**Introduction**

Type 1 diabetes (T1D) results from a progressive destruction of β cells that is triggered by environmental factors and occurs in genetically susceptible patients. Endogenous insulin secretion capacity is eventually lost and patients with T1D become fully insulin-dependent. Two possible areas of investigation exist in the search for strategies to restore endogenous insulin secretion in patients with T1D: a) restoration of β-cell mass by transplantation of β cells or β-like cells; b) preservation of residual β-cell function before/after disease onset.

In this project, we will evaluate the role of RNAs in replenishing or preserving β-cell mass in type 1 diabetes. The role of RNAs will be addressed using two innovative techniques: 1) the reproduction of pancreas embryonic development by overexpression of synthetic modified mRNAs of specific transcription factors (TFs); 2) the small interfering RNA knockdown of specific proteins implicated in the pathogenesis of type 1 diabetes.

**Aims and Results**

**-First part-**

**Direct reprogramming of HDDCs toward β-cell lineage using overexpression of key transcription factors implicated in pancreas embryonic development**

HDDCs (human pancreatic duct-derived cells) were developed for duct cell expansion and differentiation in collaboration with Pr Susan Bonner-Weir (Joslin Diabetes Center). We have demonstrated differentiation capacities of HDDCs into insulin-secreting lineages after exposure to molecule- and growth factor-based cocktails [1]. For differentiation experiments in HDDCs, we designed synthetic modified (sm)RNAs, which is more efficient and safer than DNA transfection, from key transcription factors (MAFA, PDX1, NGN3) based on a new strategy that was adapted from the work of Warren et al [2]. If PDX1, NGN3 and/or MAFA over-expression is not sufficient to improve β cell differentiation levels of HDDCs, additional transcription factors (e.g. FOXA2, SOX17, PAX6, NKX6.1) will be assayed using similar technique and following a rationale based on their respective role. A “ready-to-use” plasmid (pRTU) containing 5’ and 3’ untranslated regions (UTRs) and a cloning site in a backbone was designed to generate the templates for *in vitro* transcription (IVT).

smRNAs were modified to confer these single-stranded nucleotides the ability to escape from the immune system and for being translated to functional proteins. Modifications include the incorporation of 5’ guanine cap analogs and substitution of all cytidine and uridine residues respectively for 5-methylcytidine and pseudouridine during smRNA *in vitro* synthesis. RNA templates are then purified and phosphatase-treated to remove immunogenic residues. In our data, we successfully constructed the pRTU plasmids for MAFA, PDX1, and NGN3, and the corresponding smRNAs were highly expressed in HDDCs after transfection protocols (*Fig. 1A*). In order to evaluate the kinetics of smRNA expression inside HDDCs, we performed expression analyses in different time
Results showed that the highest expression level of smRNA located at 48h after transfection and then it decreased gradually in the following days.

The viability of transfected HDDCs revealed the cellular tolerance to our transfection technique involving JetPEI reagent and smRNAs during 7 consecutive days (Fig. 2). Groups of controls and controls+JP (JP: JetPEI) had similar viability (84.4 ± 1.7 % and 82.9 ± 2.0 %, respectively; n=3). In comparison to controls, MAFA-transfected HDDCs maintained high survival rates (69.2 ± 14.6 %; n=3). This data demonstrated the acceptable toxicity of our smRNA transfection system into HDDCs.

Since HDDCs arise from pancreatic duct cells through an epithelial-mesenchymal transition (EMT), we evaluated whether HDDCs keep their mesenchymal origin while differentiating (Fig. 3). We observed in MAFA-transfected HDDCs that there was significant downregulation of mesenchymal markers N-Cadherin and CD105, whereas only a trend toward a decreased expression was observed with αSMA. These results suggest that HDDCs proceeded with differentiation with an accompanying shift of mesenchymal phenotype. Indeed, N-cadherin is expressed at the early steps of EMT and define its beginning as the “E- to N-cadherin” switch.

Because cytoplasmic delivery of single-strand RNAs in mammalian cells may trigger cellular immune response with induction of apoptosis pathways, we evaluated whether smRNA transfection affected HDDCs in their expression profile. When analyzed after 7 MAFA smRNA transfections, HDDCs activated the expression of interferon α (INFα), protein kinase R (PKR), and retinoic acid-inducible gene 1 (RIG-1) at low levels (Fig. 4A). By immunostaining, we observed that high proportions of HDDCs co-expressed PKR and MAFA proteins (80.0 ± 4.0 %, n=3) (Fig. 4B), whereas we found only 20.5 ± 4.5 % (n=3) of MAFA+/PKR− HDDCs and no PKR+/MAFA− cells, suggesting that negligible amounts of innate immune response activation were observed in one fifth of the MAFA-transfected HDDCs.

After transfection of MAFA smRNA, HDDCs acquired specific features of β-like cells. We observed that most genes referred to β-cell markers were highly expressed in HDDCs after overexpression of MAFA smRNA (Fig. 5A) and that HDDC populations contained up to 40% insulin-expressing cells after differentiation (Fig. 5B-D). These cells also co-express synaptophysin and PDX1 proteins. Preliminary data show that MAFA-transfected HDDCs are polyhormonal with 13.3 ± 7.2 % somatostatin-positive cells and 65.5 ± 26.5 % PP-positive cells (n=3). Comparative analysis showed that NGN3 and PDX1 smRNAs also induced β-cell marker expression in HDDCs, albeit at lower levels. However, sequential or combined protocols with all 3 TFs failed to induce any phenotypic change in HDDCs.

Although current experiments are evaluating the degree of insulin secretion by MAFA-transfected HDDCs and their response to secretagogues, we are currently trying to construct plasmids for PAX6 and NKX6.1, key TFs in development of β-cell, to maximize the output of insulin+ cells from our cultures. To date, a plasmid for PAX6 was successfully constructed and will be examined in vitro as single agent and in co-incubation protocols with MAFA smRNA.
-Second part -
Small interfering RNA-based knockdown of immune mediators for β-cell mass preservation

We are constructing a multi-shRNA plasmid for the inhibition of β-cell apoptosis. This multi-shRNA plasmid contains some shRNAs of key proteins such as TNFαR, IL-1βR1, IFNγR1, FAS, NFκB and STAT1. After transfection into β cells, multi-shRNA plasmid will transcribe shRNAs for these key proteins and then the onset of inhibition of β-cell apoptosis.

So far, we are testing cloning efficiency and sequencing for backbone vector will be our next step to push forward this part of project. Once the results show up positively, we are going to produce diverse multi-shRNA plasmids and further testing plasmids in Min6 cells and mouse islets (isolation protocol is validated in our lab).

References
**Figures**

**Figure 1: kinetic expression of MAFA smRNA after in vitro transfection and**

![Image of Figure 1]

Legend: (A) high gene expressions of different smRNAs after *in vitro* transfection protocol. (B) kinetic gene expression of MAFA smRNA in time course was detected by qPCR. The expression levels reached the highest point after 48h *in vitro* transfection.

**Figure 2: Annexin V test for HDDCs viability**

![Image of Figure 2]

Legend: Annexin V test for cell viability. The batch of MAFA (MAFA-transfected HDDCs) performed 69.2% of viable cells. The other two batches (CTL+JP and CTL) revealed similar amounts of viable cells (mean 82.9% and 84.4% respectively).
**Figure 3: gene expression levels of mesenchymal markers**

Legend: mRNA expression of mesenchymal markers was detected by qPCR and compared to untransfected HDDCs as a control \( (n=3) \). N-Cadherin (N-Cadh) and CD105 were significantly downregulated 48hr after transfection of MAFA smRNA. \( P \) values compared to controls \( (**P < 0.01; ***P < 0.001) \).

**Figure 4: gene expression levels of immune response and co-staining analysis of MAFA and PKR expression**

A.
B.

Legend: (A) mRNA expressions of immune response was detected after MAFA smRNA transfection. (B) Co-staining with MAFA (Green) and PKR (Red). Pictures show that MAFA translocated into the nucleus after transfection and that PKR was activated in the cytoplasm. (Magnification: 400x)

**Figure 5: gene expression levels of β-cell markers**

A
Legend: (A) mRNA expressions of β-cell markers in MAFA-transfected HDDCs compared to untransfected HDDCs as a control (n=3). Most of the studied marker genes (β-cell markers or MAFA target genes) were induced by MAFA smRNA. (B-C) Immunostaining with INS (insulin, Green), MAFA (Red) and DPAI (Blue) in MAFA-transfected HDDCs. Results show that 40% of cell population produce insulin. (Magnification: 100X for figure B, 400X for figure C)

**Summary**

For type 1 diabetes (T1D), replacement or preservation of functional β cells is considered as a potential therapy. In our project, we generated β-like cells from human pancreatic progenitors (human duct-derived cells or HDDCs) by using over-expression of synthetic modified RNA (smRNA) related to critical transcription factors (PDX1, NGN3, MAFA). Our results show that our smRNAs (PDX1, NGN3, MAFA) were highly expressed in smRNA-transfected HDDCs and well tolerated by the host cells with low toxicity. Induction of immune response to smRNAs was observed since HDDCs expressed markers of immune activation and because 80.0 ± 4.0% of MAFA-transfected HDDCs co-expressed PKR, responsible for interferon activation. MAFA smRNA induced β-cell marker expression in HDDCs both at the gene and protein levels, and stimulated 40% insulin production. HDDCs, which are products of epithelial-mesenchymal transition, had their mesenchymal markers decreased after MAFA over-expression, suggesting a commitment to the epithelial phenotype. Our results suggest that differentiation of HDDCs into β-like cells with smRNA protocol is robust.

RNA interference is a specific and potent approach to inhibit gene expressions via small interfering (si) or small hairpin (sh) RNA. Preservation of intact β cells from T1D is using multi-shRNA plasmids against autoimmune response. In early phase of autoimmune attack, some chemokines (TNFα, IL1β, IFNγ and FASL) allow T-cell to recognize β-cell so that inducing apoptosis through NFkB and
STAT1 signalling pathway. We have created a multi-shRNA plasmid containing sh-p50, sh-IL1R2 and sh-TNF1R. Meanwhile, another plasmid including sh-IFNR1, sh-STAT1 and sh-FAS is under construction. All shRNAs will be evaluated in Min6 cells and mouse islets for their potential to avoid apoptosis from autoimmune response.

Our next perspectives are to study in vivo functionality of differentiated HDDCs and evaluate the possibility of performing in vivo intrapancreatic electroporation for multi-shRNA plasmids. We believe that these studies could help develop knowledge on immune mechanisms at play in T1D or perhaps on a new candidate for cell therapy of diabetes.