# LUDC-IRC Postdoctoral Program 2020 – Project 1

## Dissecting human pancreas development to understand type 2 diabetes heritability

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### Purpose

The purpose of this project is to understand the function of type 2 diabetes (T2D) genes during human β cell development in vivo and in vitro and changes in gene expression/function affect islet function. The lack of studies using human pancreatic tissue and species differences between human and animal pancreas represent major bottlenecks for understanding mechanisms underlying diabetes and cell therapy development. Here we aim to understand **how changes in T2D candidate gene expression influence human endocrine cell differentiation, islet morphology and β cell mass**.

### Background

Human pancreas development begins with the evagination of the dorsal and ventral buds from the embryonic foregut at around four weeks of gestation. Pancreatic buds initially consist of epithelial and mesenchymal components, with the epithelium giving rise to multipotent progenitor cells that will become mature endocrine, ductal and exocrine cells. These differentiation processes are controlled by signals from the mesenchyme, endothelial cells, nerves, and cell-cell communication within the epithelium[4-7]. Endocrine cell differentiation starts at 8 weeks post conception (PC), with scattered α and β cells detected close to the pancreatic epithelium, presumably having migrated from the multipotent progenitor cell-rich epithelium to form cell clusters that will eventually become islets of Langerhans. β cell expansion and maturation occur during the neonatal phase, establishing glucose responsiveness, and increasing insulin production.

Islet formation and β cell proliferation depend on external cues from endothelial and mesenchymal cells, but also from pancreatic nerves (Fig.1). Recent animal studies have illustrated that ablation of sympathetic nerves from the developing pancreas results in fewer and smaller islets, which was accompanied by a reduction in β cell mass[8]. Moreover, β cell migration and clustering of islets depends on neurotransmitter (NT) signaling. Our preliminary results show that **blocking of cholinergic signaling reduces cell migration towards autonomic nerve fibers and formation of β cell clusters**. Inhibition of adrenergic[8] signaling also affects β cell migration and Adra2A deficient animals have altered β cell mass[3,9] supporting a role for adrenergic signaling in islet formation. Autonomic innervation is initially observed in human embryonic pancreas from 8 weeks PC[6] with single neural cells located close to the pancreatic epithelium. We have also shown that NT and axonal guidance receptor genes are expressed in the pancreatic epithelium suggesting that a similar mechanism may be in place during human pancreas development. A notion that is further supported by **the presence of T2D risk alleles in the NT receptor and axonal guidance genes CHRNB4, P2YR1, and ROBO2** (see preliminary results).

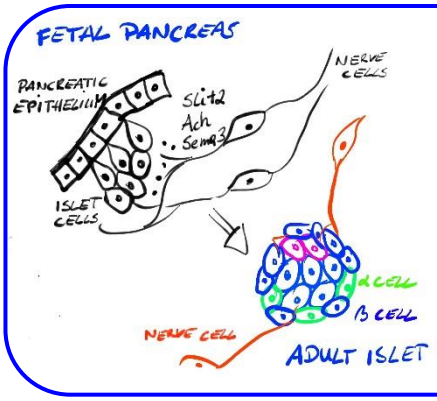



**Fig.1** NT and axonal guidance signaling (slit2, Ach, ATP, Sema3, NE) from nerve cells and the mesenchyme promote islet cell migration and β cell clustering during development. NT and axonal guidance receptor genes are expressed in developing and adult β cells and loss of function mutants have altered adult β cell mass and islet morphology[1-3].

Due to the limited availability of primary human tissue, much of our molecular understanding of pancreas development is derived from animal models. The mechanisms of organ formation are conserved, however, several important differences have been observed between rodent and human. Rodent endocrine cells differentiate in two distinct time windows, with α cells appearing during early pancreas development

followed by a wave of β cell differentiation, while human endocrine cells differentiate continuously from 8 weeks PC. These early differences in morphogenesis result in a strikingly different islet architecture, with mouse islets having a mantle of α cells surrounding a β cell core, while human islets consist of smaller β cell clusters surrounded by α cells resulting in species-specific differences in cell physiology and function. We have previously shown that the pancreatic transcription factors MafA and MafB, which are essential for adult α and β cell function, are expressed in developing mouse β cells while adult β cells only express MafA[10]. In humans, MAFA expression has only been detected in mature β cells[11], and MAFB is detected in both embryonic and adult human β cells[11], suggesting different roles of these factors in mouse and human β cell development and function. Our preliminary results show that MafB regulates NT and axonal guidance signaling in developing mouse β cells and that loss of MafB results in reduced β cell number and altered islet architecture[12]. These results suggest that responsiveness to NT signaling from developing nerves is involved in islet formation and possibly altered adult islet cell function.

## Scientific novelty and significance

Genome-wide association studies have identified >250 gene loci that are associated with the onset of diabetes[13], many of these genes are expressed in adult pancreatic islets, suggesting that defects in β cell function, cell number, and islet architecture are a main cause of the disease. Pancreatic islets containing α and β cells are produced during embryonic and fetal development, with final cell maturation occurring postnatally. Previous studies in animal models have shown that mutations in T2D genes are impacting β cell development and the islet microenvironment[14]. **However, expression and function of key pancreatic regulators differ significantly between mouse and human, creating the need to study human pancreas development to completely understanding disease-underlying changes in gene function in human β cells and to generate functional β cells for efficient cell therapy.** In this project, we will combine a single cell transcriptome analysis of embryonic pancreata with spatial transcriptomics that will allow us to determine the transcriptional relationship between neighboring cells and determine which signaling pathways are used in endocrine progenitor cells to create an islet. Moreover, we will assess how T2D risk alleles influence β cell differentiation thus expanding our understanding on T2D genetics. The single cell transcriptome/spatial transcriptomics analysis will create a unique resource for generating transplantable β cells and understanding the role of islet development in T2D heritability. As detailed expression analysis of pancreatic progenitor cells and developing β cells will be critical for assessing the efficacy of in vitro differentiation protocols for cell replacement therapy and identification of novel expression domains and gene function of T2D genes during development. No single cell sequencing or spatial transcriptomics analysis of human embryonic and fetal pancreatic tissue has been reported so far. Modeling of β cell development in iPSCs from risk allele carriers will assess if altered NT signaling affects β cell development thus providing novel knowledge on how T2D risk alleles increase T2D susceptibility.

## Preliminary results

### Nicotinic signaling promotes β cell clustering and migration towards ganglia

Recent publications have shown that autonomic innervation and specifically adrenergic signaling are required for islet formation and β cell differentiation[8], while the role of nicotinic signaling has not been investigated yet. We have assessed the role of specific NT receptors in mouse pancreas development. These results are currently under review and form the basis of our current application. Specifically, we have performed co-culture of embryonic β cells with superior cervical ganglia to test if acetylcholine receptor (nAchR) function is required for β cell migration towards nerve cells, as observed during pancreas development. Co-culture of superior cervical ganglia (NT source) with β cells resulted in formation of β cell clusters, which were connected to nerve fibers, and migration of β cells to the centrally located ganglion. In contrast, treatment of co-cultures with mecamylamine (inhibitor) and nicotine (agonist) resulted in reduced cell migration and β cell clustering. The reduced cell migration in response to nicotine is most likely due to receptor desensitization and the presence of an activator throughout the medium which prevents directed migration. These preliminary data suggest that nAchR expression is important for β cell migration to pancreatic nerves and β cell clustering which is required for proper islet formation and establishing a functional β cell mass.

### NT and axonal guidance genes are expressed in the developing human pancreas

Autonomic innervation is initially observed in human embryonic pancreas from 8 weeks PC[6], with parasympathetic and sympathetic nerve fibers detected close to the pancreatic epithelium. The appearance

of autonomic nerve cells coincides with the onset of β cell neogenesis suggesting that neural signals play a role in human pancreas development. A notion that is further supported by our discovery of T2D risk alleles in the NT and axonal guidance genes ADRA2A[15], CHRNB4[16], ROBO2, PLXNA3, and P2RY1. These genes are expressed in the developing human pancreas[17] and adult human islets with expression quantitative loci (eQTL) influencing islet expression.

## Research program

**1) Define the transcriptional landscape of the developing human pancreas**

**Rationale:** Islet development is a dynamic process with endocrine progenitor cells migrating from a multipotent progenitor-rich epithelium to form endocrine clusters and eventually fully functional islets of Langerhans which are connected to blood vessels and nerve cells. Most of our knowledge on pancreas development is derived from animal studies. However, islet morphology and development differ significantly between species which may explain the limited success of generating transplantable β cells and understanding the heritability of T2D in humans. Here we aim to determine the transcriptional landscape of the developing human pancreas to identify expression domains of known T2D genes.

**Methods:** Human pancreas anlagenwill be dissected and a single cell suspension will be made. 8000 cells/sample will be treated with Chromium Single Cell 3' solution on a 10x Genomics platform which allows for single cell labeling using a droplet-based approach. Single cell libraries will be sequenced using Novaseq sequencer (with approximately 50 000 reads/cell).

In parallel, samples will be snap-frozen in cryo-mounting medium and sectioned for spatial transcriptomics at SciLife lab, Uppsala. In this approach, single pancreatic sections will be mounted on glass slides containing a grid with bar-coded primers that fuse with attaching cells[18]. cDNA from bar code-labeled cells will be extracted and processed for sequencing. The transcriptomics data will then be aligned according to the spatial information from the section/grid to allow for the analysis of transcriptomes from neighboring cells and analyze potential lineage relationships[19].

**Outcome:** The proposed experiments and analyses will generate a comprehensive transcriptional profile of the developing human pancreas on a single cell level. The combination with spatial transcriptomics will allow us to determine which single cells are located next to one another and thereby add structural information that will aid in determining the lineage relationship of neighboring cells and to identify signaling pathways active in endocrine progenitor and neighbouring cells. A similar approach has been recently published for the developing heart[19].

**2) Evaluate how T2D risk alleles in NT and axonal guidance receptor genes influence human endocrine cell differentiation and islet morphology**

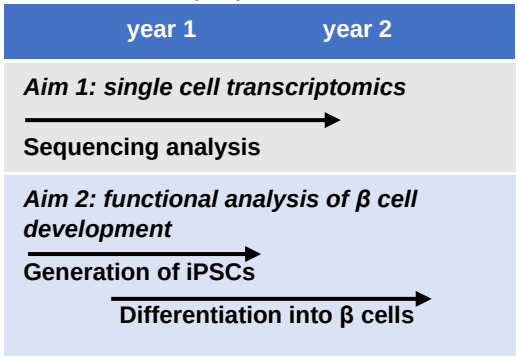
**Rationale:** In vivo and in vitro studies in animal models have been used in an attempt to understand the function and underlying disease mechanisms of T2D genes. Though many of these studies implicate defects in pancreatic β cell function, the mechanisms whereby these T2D risk alleles exert their negative effects remain largely unknown[20]. This could be partially accounted for by species-specific differences in gene expression and yet unidentified effects on the generation of β cells *in utero*. The emergence of iPSCs and β cell differentiation protocols[21] provides the opportunity to model human development in vitro, using cells from both risk allele carrying donors and control subjects. Here we aim to study how T2D risk alleles of NT and axonal guidance receptor genes which are expressed in β cells affect β cell differentiation/proliferation in human iPSC models of β cell development and in adult islets.

**Methods:** Endocrine cell differentiation: iPSC lines from risk allele carrying donors and isogenic controls will be generated at the StemTherapy iPSC/genome editing core facility. The core uses state-of-the-art procedures, Sendai viruses as vectors for the expression of molecular cues required for reprogramming of fibroblasts into iPSCs. This approach ensures high-efficiency reprogramming but also the removal of vector DNA from the host genome after the iPSC lines have been established thereby reducing the risk of tumor formation. CRISPR/Cas9 genome editing has been established. iPSCs will be differentiated towards insulin-secreting cells[22]. Once endocrine progenitors are detected, a 3-D cell culture will be employed to ensuring better yield and successful differentiation[22]. Cell differentiation and proliferation will be assessed by BrdU incorporation and analysis of marker gene expression by RT-qPCR and Western blotting/ immunocytochemistry. Cell proliferation, candidate gene expression, and differentiation will be assessed using immunocytochemistry for cell quantification.

Assessment of adult islet morphology of risk allele carrying donors: Human pancreatic sections from risk allele carrying (and control donors will be stained for islet hormones (insulin, glucagon, somatostatin, PP and

ghrelin) to assess endocrine cell distribution and islet morphology. RNAseq data from islet donors will be analyzed for the levels of islet hormone gene expression to evaluate if reduced gene expression alters islet cell composition and determine if reduced NT or axonal guidance receptor gene function impairs islet morphology.

**Outcome:** The proposed experiments will determine if T2D risk alleles of NT receptor genes, affect β cell differentiation, proliferation and function. Initially, we will focus on studying the effect of NT and axonal guidance genes which are expressed in adult β cells and the embryonic pancreas and which are associated with T2D. Adra2A and Robo2 have been shown to affect β cell mass and pancreas development, respectively in animal studies[3,23], further suggesting a role of these receptors in regulating β cell differentiation and islet morphology in the human pancreas. The differentiation studies of risk allele iPSCs will be complemented with siRNA knockdown of candidate genes and NT agonist treatment to assess the responsiveness of risk variant containing cell lines to NT signaling. Islet morphology studies will assess if altered gene expression affects islet composition which may ultimately affect endocrine cell function. The P.I. has experience with stem cell culture and the proposed methods[24].




This is a collaborative project between Islet Biology and Type 2 Diabetes Genetics groups (Rashmi Prasad/Ola Hansson (bioinformatics) and Hindrik Mulder/Malin Fex (iPSC biobank and β cell differentiation)). Interactions with these research groups will aid the applicant with technical support from my group to complete this project in a timely fashion. An outline of the time plan is presented in Fig.2. Human embryonic tissue has already been collected and sequenced and is awaiting bioinformatics analysis.

Fig. 2 illustrates the time frame and aims we will perform to **understand human pancreas development and *how T2D risk alleles that alter NT and axonal guidance receptor gene expression have impaired* β *cell differentiation***.

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