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Generation of islet-like cells from mouse gall bladder by direct *ex vivo* reprogramming

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Abstract Cell replacement is an emerging therapy for type 1 diabetes. Pluripotent stem cells have received a lot of attention as a potential source of transplantable β-cells, but their ability to form teratomas poses significant risks. Here, we evaluated the potential of primary mouse gall bladder epithelial cells (GBCs) as targets for *ex vivo* genetic reprogramming to the β-cell fate. Conditions for robust expansion and genetic transduction of primary GBCs by adenoviral vectors were developed. Using a GFP reporter for insulin, conditions for reprogramming were then optimized. Global expression analysis by RNA-sequencing was used to quantitatively compare reprogrammed GBCs (rGBCs) to true β-cells, revealing both similarities and differences. Adenoviral-mediated expression of *NEUROG3*, *Pdx1*, and *MafA* in GBCs resulted in robust induction of pancreatic endocrine genes, including *Ins1*, *Ins2*, *Neurod1*, *Nkx2-2* and *Isl1*. Furthermore, expression of GBC-specific genes was repressed, including *Sox17* and *Hes1*. Reprogramming was also enhanced by addition of retinoic acid and inhibition of Notch signaling. Importantly, rGBCs were able to engraft long term *in vivo* and remained insulin-positive for 15 weeks. We conclude that GBCs are a viable source for autologous cell replacement in diabetes, but that complete reprogramming will require further manipulations.

Abbreviations: PSCs, pluripotent stem cells; GBC, gall bladder cell; RA, retinoic acid; DBZ, dibenzazepine; GSIS, glucose-stimulated insulin secretion; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR; FACS, fluorescence-activated cell sorting.

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Introduction

The initial success of the Edmonton protocol highlighted the potential of cell replacement therapy in type 1 diabetes (Shapiro et al., 2000). However, wider application of this approach is severely limited by the shortage of transplantable pancreatic β-cells (de Kort et al., 2011). In addition, the transplantation of cadaveric islets requires life-long immune suppression (Ricordi and Strom, 2004). Ideally, therefore, a source of transplantable β-cells would be both autologous and abundant. Because of their *in vitro* growth capacity, pluripotent stem cells (PSCs) are an attractive potential source of transplantable β-cells. While significant progress has been made, the generation of true β-cells *in vitro* has remained elusive thus far (Alipio et al., 2010; D'Amour et al., 2006; Nostro et al., 2011). In addition, PSCs are capable of forming teratomas and represent an unknown risk in terms of tumorigenesis (Fujikawa et al., 2005; Kroon et al., 2008).

Direct genetic reprogramming of postnatal primary cells by forced expression of key developmental transcription factors has emerged as an alternative to *in vitro* differentiation of PSCs. This strategy has been used successfully to produce functional cells, including neurons, hepatocyte-like cells and cardiomyocytes from fibroblasts (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010). Similarly, *in vivo* reprogramming of hepatic cells by expressing different pancreatic transcription factors, including *Neurog3* and *Pdx1*, was able to restore euglycemia in hyperglycemic mice (Ferber et al., 2000; Wang et al., 2007; Vijay Yechoor et al., 2009). Additionally, *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 (Zhou et al., 2008). Furthermore, overexpression of *Neurog3* and *Pdx1* has been shown to enhance pancreatic differentiation of embryonic stem cells (Kubo et al., 2011). Other cell types have also been tested for amenability to reprogramming towards the β-cell fate, including adipose tissue-derived stem cells, placenta-derived multipotent stem cells, hepatocytes, intrahepatic biliary epithelial cells and gall bladder cells (Chandra et al., 2011; Chiou et al., 2011; Coad et al., 2009; Motoyama et al., 2009; Nagaya et al., 2009; Shigeru et al., 2007).

The extrahepatic biliary tissue, including the gallbladder, is a particularly appealing source of cells for reprogramming to the pancreatic fate. The extrahepatobiliary system shares a common developmental origin with the ventral pancreas, from a cell termed the pancreatobiliary progenitor (Spence et al., 2009). Segregation of these distinct lineages is partly regulated by the Notch effector *Hes1*. Recently it was demonstrated that inhibition of Hes1 in cultured gall bladder cells (GBCs) was sufficient to induce some insulin expression (Coad et al., 2009). While this work highlighted the potential of GBCs as a source of transplantable β-cells, the full spectrum of β-cell expressed genes or the *in vivo* functionality of these cells was not determined. Moreover, the cells showed only limited proliferative potential under the culture conditions used. For GBCs to be a viable substrate of future β-cell replacement therapies, they would have to be robustly expandable (Yechoor and Chan, 2010). Therefore, the true utility of GBCs as a source of transplantable β-cells remains unknown.

In this study, we investigated if mouse GBCs significantly expanded *in vitro* can still be reprogrammed towards the β-cell fate by using a combination of positive instructive signals as well as Notch inhibition. GBCs were transduced with adenoviruses expressing the transcription factors *NEUROG3*, *Pdx1* and *MafA* and treated with retinoic acid and Notch inhibitors, resulting in their differentiation into islet-like cells. Reprogrammed cells had the ability to engraft, survive and remain insulin-positive up to 15 weeks post-transplantation. However, there were also differences between the reprogrammed GBCs and true β-cells. Our findings confirm that the gall bladder represents a promising source of autologous reprogrammable cells for the treatment of type 1 diabetes mellitus.

Materials & methods

Mouse gall bladder cell isolation and culture

Gall bladders from C57Bl6/6J-MIP-GFP male and female mice between the ages of 4 and 8 weeks were removed by a surgical incision and bile released by making a single cut in the wall. Gall bladders were rinsed twice in DPBS (Life Technologies, Grand Island, Ca) and then cut into several pieces. This material was then incubated at 37 °C with 0.25% Trypsin/EDTA (Life Technologies, Grand Island, Ca) for 45 min to obtain a cell suspension. Cells were cultured using a modified protocol to that previously described (Manohar et al., 2011). Briefly, cells were plated on a 70–80% confluent irradiated LA7 rat epithelial feeder layer that had been previously irradiated at 60 Gy. Cells were cultured in DMEM/F12 (Life Technologies, Grand Island, Ca) supplemented with 0.5% FBS (Thermo Fisher Scientific, Cambridge, MA), 1% insulin–transferrin–selenium (Roche, Indianapolis, IN), 15 mM HEPES (Thermo Fisher Scientific, Cambridge, MA) and antimicrobials (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Amphotericin B; Cellgro, Manassas, 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, Grand Island, Ca) at 37 °C, followed by incubation with DNaseI at 37 °C for 10 min to obtain a single cell suspension.

Fibroblast culture

For initiating fibroblast cultures, mouse tail-tips from euthanized C57Bl6/6J-MIP-GFP mice were washed with DPBS, cut into several pieces, and digested with 0.25% Trypsin/EDTA for 60 min at 37 °C with regular mixing. Upon inactivation of trypsin by addition of serum, the tissue mix was spun at 1000 rpm for 5 min, 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 E1-deleted adenovirus (serotype 5) consisted of the full-length cDNA (human *NEUROG*, 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