGGCATGTC
Nkx6-1	GGATGACGGAGAGTCAGGTC	CGAGTCCTGCTTCTTCTTGG
Rfx3	CGGGCCTCCCCAGCAACAATTG	TCGGCAGGCTCACTCCCTCC
Gck	CTGGATGACAGAGCCAGGAT	GGCTCATCACCTTCTTCAGG
Nkx2-2	CCCGGGCGGAGAAAGCATTTCA	GGACACTATGGGCACCGCAGC
Pax4	CCTGGAATTCCCACCTTTTT	ACAGAAGGACAGGAAGCCAA
Ppy	CTGGGCCCAACACTCACTA	CAGAGCCACCCAAGTGGATA
Ghrl	CCCAGAGGACAGAGGACAAG	GCCATGCTGCTGATACTGAG
Gcg	TTACTTTGTGGCTGGATTGCTT	AGTGGCGTTTGTCTTCATTCA
Sst	TCTGGAAGACATTCACATCCTG	AGTTGAGCATCGGGGGCCA
Irx2	ACGCACACCACCGGAATG	ATGGATAGGCCGCACTGC
Muc1	TACCAAGCGTAGCCCCTATG	TGCTCCTACAAGTTGGCAGA
Aqp1	GGCATCACCTCCTCCCTAGT	TGCAGAGTGCCAATGATCTC
Hes1	GAAAGATAGCTCCCGGCATT	GGTATTTCCCCAACACGCT
Krt19	GGACCCTCCCGAGATTACAACCA	GCCAGCTCCTCCTTCAGGCTCT
Sox17	CGAGCCAAAGCGGAGTCTC	TGCCAAGGTCAACGCCTTC
Prss1	ACCAGGTGTCCCTGAACTCTGGC	ACTTGGATGCGGGTCTTGTAGCA
Cela2a	GCCTTGGTGGCTGGAGCCCTCA	GACCTGCCAGGGCCAGGTGTT
Cpa2	GACCCGGTCTAAACGGTCGGGA	GCTACTGGCTCCAGGTCCTCCG
Pcsk1	GACCTGCACAATGACTGCAC	GGTCCAGACAACCAGATGCT
Pcsk2	CTCAGAAGCACTAAGGTTCCG	AGGGAATGTTACAAGGTGCCA

Table 3-2. Summary of transplantation experiments.

Experiment	GBC passage	Days of reprogramming	+ RA?	Recipient Mouse Strain	Number of mice transplanted	Number of insulin-positive grafts
PW0	**9**	**3**	**No**	**NSG**	**4**	**1**
PW3	**0**	**3**	**No**	**NSG**	**4**	**2**
PW4	**0**	**4**	**No**	**NSG**	**5**	**1**
PW5	**0**	**3**	**Yes**	**NRG**	**6**	**4**

DISCUSSION

With the global worldwide prevalence of diabetes estimated to be 7.7% by the year 2030, there is an ever-increasing need for an abundant source of transplantable pancreatic beta cells to be generated (39). We hypothesized that the gall bladder could be used a source of reprogrammable cells for two main reasons. Firstly, the gall bladder is developmentally closely related to the pancreas, and in theory this should make the cells more amenable to reprogramming to the beta cell fate than a non-related cell type like a fibroblast. This has been previously demonstrated by *in vivo* reprogramming of pancreatic exocrine cells into beta cells (118). Secondly, and importantly for future human studies, removal of the gall bladder can be routinely performed by an endoscopic procedure, with few negative side effects associated with the procedure (139, 140). As immune suppression is a necessary requirement for current islet transplantations, and for potential ESC-derived transplantations, the gall bladder presents an opportunity for autologous cell therapy in the future.

A key requirement for cell therapy is the presence of a reprogrammable cell in sufficient quantity. Our results build upon those of Manohar et al. who recently demonstrated robust expansion of cells from the gall bladder (122). We chose a genetic approach to differentiate these GBCs towards the pancreatic lineage. The expression of key transcription factors has been shown to be a successful method to reprogram one cell type directly to another, without the need to go through a pluripotent intermediate. It is interesting that the three transcription factors needed

for reprogramming GBCs into beta-like cells are the same as those required for *in vivo* reprogramming of exocrine pancreas into endocrine pancreas (118). Therefore, together these results implicate *Neurog3*, *Pdx1* and *MafA* to be the key genetic reprogramming factors needed for the *de novo* generation of mouse beta cells.

In addition to expressing these three transcription factors, we were able to augment the frequency of reprogrammed GBCs in the cultures by the timed addition of RA and by inhibiting notch signaling by addition of DBZ. These factors have also been used to differentiate pluripotent stem cells towards a pancreatic progenitor fate (RA & DBZ) and a differentiated beta cell fate (DBZ), indicating manipulation of both RA-responsive genes and notch signaling to be critical for proper pancreatic and beta cell differentiation (56).

To our knowledge, there has yet to be demonstrated a more thorough and rapid *in vitro* beta cell differentiation protocol than that presented here. Within 48 hours of genetic manipulation of these cells by transduction with *Neurog3*, *Pdx1* and *MafA*, de-repression of the insulin loci occurs as evidenced by expression of both *Ins1* and *Ins2* mRNA transcripts. Other rapid gene expression changes occur that indicate that these cells are taking on a beta cell fate, including increased expression of the transcription factors *Neurod1*, *Nkx2-2*, *Pax4*, *Pax6*, *Isl1* and *Nkx6-1*. In addition, these reprogrammed GBCs increase expression of the proprotein convertases *Pcsk1* and *Pcsk2* that are needed to process the immature proinsulin into insulin and c-peptide. In line with these gene expression changes, insulin protein can also be detected by immunocytochemistry and by ELISA.

It is evident, however, that these cells, *in vitro* at least, are not fully mature, fully functioning beta cells. Firstly, the expression of both *Ins1* and *Ins2* mRNA is at a reduced level compared to that seen in pancreatic islets. Secondly, these cells appear to lack the ability to respond to changes in glucose concentrations in the media as evidenced by the fact these cells do not increase insulin secretion in response to glucose stimulation, a common problem for *de novo* generated beta cells (56, 141). The potential underlying genetic problem is diagramed in figure 3-13 and discussed in more detail in chapter four. Thirdly, the transplanted cells are unable to reverse the hyperglycemic state of diabetic mice after transplantation as would be expected by transplanted fully functioning islets. However, it is possible that these cells are simply too few in number to have a substantial effect on blood glucose levels. In fact, insulin-positive cells can be detected in the grafts of transplanted mice. A number of possibilities exist to explain this, however the most likely explanation is that although a sufficient number of GFP+ reprogrammed cells were transplanted that would be expected to reverse hyperglycemia, not all transplanted GFP+ cells are equal and only a percentage of these cells are truly beta-cell like. Further experiments will need to be performed to ascertain whether different sub-populations exist within the GFP+ population of reprogrammed GBCs.

In conclusion, the data presented here outlines a novel strategy to generate insulin-secreting pancreatic beta-like cells. The gall bladder is a novel and expendable tissue that contains cells amenable to large-scale expansion and reprogramming to a pancreatic fate. Our experiments show that these GBCs turn on a number of key pancreatic genes, allowing for the processing and secretion of

insulin. Importantly, these cells can be successfully transplanted, however, further experiments are needed to optimize the transplantation in order to reverse hyperglycemia in diabetic mice. Overall, therefore, the results outlined here will be useful for further experiments aimed at generating a direct differentiation-based cell therapy for type 1 diabetes in human patients.

CHAPTER 4

Conclusions & Future Directions

AAV-mediated gene targeting

Adeno-associated virus (AAV) vectors have single stranded DNA genomes and have proven a powerful tool for gene targeting in multiple different cell lines and species (142). The multitude of AAV serotypes available has allowed the spectrum of cell tropisms to constantly expand. The wild-type single-stranded genome of AAV is around 4.7 kb, consisting of approximately 145 nucleotides of inverted terminal repeats (ITRs) surrounding the two viral genes *rep* and *cap* (143). The discovery that all the *cis*-acting elements required for genome rescue, replication and packaging were situated in the ITR regions, permitted the development of AAV to be used as a genetic vector. Effectively, this meant that an entirely new 4.7kb of DNA material could be inserted between the ITRs, and provided that all the elements required for replication and packaging are provided in *trans*, a recombinant AAV-targeting vector could be generated (142, 143).

We used AAV as our targeting vector of choice for several reasons, specifically the AAV-DJ serotype (102). This vector could efficiently transduce the cell of choice (pig fetal fibroblasts) at a low multiplicity of infection, as evidenced by flow cytometry analysis of AAV-DJ-GFP infected cells. Using a PCR-based strategy, we designed a 4.7 kb construct consisting of 1.5 kb of homology on either side of exon 5 of the *Fah* gene with a 1.7 kb Neo disruption cassette located in the center of the homology arms. This targeting structure has been shown to induce the highest rate of homologous recombination between the vector and target genome (112). Additionally, the work by Rogers et al. confirmed that AAV-gene targeting in pig

fetal fibroblasts to be much higher than traditional gene targeting methods (85). Moreover, the frequency of gene targeting detected in our study was even greater than that observed in the CFTR pig study (144).

This study, and that of Rogers and colleagues, adds credence to the idea that for efficient and relatively simple generation of future targeted knockout models, in pigs and other species, AAV will play an important role. This includes generation of gene-knockouts by targeted disruption of exons, but also for introducing single point mutations or small insertions and deletions to mimic specific human monogenic disorders. Undoubtedly, there is potential to improve the gene targeting efficiency of AAV even further. Although in our study the rate of targeted gene knockout was relatively high, in other contexts the efficiency with AAV alone has been as low as 10^{-5} (142). To improve these frequencies, different methods have been devised to induce double strand breaks to increase the rate of homologous recombination at a specific locus. For example, zinc finger nucleases (ZFNs) can induce a double strand break by binding to specific sequences and creating a double strand break. When homologous sequences are subsequently introduced along with the ZFN (for example with AAV), a recombination event can be initiated, allowing insertion of a desired sequence at a higher frequency than introducing AAV alone (145). Although the use of ZFNs are currently associated with increased genotoxicity due to on- and off-target cleavage, undoubtedly their combined use with AAV would be a worthwhile endeavor to improve gene targeting efficiencies for future gene knockout studies.

Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) is arguably the definitive demonstration of cellular phenotypic plasticity. The work of Gurdon, Wilmut and others has exquisitely demonstrated that a fully differentiated cell can be completely reprogrammed to give rise to an entire organism by transferring a somatic nucleus into an enucleated egg, thereby reinitiating normal zygote development (63-65). SCNT has now been used to generate sheep, cattle, goats, horses, cats, rabbits, rats and mice. The first cloned pig from a cultured adult somatic cell was reported in 2000 (96). Pigs have been used extensively in biomedical research as large animal models of human disease due to shared anatomical and physiological characteristics. In cardiovascular research, for example, the pig is the preferred model for the studies of atherosclerosis and myocardial infarction (146). The pig is also commonly used for studies of liver disease, most notably acute liver failure, but also for interventional procedures and surgical manipulations of the liver because of its similarity in size and anatomy to the human liver (147, 148).

Our research builds upon that of others in developing methodologies to generate gene knockout pigs to model human disease by using gene targeting and SCNT. Following on from the production of GFP-expressing pigs derived by nuclear transfer of transgenic fibroblasts, the first gene knockout pig to be generated by SCNT was the α-1,3-galactosyltransferase knockout animal (103). Although this animal was not created for modeling disease, the idea was that organs from this animal could be used for transplantation into humans. Humans do not possess the

galactosyltransferase activity seen in pigs, and naturally develop antibodies against the alpha1,3Gal epitopes present on pig cells. Therefore, by removing these epitopes from pig organs using the transgenic animals, the hypothesis was that transplantations of these organs would not be subject to the hyperacute rejection that is a major obstacle for successful graft survival. The first knockout pig to model a specific human disease using SCNT was the CFTR pig model, as previously discussed (85).

It is becoming more evident that better models of human disease are needed if successful therapies are to be generated and implemented in the clinic. Undoubtedly, the thousands of transgenic mice that have been created so far have allowed an enormous insight into human genetics and disease to be achieved. However, in many cases, estimated to be 10-15%, the evolutionary distance between mouse and human can be sufficient that knocking out the causative human disease gene in mice does not give the expected phenotype (149). This fact is well exemplified by the numerous knockout mouse models of CFTR which all failed to fully recapitulate the human disease phenotype (85). In contrast, the generation of CFTR-knockout pigs has allowed a more thorough understanding of the pathophysiology of the disease to be studied, and will permit testing of genetic and biochemical therapies for eventual use in clinical trials for humans (150, 151). Nonetheless, a pig is not a human and although generation of knockout pigs using SCNT will continue to produce valuable human disease models, the field may navigate eventually towards knockout primate models, utilizing methodology already developed to generate primate embryonic stem cells by SCNT (66).

The Fah null pig as a model of HT1 and HCC

Along with the potential to serve as a permissive recipient for hepatocyte expansion, the FAH-knockout pig could serve as a large animal model of both hereditary tyrosinemia type 1 (HT1), cirrhosis and hepatocellular carcinoma (HCC). HT1 is an autosomal recessive inborn error of metabolism resulting from a deficiency in FAH, the catalyst of the last step of tyrosine degradation (76, 107). HT1 is characterized by the early development of cirrhosis and HCC in humans. Though the clinical severity of this disorder is variable, HT1 is the most serious and the most common of the genetic defects in tyrosine metabolism. Treatment of HT1 consists of a restricted diet limiting intake of phenylalanine and tyrosine, and more recently the addition of NTBC. NTBC therapy inhibits the tyrosine pathway at a stage preceding FAH, thus preventing the formation of the toxic metabolites responsible for liver injury (Figure 2-1). Failure of diet and NTBC therapy to treat patients requires that a hepatectomy with orthotopic liver transplantation is performed. In one recent study, despite treatment with NTBC, 4 out of 45 patients had to undergo liver transplant surgery due to either failure of therapy or due to onset of cirrhosis or HCC (152). Therefore, alternative therapies are still needed in the treatment of HT1, thus requiring an appropriate large animal model to perform these studies on.

Additionally, there is a need for reliable and appropriate large animal models of HCC to study the pathophysiology of this cancer. There are no gross abnormalities in histology of livers from *Fah*$^{-/-}$ mice at birth (38). However, the livers of *Fah*$^{-/-}$ mice fail acutely after birth in the absence of NTBC. At partial therapeutic doses of NTBC,

acute liver failure is avoided and HCC occurs within several months of life. Observations of acute liver failure and HCC made in *Fah*$^{-/-}$ mice and HT1 humans are expected with the creation of *Fah*$^{-/-}$ pigs. Furthermore, there is a requirement in the hepatology field for a large animal model of HCC to conduct pre-clinical trials of novel therapeutic agents (153). The demand for improved therapies is highlighted by the fact that the five-year survival rate of individuals with liver cancer is below 10%, making it the second most lethal form of cancer after pancreatic ductal adenocarcinoma (154). The lethality of liver cancer is related to its resistance to existing anticancer agents, lack of biomarkers allowing early detection of surgically resectable disease, and the underlying liver disease that limits the use of chemotherapeutic drugs. Therefore, the availability of new large animal models of HCC would allow an improved understanding of this disease and provide preclinical testing of novel therapeutic agents.

The Fah null pig as a method to expand human hepatocytes

The two principal barriers to expanding human hepatocytes in mice are immune rejection of the transplanted cells and the size of the animal, which limits the amount of cells that can be propagated. The former barrier is more easily overcome, thanks to the thousands of gene knockout mouse models available (155). By breeding FAH-deficient mice with *Rag2* and *Il2rg* knockout animals, an immunodeficient triple knockout mouse was generated that allowed the engraftment, survival and propagation of human hepatocytes (37). As discussed

earlier, the advent of gene knockout pigs is less than ten years old and no immunodeficient pigs are available for cross breeding. However, the induction of tolerance in fetal pigs is well established and there are several reports of engraftment of human cells in immune-competent pigs (156, 157). More recently, our collaborators at Mayo Clinic have developed a technique to transplant human hepatocytes directly into the liver of fetal pigs, prior to the full development of its immune system. The procedure consists of a midline laporotomy under general anesthesia and ultrasound-guided direct injection of cells into the developing liver of fetal pigs. The procedure is safe, and long-term engraftment of the human cells has been achieved (Scott Nyberg, Mayo Clinic, personal communication). However, without a selective growth advantage, these cells will not robustly expand. Therefore, similar to the situation in mice, it is expected that FAH-deficiency in pigs will allow robust expansion of transplanted cells.

The potential limitations of the Fah null pig

At the time of writing this thesis, *Fah* heterozygous knockout pigs were being bred in order to generate the first homozygous knockout animals. While it is hypothesized that the $Fah^{-/-}$ pigs will exhibit a phenotype comparable to that seen in $Fah^{-/-}$ mice and humans with HT1, it is not beyond the bounds of possibility that these animals will display an unpredicted phenotype. Since the advent of gene targeting and generation of mouse mutants in the late 1980's, there are many examples of unexpected lethal phenotypes (prenatal, perinatal and neonatal) due to

genetic disruption of specific genes. The liver is particularly susceptible to defects in homeostasis, owing to the multitude of roles it fulfills, including energy homeostasis. For example, poor neonatal survival is observed in mice defective in glycogen accumulation caused by null alleles of either *Cebpa* or *eIF2a* (158). Similarly, with *Fah*$^{-/-}$ mice, pups die shortly after birth if not given the drug NTBC (38). Thus, it is conceivable that FAH deficiency in pigs will lead to either neonatal lethality, or even NTBC-refractory therapy. On the other side of the coin, knocking out the *Fah* gene in pigs may have no overt phenotype. Theoretically, this could be caused by duplication of the gene during evolution of the order Artiodactyla, from which pigs are derived, thereby leading to two functional *Fah* genes. Again, although unlikely, an appropriate example is the presence of one insulin gene in humans, whereas in mice and rats two insulin genes exist, which occurred when *Ins1* was retroposed from the partially processes mRNA of *Ins2* (159). When either the *Ins1* or *Ins2* gene is knocked out in mice, there is a compensatory response that results in increased production of insulin from the other locus, causing no overt phenotype in either knockout mouse (160).

In addition to an unpredictable phenotype at birth, the onset of the symptoms of human HT1, as well as HCC, will need to be assessed. Once again, the *Fah*$^{-/-}$ mouse exemplifies a cautionary tale. In humans with HT1, the severity of the disease correlates with the residual amount of liver FAH activity (dependant on the type of mutation in the genome) that leads to either the acute or chronic form of the disease (76). In *Fah*-null mice, and as is likely with the pig model, there is no FAH enzyme activity, which is responsible for the neonatal lethality in mice (38). In order

to study the chronic form of the disease, mice must be kept on NTBC, which inhibits accumulation of the toxic metabolite fumarylacetoacetate (107). When this is done, mice can survive over a year, and develop many of the symptoms of human HT1, including elevation of alpha-fetoprotein levels and development of liver tumors (107). However, not all the human symptoms are recapitulated in the mouse model, including, most importantly, cirrhosis. As no reproducible large animal model of cirrhosis exists, a pig model of cirrhosis would be highly desirable (161). Whether FAH deficiency in pigs will provide that genetic model is to be determined.

Another potential obstacle for further studies in the FAH knockout pig is the ability to expand human hepatocytes, and the impact of the pig liver environment for their survival and genetic integrity. As discussed previously, human hepatocytes can engraft in the liver of wild-type pigs and other cell types (from the hematopoietic lineage) can survive long term in immune-competent pigs (156). The next question then is whether under selective pressure these human cells can expand and repopulate the host liver. Based on the humanized mouse studies, human hepatocytes can proliferate successively in the mouse liver niche. Moreover, the liver environment of HT1 patients does not preclude spontaneously corrected hepatocytes forming regenerative nodules within the same liver (162, 163). Once again, only time will tell whether the environment of the FAH-deficient pig liver will be conducive to human hepatocyte expansion.

Finally, even if the FAH-knockout pig recapitulates the phenotype of human HT1 and is conducive to large-scale expansion of human hepatocytes, there are additional barriers to using these hepatocytes for transplantation into actual

patients. The principal known concern currently is the existence of the porcine endogenous retrovirus (PERV). These DNA elements are related to infectious retroviruses and can be transmitted genetically in a Mendelian manner, but can also be transmitted by infection if the DNA sequence contains a full open reading frame and functional LTRs (164). The pig contains several of these sequences, which have been shown to have the capability to infect human cell lines *in vitro*, as well as when pig islets are transplanted into immunodeficient mice (165, 166). However, to date no PERV-related infections have been detected in any human or non-human primates after exposure to pig xenografts (167). Nonetheless, the susceptibility of infection by these elements is possible and will require a thorough molecular analysis of expanded hepatocytes to determine if any exogenous retroviral DNA has been transmitted to the human cells.

The gall bladder as a novel source of reprogrammable cells

The ventral foregut endoderm of mammals gives rise to a spectrum of organs, including the liver, pancreas and gall bladder. Specifically, it has been demonstrated elegantly in developmental studies that the liver, biliary system (which includes the gall bladder) and ventral pancreas arise from the posterior region of the ventral foregut (62). However, until recently, it was not known what the exact lineages and progenitors were that were responsible for giving rise to the specific organs. Then, in 2009, Wells and colleagues demonstrated key roles for *Sox17* and *Hes1* in organ lineage segregation of ventral foregut progenitor cells

(119). Moreover, they showed that coexpression of Pdx1 and Sox17 marked a pool of pancreatobiliary progenitors that gives rise to Sox17+ biliary tissue and Pdx1+ pancreatic tissue. Importantly, this was the first demonstration of how close both the gall bladder and pancreas are to each other developmentally, and how the gall bladder could theoretically make an ideal population of cells to reprogram to the pancreatic lineage.

This study was not the first to postulate the close lineage sharing between the gall bladder and the pancreas. *Hes1* deficient mice undergo agenesis of the gall bladder and generation of pancreatic tissue in biliary tissue, suggesting a single gene, *Hes1*, can have profound effects on the development of the gall bladder and pancreas (120). Even more recently, inhibition of Hes1 using a dominant negative Hes1 in cultured mouse gall bladder cells was sufficient to induce modest pancreatic gene expression changes in these cells (121). Further antidotal evidence also exists in the literature with clinical reports of ectopic pancreas discovered in surgically removed gall bladders, hinting at a conserved developmental process in humans as in mice (168).

An essential factor for autologous cell therapy is availability of a tissue as a source of cells to reprogram. In a theoretical example, a patient with type 1 diabetes who is undergoing insulin therapy could donate several tissue samples for isolation of cells for expansion. Foremost of these would be skin fibroblasts or blood lymphocytes that have been used to generate patient-specific iPSCs (169, 170). However, although fibroblasts have been shown to be reprogrammable into several cell types, including neurons and cardiomyocytes, the experiments presented in this

thesis demonstrate a more efficient way of generating developmentally lineage-related cells. Moreover, removal of the gall bladder is a routine surgery with minimal side effects (139). Preliminary studies have also conclusively shown that human gall bladder cells can be expanded indefinitely, permitting generation of a large pool of developmentally related cells that can used for human reprogramming experiments (Feorillo Galivo, OHSU, personal communication).

One additional consideration makes a preliminary, but compelling, argument against the use of iPSCs for autologous cell therapy. Several reports have now demonstrated that iPSCs harbor many mutations as a result of the process of becoming pluripotent and being maintained in culture, including chromosomal aberrations, copy number variations and point mutations, including within cancer-associated genes like *Atm* (171, 172). Although this could be caused by forced expression of the pluripotency factors, which include proto-oncogenes, the authors argue that the genetic instability is more likely a result of replicative stress and the time the cells spend in culture. Perhaps even more worrying, though, was a report demonstrating immunogenicity of iPSCs following transplantation into recipient mice (173). Of course, direct reprogramming of gall bladder cells *in vitro* may not obviate the occurrence of mutations in induced beta cells. However, based on the experiments detailed within this thesis, these cells remain in culture many weeks shorter than iPSCs do and there is no need for over-expression of proto-oncogenes like *c-myc* and *Klf4*. A more thorough genetic analysis of these gall bladder-derived cells, specifically whole genome sequencing and molecular karyotyping, will need to be performed in future studies.

Future work on deriving functional gall bladder-derived beta cells

The ultimate goal of this work was to reprogram gall bladder cells *in vitro* and transplant these beta-like cells into diabetic mice to reverse the hypoinsulinemia and hyperglycemia phenotypes that are characteristic of this disease. This goal was not achieved. Firstly, although these gall bladder-derived cells have differentiated towards the beta cell fate, with concomitant loss of the gall bladder phenotype, these reprogrammed cells are not true pancreatic beta cells based on the RNA-Seq and gene expression results. Secondly, *in vitro* these cells are unable to secrete insulin in response to glucose stimulation; instead they appear to secrete insulin only tonically. Thirdly, upon transplantation these cells are able to engraft, survive and produce insulin; however, either the limiting cell number or amount of insulin secreted is insufficient to reverse the hyperglycemia. There are many avenues of investigation to pursue in order to achieve the objective of a successful cell therapy for diabetes in the mouse model, ultimately paving the way for a novel cell therapy approach for human studies.

No reprogramming effort to date, either in mouse or human studies, has produced *in vitro* glucose responsive insulin secreting cells. Therefore, it is imperative to speculate what factors are missing to instigate glucose sensing, insulin packaging and insulin secretion. The comparison of these cells to that of beta cells in type 2 diabetic patients is appropriate. Type 2 diabetic patients exhibit defective insulin secretion, exemplified by the loss of glucose-stimulated insulin secretion. A recent study investigated the cellular cause of this condition in mice, and implicated

non-membrane localization of the glucose transporter Glut2 (encoded by the gene *Slc2a2*) to be an essential deficit in these cells (174). The inability of Glut2 to be located on the membrane was caused by impaired glycosylation of Glut2, which inhibited maintenance of glucose transporter expression and glucose transport. Based on RNA-Seq analysis of GFP+ GBCS, *Slc2a2* mRNA is not even transcribed in these cells, raising the simple hypothesis that failure of reprogrammed GBCs to secrete insulin in response to higher glucose concentrations is their inability to detect changes in glucose concentrations. However, an additional factor that was significantly downregulated in reprogrammed GBCs relative to beta cells was the transmembrane protein 27, encoded by *Tmem27*. Also know as Collectrin, this factor controls insulin exocytosis and has been implicated as a key component of glucose-stimulated insulin secretion (175). Decreased *Tmem27* gene expression has also been detected in islets from type 2 diabetic patients (176). Other genes that are not expressed at comparable levels to actual beta cells are *Slc30a8* and *Nnat* that are involved in vesicle maturation and insulin secretion respectively (177).

It is appealing to speculate then that the pathways involved in differentiation to the beta cell fate and expression of insulin are independent of the pathways involved in glucose sensing and insulin secretion. Three candidate transcription factors that may be involved are *Hnf1a, MafB* and *Foxa1*. *Hnf1a* plays a key role in the development of a number of organs, including the liver and pancreas (178). Defects in Hnf1a function have been implicated as one of the primary causes of maturity-onset diabetes of the young (MODY) (179). Interestingly, Hnf1a has also been demonstrated to be a key transcription factor involved in glucose-stimulated

insulin secretion and it has been shown to have a direct effect on expression and function of Tmem27 and Glut2 (174, 175, 180). Based on RNA-Seq data, *Hnf1a* expression is not changed in control and reprogrammed GFP+ GBCS. Therefore, one appealing hypothesis would be that overexpression of this transcription factor in GBCs would induce proper glucose sensing and insulin exocytosis, allowing the cell to regulate insulin secretion in response to changing glucose concentrations. Similarly, *MafB* is not differentially expressed in control and reprogrammed GBCs. *MafB* is a key transcription factor involved in early development of pancreatic islets and beta cells, but is not expressed in adult beta cells, instead being restricted to alpha cells (181). Interestingly, it has been shown that the absence of *MafB* in developing islets leads to normal levels of transcription factors involved in beta cell differentiation (such as *Neurod1*, *Pax4* and *Isl1*) but decreased levels of genes involved in mature beta cell function such as glucose sensing (*Slc2a2*), vesicle maturation (*Slc30a8*) and insulin secretion (*Nnat*) (177). *Foxa1* has also been implicated as a key component of pancreas function. In fact, *Foxa1*-deficient islets have severely impaired glucose-stimulated insulin secretion without a deficiency in actual beta cell number, indicating *Foxa1* may be involved in proper beta cell functioning rather than beta cell differentiation (182). It is appealing to speculate that a similar deficit is apparent in reprogrammed GBCs, and that expression of *Foxa1* may ameliorate mature beta cell functioning in these cells. Whether the expression of any of these factors in GBCs will lead to a more mature functioning beta-like cell will need to be determined.

Then again, a more simple explanation is that if these cells were given more time to mature *in vitro*, this alone may be sufficient for a mature, fully functioning beta cell to be derived. This experiment has been difficult to perform, as these cells are unable to survive in the current growth conditions past four to five days. Although it is possible that an *in vivo* milieu is required for maturation, it would be worth testing different media conditions, inclusion of pro-survival/anti-apoptosis factors, or different culture surfaces to investigate if any of these can both prolong survival *in vitro* and improve differentiation to a mature beta cell. A parallel experiment would be to determine if the insulin-positive grafts that were detected in the transplanted mice were also positive by IHC for markers like Glut2 and Collectrin. Moreover, the most informative experiment would be to isolate these transplanted cells (by FACS-sorting of GFP+ cells) and perform RNA-Seq analysis on these cells to determine the global transcription profile of these cells after *in vivo* differentiation.

There has been only a single paper to date that shows rapid reversal of hyperglycemia in diabetic mice following transplantation of induced beta-like cells, in this case using mouse iPSC-derived beta cells (54). Interestingly, despite the large number of groups working towards a cell therapy for diabetes, these results have not been replicated. Although a return to euglycemia is the standard following transplantation of whole islets into diabetic mice, both published and personal communication would indicate that transplanted stem cell derived beta cells do one of two things: they either fail to engraft; or they engraft and three to four months later become functioning insulin-producing cells that can rescue the hyperglycemic

phenotype of streptozotocin-treated mice (55, 183). Although preliminary, and potentially only of anecdotal note, this time frame of *in vivo* maturation may also be representative of what is occurring with GBC reprogramming. In one cohort of mice, all transplanted on the same day with the same cells, 4/4 mice histologically examined after 10 weeks contained insulin-positive cells, whereas 2/2 mice examined after 7 weeks were negative for insulin by IHC. Why exactly it requires such an extended period of time for maturation of beta cells *in vivo* is a key unresolved question in the field. Somehow, the lack of developmental cues that would normally be present in the developing embryo as differentiation of the pancreas occurs, sufficiently inhibit the proper maturation of beta cells in ectopic transplant sites, despite the significant suite of pancreatic genes already expressed in these cells. The necessity of the niche has been best studied in the hematopoietic field, where proper function requires the bone marrow microenvironment (184). Based on the success of whole islet transplantation in the mouse, and to a lesser extent in humans, it is probable that a new strategy for cell therapy for diabetes may be needed, one in which the desired end product is not a homogenous beta-like cell population but a heterogeneous islet-like cell population that includes all the cellular constituents of a typical islet. This is further supported by transplantation experiments in diabetic mice in which single cell suspensions of insulin-producing cells were found to be less functional than comparable number of cells contained within an islet structure (123).

The limitations of cell therapy for type 1 diabetes

Regardless of the molecular approach to generate new sources of transplantable cells for diabetes therapy, there are numerous, inherent obstacles that will need to be overcome before a clinically relevant cell therapy is viable. Currently, the gold standard for cell therapy of diabetes is infusion of islets from cadaveric donors to the liver of the patient. However, while this results in rapid insulin independence in the patient, the effect is short lived and within five years the patient returns to full insulin dependency (117). Although there are several hypotheses as to why these transplantations eventually fail, common postulations are that it is due to graft rejection, recurrence of autoimmunity to transplanted islets, or a combination of both (48). Autologous gall bladder or iPSC-derived beta cells may obviate the need for immune suppression against the graft rejection, however it is not clear whether longer term autoimmune-mediated graft destruction will also require immune suppression.

An interesting alternative but complementary approach using cell encapsulation has had encouraging initial success in rodent models of diabetes. Cell encapsulation has come about as a result of some of the limitations of islet transplants discussed earlier, primarily the short term effect of islet transplantation and a return to insulin dependency within five years (117). Currently, encapsulation performs three functions. The first of these is to provide protection against attack by the host immune system, thought to be the primary cause of islet transplantation failure. The second function is to provide a protected environment for the immature

beta cells to mature. This is currently an obligatory step for complete differentiation to a mature, glucose-sensing beta cell to occur. Thirdly, and specifically for pluripotent-derived beta cells, encapsulation provides a structure to contain any tumors that may develop from the pluripotent stem cells. In one study using ESC-derived beta cells, 5% of the transplanted mice developed tumors (55). By trapping these cells inside a retrievable device, tumors can be isolated and removed without the risk of metastasis to other organs.

The first studies used beads made of alginate for cell encapsulation and have even resulted in one clinical trial in Moscow in which pig islets were transplanted into seven patients with type 1 diabetes. Alginate micro-encapsulation protects transplanted cells from contact and attack by immune cells while still permitting accessibility of the cells to smaller molecules like glucose and insulin (185). Alginate consists of linear copolymers of beta-D-mannuronic and alpha-L-guluronic acids that solidify around the islets in a divalent cationic solution (186). Early success was reported in restoring euglycemia in rodent models of diabetes, but more recently larger animal studies have highlighted the limitations of this technology for long-term restoration of euglycemia (186). Other non-alginate devices have been tested, again with various degrees of success. One of the most commonly used is the Theracyte macroencapsulation device that consists of a bilaminar poly-tetra-fluorethylene membrane system (187). Similar to other methods, this device has been reported to prolong islet survival and enhance islet maturation *in vivo*. In addition to immune protection, these devices allow cells to be transplanted by

minimally invasive procedures, with the opportunity to retrieve the cells at any time.

It is likely that cell encapsulation devices will play a prominent role in cell therapy for type 1 diabetes. The exact composition of the ideal device will need to be determined based on long-term survival *in vivo*, structural stability *in vivo*, and the ability of the device to support islet function for an extended time period (years). However, a more important question will be what cells will reside within the capsule. The fact that these devices are manufactured to protect against immune attack by the host system would argue that the origin of the encapsulated beta cell is unimportant. This has been demonstrated in various studies in which encapsulated rat and human islets can survive and function in immunocompetent mice (188). However, other reports have highlighted that while both micro- and macro-encapsulation devices block entry of immune cells into the device, if an immune response in stimulated it can cause impairment of transplanted islet function (189). Based on this fact, the inefficiency of pluripotent stem cells to fully differentiate into mature cells, the long time period needed for *in vivo* differentiation, and the risk of teratoma formation and escape, the current focus on pluripotent stem cell-derived beta cells should be guarded, and the importance of autologous cell therapy, even in the realm of cell encapsulation, should be appreciated.

Final thoughts

Cell therapies for both liver disease and type 1 diabetes are currently only experimental procedures. However, initial human transplantation studies have demonstrated that these procedures can be performed safely and have beneficial therapeutic effects. There are many obstacles that limit the more widespread and effective use of cell therapy for these disorders, with the primary restriction currently being the lack of transplantable cells. My thesis has focused on two very different efforts to generate hepatocytes and pancreatic beta cells. Although both these methods are at an early time point in their path to possible use in the clinic, the preliminary data is encouraging. The *Fah*-null homozygote pig has the potential to generate billions of human hepatocytes that could be used to treat myriad liver disorders that are currently only treatable by liver transplantation. Similarly, type 1 diabetic patients could benefit from the transplantation of autologous gall bladder-derived beta cells if the experiments described here could be translated to human gall bladder cells. Regardless of the outcomes of either of these approaches, cell therapies for these disorders will need to be generated by some method as the waiting lists for liver and pancreas transplantations continue to grow longer every day.

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PUBLICATIONS

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