Open Access

OBM Transplantation

LIDSEN Publishing Inc.

Review

Single Cell Transcriptomic Analysis of Pancreatic β Cell Development and Differentiation from Pluripotent Stem Cells

Chunhui Xie, Lillian Ye, Yeh-Chuin Poh, George Harb *

Semma Therapeutics, Inc. 100 Technology Square, Floor 3 Cambridge, MA 02139 USA; E-Mails: c.xie@semma-tx.com; l.ye@semma-tx.com; poh.yehchuin@gmail.com; g.harb@semma-tx.com

* **Correspondence:** George Harb; E-Mail: g.harb@semma-tx.com

Academic Editor: Tatsuya Kin

Special Issue: Human Islets for Diabetes Research and Transplantation

OBM Transplantation
2019, volume 3, issue 3
doi:10.21926/obm.transplant.1903072

Received: May 06, 2019
Accepted: July 08, 2019
Published: July 17, 2019

Abstract

Single cell genomics is a powerful tool to study cellular heterogeneity and discover novel cell types. Recent studies used single cell RNA sequencing (scRNA-seq) to analyze the transcriptomes of individual pancreatic islet cells. Islets are a complex mixture of endocrine cells and therefore represent an ideal tissue type for single cell transcriptomic analysis. Adult human islets consist of five known endocrine cell types (α, β, δ, γ, ε) and multiple less well-defined non-endocrine cells. In this review, we discuss the scRNA-seq studies performed on human fetal, adult, diseased and pluripotent stem cell-derived islets in recent years. Since 2015, ~30,000 adult human islet cells have been analyzed using several scRNA-seq technologies. Studies provide a complete catalogue of all islet cell types and subtypes found throughout human development from fetus to adulthood. Islets from patients with Type 2 diabetes have also been analyzed with scRNA-seq unraveling multiple mechanisms of islet dysfunction. Advances in stem cell differentiation protocols and cell therapy manufacturing are bringing stem cell-derived islets (SC-Islets) closer to clinical trials. In 2018, more than 60,000 SC-Islet cells were analyzed using scRNA-seq technologies. Lessons learned include SC-Islet cell populations, lineage trajectories and comparative analyses to adult human islet

cell transcriptomes. Studies have also identified and characterized the non-islet, off-target cell populations revealing potential strategies for their elimination.

Keywords
Islets, β cell; single cell RNA sequencing (scRNA-seq); transcriptome; differentiation; pluripotent stem cells; hPSCs

1. Introduction

The global number of patients with diabetes has risen to over 400 million [1]. Alternatives to insulin therapy and anti-hyperglycemic medications are urgently needed. Cadaveric donor derived islet cell therapies have demonstrated impressive proof-of-concept outcomes in clinical trials with brittle Type 1 diabetes patients that are C-peptide negative and immunosuppressed. However, with a lack of sufficient allogeneic islets available, the number of islet transplants available for patients remains limited. The successful development and delivery of novel β cell replacement therapies produced from human pluripotent stem cells (hPSCs) could provide an unlimited therapeutic supply of β cells for diabetic patients. Clinical trials are now underway to evaluate first generation β cell progenitor therapies [2]. Early implementation of scRNA-seq analysis in the development of hPSC-derived β cells could aid in improving the composition of manufactured β cell therapies by identifying surface marker-enabling selection of cell populations.

Recently developed single cell genomic tools that are high throughput at large cell numbers, with unsupervised cell clustering have been used to study pancreatic islet β cell transcriptomes at single cell resolution. Commercially available, bench top single cell technologies can process tens to hundreds of thousands of individual cells simultaneously to define their transcriptional profiles. The Human Cell Atlas Project has initiated an effort to apply single cell genomics technologies to every tissue in the human body to create a comprehensive reference map of all $2x10^{13}$ nucleated human cells [3]. The cell atlas will collect multiple data sets including DNA, RNA, epigenome and protein. High dimensional single cell genomics is a powerful tool that enables comprehensive characterization of all cell types and subtypes within human tissues. Single-cell RNA sequencing (scRNA-seq) is now routinely used to measure the genome-wide expression profile of individual cells. scRNA-seq differs from bulk, whole transcriptome RNA-seq, which is a gene expression average of all cells. From each cell, mRNA is isolated, and reverse transcribed to cDNA for high-throughput sequencing [4]. Separation and unique bar coding of individual cells can be accomplished using flow cytometry or microfluidics-based platforms such as Fluidigm C1 [5] and droplet-based technologies such as inDrops or 10X Genomics [6, 7]. scRNA-seq can reveal the expression profile of each individual cell in response to various phenomena such as differentiation, disease progression and cell fate reprogramming.

Single cell RNA sequencing technologies first emerged in 2009 [8, 9] and was later used to study the adult human pancreatic islet transcriptome in 2015 (reviewed by [10]). This review will discuss the application of scRNA-seq technologies in studying islet cell subtypes in healthy and diabetic donors, development and islet derivation from pluripotent stem cells. Single cell expression profiling of islet transcriptomes relies on a method of single cell isolation: Either fluorescence

activated cell sorting or commercially available emulsion droplet-based platforms (Fluidigm C1, inDrops, 10X Genomics), cDNA library synthesis (CEL-Seq2 or SMART-Seq2) and sequencing (Figure 1) [9]. Single-cell RNA sequencing generates high-dimensional, high-throughput single-cell data which requires complex computational, modeling and visualization techniques. Data set analysis workflow typically includes genome alignment, quality control, variant calling, dimensionality reduction and pseudotime analysis. Dimensionality reduction techniques such as principal component analysis and t-distributed stochastic neighbor embedding (tSNE), are used to resolve cell type populations in islets. Other computational tools such as spatial transcriptomics [11] and Seurat [7] have been developed to link subpopulations and spatial positioning. Pseudotime analysis has been used to infer trajectories of cellular events, such as proliferation, differentiation, stress, disease, and reprogrammed cell fate [12].

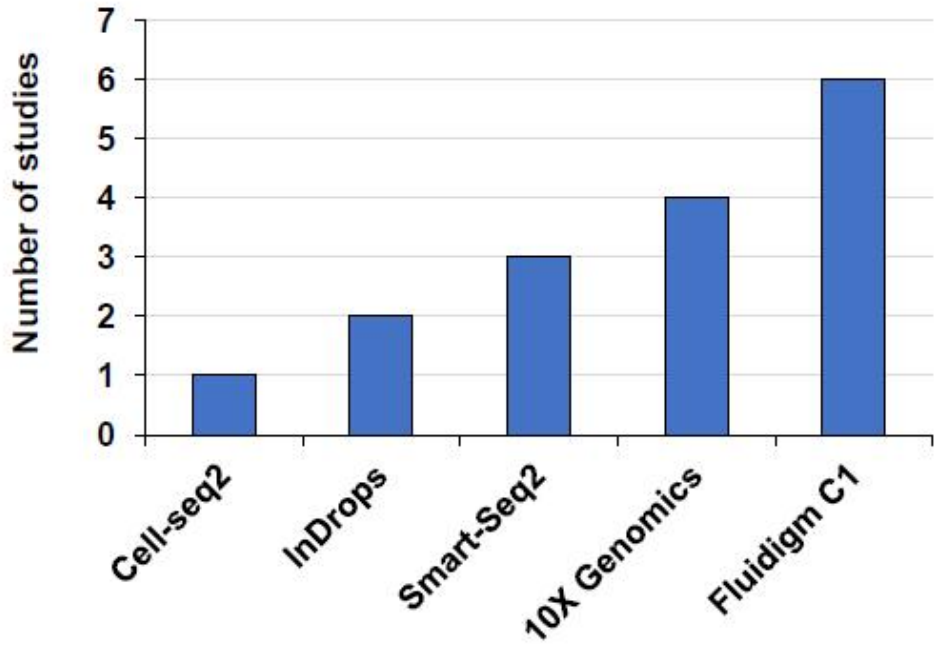

Figure 1 Single cell RNA sequencing platforms for studying islet cell transcriptomes (2015 to 2018).

Despite the continual evolution of computational methods and the generation of ever larger and complex data sets, challenges remain. Analysis of scRNA-seq data is complicated by excess zero counts and high drop-off due to low abundance mRNAs sequenced within individual cells. High dropout rates mean only up to 3-5% of the transcripts inside of cells are analyzed. The use of standardized controls such as External RNA Control Consortium (ERCC) spike-in are not always reported. Normalization is critical and challenging [13]. There are no established standards for data pre-processing and noise removal, which can influence data interpretation. Methods to cope with the inherent noise and the high dimensionality of single-cell data are still being evaluated and proper application of these strategies is crucial to not only discern genuine gene expression from technical artifacts but also to compare data across different laboratories and instruments.

2. Adult Islets

Bulk transcriptomic studies have provided important insights into the gene expression profiles of adult human islets [14, 15], but these analyses lack the resolution of single cell analysis. Human islets are naturally heterogenous in cellular composition depending on the anatomic location of the islets in the pancreas. The heterogeneity may also vary according to genetic variation, diet, and other factors. Therefore, single cell genomic profiling is best suited to unravel islet cell heterogeneity in detail. The variation that exists may help inform the upper and lower limits of what is defined as normal healthy islet cell gene expression signatures and serve as a benchmark to which diseased or in vitro generated islets can be compared. To define all the cell types within human pancreatic islets, twelve studies have so far profiled islets donated from 71 individual adult donors (Table 1) [16]. The donors ranged in age, ethnicity and health status (diabetic or non-diabetic). There were differences in islet cell sample preparation including the enzymatic isolation process, culture media and incubation time (0-12 days). Despite these differences in processing across the studies, there were no significant changes in gene expression profiles between islet cell from across donors [17]. Sample preparation variability applies to all islet samples regardless of donor source or in vitro production. Enzymatically dissociated single islet cells are then captured for subsequent reverse transcription and cDNA synthesis [18, 19]. Using several commercially available scRNA-seq platforms (Figure 1), academic and industry groups profiled the transcriptomes of human islets from 52 non-diabetic (ND) donors between the ages of 17 and 68 (Figure 2) and identified multiple cell types. Islets from Type 2 diabetic adult donors (n=15) were also studied (age range: 37 - 57 years) [17, 19-21]. Altogether, 33,271 human islet cells were sequenced at depths of up to 13 million reads (Figure 3). Donors were from multiple ethnicities including African American, Caucasian, Hispanic and Asian. In each study, islet batches from between 1 and 18 adult islet donors were analyzed (Figure 4). Islet cell heterogeneity, enzyme and surface marker expression, transcription factors and signaling receptors are described. All studies analyzed islet cells without prior cryopreservation, although successful transcriptomic analyses have been performed on previously cryopreserved cell lines and primary cells such as tumor cells, PBMCs and human islet cells [22, 23].

Table 1 Adult human islet single cell RNA-seq studies.

	Study	**Single cell technology** (FACS, microfluidic)	**Reference**
1	Non-diabetic islets	FACS	[16]
2	Non-diabetic islets	InDrops	[24]
3	Child, adult non-diabetic, T1D, T2D islets	Fluidigm C1	[17]
4	Non-diabetic islets	FACS	[18]
5	Non-diabetic and T2 diabetic islets	FACS	[20]
6	T2 diabetic islets	Fluidigm C1	[21]
7	T2 diabetic islets	Fluidigm C1	[19]
8	Non-diabetic East-Asian islets	Fluidigm C1	[25]
9	ε cells (transcriptome)	10X Genomics	[26]
10	α cells (subtypes and transcriptome)	10X Genomics	[27]
11	β cells (subtypes and transcriptome)	10X Genomics	[28]
12	Non-diabetic islets	Fluidigm C1	[29]

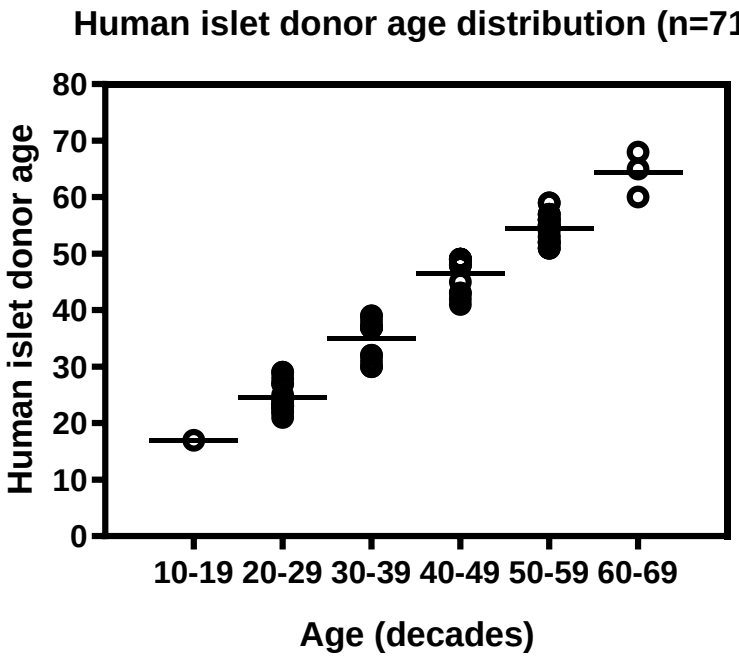

Figure 2 Human islet donor ages analyzed by scRNA-seq. The majority of adult human islets sequenced were from donors aged 50-59 (n=23).

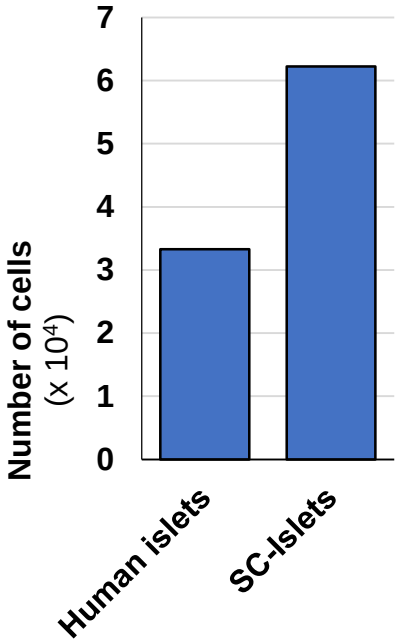

Figure 3 Number of cadaveric versus stem cell-derived islet cells analyzed by scRNA-seq (2015 to 2018).

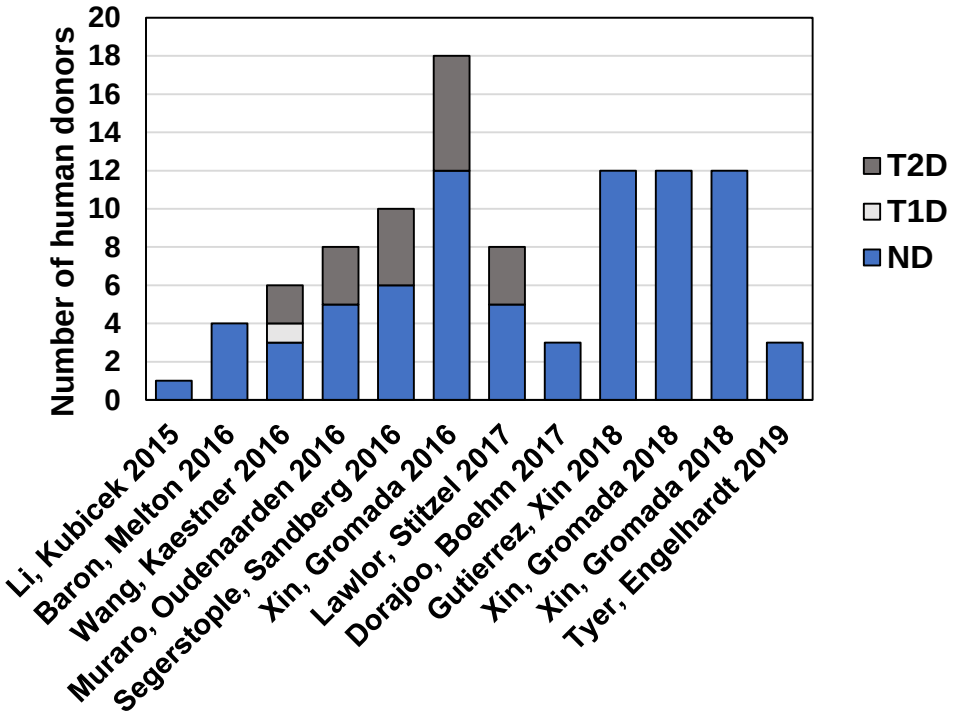

Figure 4 Breakdown of healthy non-diabetic (ND) versus diabetic (T1D, T2D) islets analyzed by scRNA-seq from a total of 73 adult human donors.

Adult human islets isolated using enzymatic digestion of the pancreas were found to contain multiple cell types classified as either endocrine or non-endocrine. Endocrine cells comprise α (11-67%), β (10-19%), δ (1-10%), γ (1-10%) and ε (0.4-1.4%) cells [20, 26]; the non-endocrine cells are acinar (*PRSS1*), ductal (*SPP1, KRT19*), stellate cells (TIMP1, FN1), endothelial cells (PLVAP), immune cells [macrophages (*CD74, CD86*), mast cells (*TPSAB1, CD31*), and antigen presenting cells (HLA-DPA1, HLADPB1, and HLA-DRA)] and unclassified cells. In other words, 9 non-endocrine cell types are infused into patients in each intraportal allogeneic islet infusion. The impact of these cell types on islet engraftment, glycemic control and long-term graft survival are currently unknown. In addition to the variations in islet cell composition, subpopulations of islet cell types such as α and β cells exist and are still under active investigation [16, 17, 20, 24, 27-30]. Some groups have described β cell subtypes that exist in healthy adult humans [18, 20, 24], whereas others did not find evidence for β cell subpopulations [19]; α and δ cell heterogeneity have also been reported [17, 20].

Glucose responsive β cell function is controlled by genes associated with glucose uptake, metabolism and insulin secretion. A key regulator of glucose metabolism is the glycolytic enzyme 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2 (*PFKFB2*) [31]. This enzyme has been identified in several scRNA-seq studies as uniquely expressed in β cells [18, 24, 25]. The inhibition of PFKFB3 has recently been described as an experimental diabetes treatment mediated through the inhibition of glycolysis and reduction in ER stress-induced β cell death [32]. Novel cell surface markers have also been discovered for labeling of islet cell types. Antibody mediated enrichment strategies have been used to purify islet cell populations such as based on surface marker expression such as CD39L3 for β cells [33], CD24 for acinar cells, CD44 for duct cells and TM4SF4 for α cells [18]. Pharmacologically druggable GPCRs, ion channels, expressed by islet cells include *FFAR4/GPR120, GPR119*, *LEPR, GHSR, SLC6A4, CASR* and *GLP1R* [18, 20, 24].

β Cell Heterogeneity. It has been known for three decades that functionally diverse β cell subtypes with different glucose responsive thresholds for insulin release exist [34]. Single cell RNA sequencing analysis can leverage gene expression variation within islet cell populations to reveal underlying transitory states and heterogeneity. Evidence for β cell heterogeneity exists from both functional and gene expression analyses [35]. For example, the surface markers ST8SIA1 and CD9 were recently identified to label four β cell subtypes in human islets [30]. Segerstolpe et al. found five β cell subtypes based on varying *RBP4, FFAR4/GPR120, ID1, ID2*, and *ID3* expression [20]. Across the five subtypes, insulin gene expression did not differ. However, Xin et al. reported that β cell subtypes do segregate based on insulin gene expression as well as stress response [28]. Activation of the unfolded protein response (UPR) in β cells with low insulin expression is thought to be a stress coping mechanism [28]. Four scRNA-seq studies have identified human β cell subtypes with an active UPR signature and low insulin expression [18, 20, 24, 28]. Genes enriched in this subtype express elevated *DDIT3, FTL, FTH1, FTH1P3, HSPA5, SRXN1* and *SQSTM1*. Increased expression of these UPR genes suggests activation of a stress response which is also associated with lower β cell transcription factor expression (e.g. *MAFA* and *SIX3*) [28]. Antioxidant defense programs driven by superoxide dismutase (*SOD1* and *SOD2*), *NRF2* and genes involved in glutathione and thioredoxin metabolism, quinone detoxification and iron storage are also active as is the high metabolic demand in these cells (e.g. glycolysis, TCA cycle, pentose phosphate pathway). β cell subtypes with active UPR are more likely to be associated with elevated cell cycle gene expression (e.g. *ZNF143, CDKN1B, HES4*) [28]. From a root state, pseudotime trajectory

analysis revealed that β cells branch into either average INS or high INS β cell subtypes. The fluctuations in the INS gene expression level and activation of UPR stress response pathways may reflect the inherent responses of individual β cells to increased metabolic demand placed on the cells.

α Cell Heterogeneity. In both non-diabetic and Type 1 diabetic human donors, a highly proliferative α cell subtype has been identified [27, 36]. Although the majority of α cells display a fully differentiated, non-proliferative phenotype (α1 and α2), a rare subpopulation (α3) present at only 1-2% is marked by high levels of cell cycle genes such as *Ki67, TOP2A, CDK1*, and a variety of cyclin genes (E2, A2, B1-B2) [27]. These cells also display increased *FOXM1* expression, a transcription factor shown previously to increase human islet cell proliferation [37]. The mechanism of cell cycle control in the α3 subgroup is regulated by the PRC2 complex which consists of EZH2, and other epigenetic components already implicated in β cell proliferation [38]. The proliferating α cells also express lower levels of the cell cycle inhibitors *p21* (*CDKN1A)* and *p57* (*CDKN1C*). Pseudotime analysis confirmed the proliferation trajectory of non-proliferative α1 and α2 cells into proliferating α3 cells [27]. The importance of this proliferating α cell subtype in health and diabetes remains unknown.

3. Diseased Islets: Type 2 Diabetes

In patients with Type 2 diabetes (T2D), hyperglycemia results from impaired islet cell function and insulin insufficiency [39, 40]. Studies analyzing T2D islets revealed abnormalities in islet cell proportions and gene expression patterns compared to non-diabetic islets [17, 19-21]. However, as much as 30% of the genes affected by T2D have no known function [21]. In T2D patients, the proportion of islet cells may be significantly altered [20] or not [19]. The proportion of insulin-producing β cells can be reduced by as much as 50% in Asian T2D patients with a mean disease duration of only 5 years [40]. Between 2016 and 2017, four studies used scRNA-seq to analyze islets from 15 T2D islet donors [17, 19-21]. The studies examined the expression patterns of insulin and stress response pathways in β cells and how they may influence disease susceptibility. Other groups have reported altered gene expression patterns in all islet cells and identified dysfunctional gene expression profiles in α, δ, γ cells. Transcriptomic analyses have identified altered gene expression patterns in multiple islet genes including *INS, DLK1, FXYD2, SLC30A8, MTNR1B, TCF7L2*, and *KCJN11* (Table 2).

Table 2 Genes differentially expressed between single T2D and non-diabetic β cells.

Study	Upregulated genes in single T2D beta cells
[17]	ST6GAL1, USH2A, PLEKHO2, STS, NLE1, LOC441666, CFB, PRKD1, LAD1, DNAJB7, SLC4A4, AHNAK, NHSL2, IGSF5, HSP90AB4P, HLA-G, GPSM3, CCDC64, ZNF493, EPPK1, RARRES2, HLA-J, LOC439949, TRPC4AP, LOC678655, BARD1, CDKN2B, HLA-L, ELFN2, ID2B, JUNB, C9orf16, SNORD116-29, UBD, LPCAT1, TSPAN8, ZNF208, PIAS4, PRKCSH, DUOX2, CSNK1A1L, PDXDC1, NACAP1, PYCARD, MZT2B, AES, MTRNR2L8, RPL30, HSP90AB3P, B2M, HSP90AA1, ATP5EP2
[20]	GPD2, XPO7, ANXA2P2, NOL8, CCDC90B, LEPROTL1, FUCA1, NFE2L2, DZIP3, CMTM6, MEIS1, TMEM167A, COPS8, SSBP2, CNTN1, DNAJC10, PNRC2, AZIN1,MMADHC, SPAG16, VWA5A, TTC37, HSP90B1, ARL1, MEG3, SEPT2, HIF1A, RPL15, PREP, EIF4G2, ITFG1, CD46, LAMTOR3, UBE2N, COPB1, GPBP1, UNC50, TUBA1A, SCOC, TSPAN13, EIF4E, CAPZA2, TTC3, VDAC1, RAB10, YWHAZ, BTF3L4, DDX5, REEP5, NDFIP1, MAP1B, TMOD1, EPCAM, TMEM59, ARPC3, TUBA1B, YWHAQ, PRKAR1A, LINC00657, APLP2, ERO1LB, STXBP1, CAP1, ATP5B, HNRNPC, LOC644936, SKP1, DYNLL1, TMBIM6
[28]	LOC100873065, PAXBP1-AS1, APOL4, RPS3AP18, FXYD3, GLS2, FZD6, RN7SK, LAMC1, TAB3, LINC00478, LINC00486, RPL34-AS1, LOC101927317, TAPT1-AS1, LOC100128045, ANKRD36C, CD47, SUN1, RIN2, RPL7, SGSM2, FRG1B, SLC7A8, TTR, SNHG5, TUBA1C, FTL, MTRNR2L1, RNF216, FXYD2, PSPHP1, MIR4458HG, PPP1R1A, LOC100616530, SEC24C, DCAF16, DNPH1, NUP93, GLRA1, PVRL1, ATP5SL, MCOLN3, COTL1, IGFBPL1, ZNF397, LAMB1, LOC100128906
[29]	SPP1, PSAT1, ST6GAL1, CCDC67, PRKAR2B, PCCB, CDH6, SMC4, EXOC2, KIF27, GSTT1, PAK1, SCN9A

Gene list: T2D.vs.NonT2D.Beta logFC > 3 [29].

Variations in INS gene expression across individual β cells are known to be more heterogenous than previously thought [19]. β cell expression of insulin can be modulated by the severity of the stress response present [28]. In β cells with low stress, INS gene expression levels are similar to non-diabetic control levels. But in β cells with high levels of ER- and oxidative stress, the median value of INS gene expression per β cell is lower overall. The major stress response pathways implicated in mediating β cell stress include death receptor TNFR1, mitochondrial BAX, the cysteine proteases CAPN1- and CAPN2 and p53. There was also no evidence of de-differentiation occurring in islets from T2D donors, as seen from the lack of change in expression levels of *FOXO1, NANOG, POU5F1, NEUROG3* and *MYCL* [19, 39].

Single cell genomics has been used to identify diabetes susceptibility genes in a specific Asian population [25]. The scRNA-seq study examined a small set of East-Asian islet donors and could identify susceptibility to islet cell death based on the gene expression profile of the β cells [25]. Significant upregulation of the protein ubiquitination pathway genes (*OLMALINC, RNR2, SCD*, *MAP1B, DNAJC3, SEC11C* and *UCHL1*), was found in β cells of East-Asians despite normal HbA1c (5.9%, 5.8%, and 5.1%). In non-Asian T2D patients however, the protein ubiquitination pathway

was not elevated in β cells, despite an average HbA1c of 7.0±0.4% (n=6) [21]. This suggests East-Asian β cells may be inherently vulnerable to protein misfolding and ER stress [41, 42]. Increased susceptibility to cell death may be why East-Asians develop T2D at a younger age and at lower BMI levels compared to Europeans.

4. Fetal and Juvenile Islets

The human pancreas first emerges when the foregut evaginates into dorsal and ventral buds at 4 weeks of development (WD). Pancreatic buds contain multipotent progenitors expressing *PDX1, NKX6.1* and *SOX9* which proliferate and differentiate into all pancreatic lineages (acinar, ductal and endocrine) [43]. Endocrine cell differentiation starts at 8 WD beginning with the expression of neurogenin 3 (*NEUROG3*) and the coincident expression of insulin and glucagon. At 10 WD, insulin-positive cells begin to form clusters. Islet-like clusters become penetrated by vasculature and other endocrine cell types including glucagon-, somatostatin-, and pancreatic polypeptide-positive cells by week 12. The understanding of human pancreas development has relied primarily on immunohistochemistry and bulk gene expression analyses. Ramond et al. recently studied endocrine cell induction during fetal development using 9-week-old human pancreases from three individual donors [44]. Distinct progenitors with subpopulations of intermediates were identified in the human fetal pancreas and designated Populations A, B, C and D [44, 45]. The 9 WD timepoint was chosen for a comparative analysis of human endocrine induction and in vitro pluripotent stem cell-derived endocrine induction. Comparing the transcriptomes of in vitro derived progenitors with their human fetal pancreatic counterparts helps to pinpoint where in vitro progenitors deviate and how differentiation can be improved. Access to human fetal samples are necessary for this type of transcriptomic profiling analysis given the differences that exist between rodent and human endocrine specification and overall pancreas development.

Four populations of epithelial progenitor cells (EPCAM+) in 9 WD pancreases were designated: multipotent pancreatic progenitors (A), early endocrine pancreatic progenitors (B), NEUROG3-positive endocrine progenitors (C) and early endocrine cells (D) based on the expression pattern of three surface markers (ECAD, CD142 and SUSD2). Population surface marker expression patterns: **A**: ECAD+CD142+, **B**: ECAD+CD142−, **C**: ECADlowCD142−SUSD2+, **D**: ECADlowCD142−SUSD2−. Expression of SUSD2 in population C correlated with peak NEUROG3 expression and is the population in which *INS* is first detected followed by a 13-fold increase in expression in Population D by 10 WD. Populations A and B cluster together as pancreatic progenitors (*PDX1, SOX9, ONECUT1*), whereas Population C contains endocrine progenitors (*NEUROG3, FEV*) and Population D contains more mature endocrine cells (Table 3). The pancreatic progenitors in Population B are predominantly duct-like progenitors (CD133+, CFTR+) but also contain a smaller population of endocrine restricted NEUROG3+ progenitors. Most of the endocrine progenitors in Population C express NEUROG3 and Population D cells become committed endocrine cells expressing genes involved in hormone regulation and secretion such as *ISL1, CHGA, MAFB, PAX6, PCSK1, GCG, SST, GHRL* and INS [45]. *PAX4* was present only in population C and was absent from D. The β cell specific genes such as *MAFA, PCSK1, IAPP, G6PC2, FFAR1, SLC30A8* are more enriched in population D than in C. Pseudotime construction of the developmental trajectories within the non-ductal portion of Population B (CD133-negative) revealed three branch points for determining cell fate choice. The first branch point represents polyhormonal endocrine cells (*INS, GCG, PPY*), the

second branch diverges into endocrine progenitors (*NEUROG3, ARX*) and the third branch diverges into two endocrine populations (β and δ cells). The INS+ cells in Population D express mature β cell markers including *DLK1, MAFA* and *HADH*.

Table 3 Genes differentially expressed between Populations A to D [44].

A	B	C	D
SPINK1	SLC4A4	GCG	MAP1B
AMBP	BICC1	NNAT	CPE
SERPINA1	DCDC2	TUBA1A	MFAP4
TM4SF1	HMGA2	GHRL	QPCT
CLU	CFTR	SCGN	SLC7A8
RBPJ	ID2	MLLT11	TTR
IGF2	ANXA4	UCHL1	APLP1
ELF5	AKAP7	TAGLN2	MYT1L
HNMT	TGIF1	TUBA4A	RP11-521D12.5
CLDN6	FXYD2	IGFBP5	PIPOX
PHGDH	ANXA2	CACNB2	SCG5
ERP27	VEPH1	SCG3	FAM92B
CPA2	C14orf105	RAB3B	GRIA2
DLK1	ATP10B	KIF1A	KIAA1644
EFEMP1	UPK1B	A1CF	HADH
GABRB2	SDC4	ABCA5	ABCC8
GATM	FRAS1	LINC00643	CDH8
PDPN	FREM1	PDE4DIP	PAX6
ATP1B1	HKDC1	UCP2	GNAS
UGT2A3	RP11-834C11.4	ASNS	JAKMIP2
KRT18	S100A10	MAP2	MIAT
SOX9	ONECUT1	NEUROG3	INS

After birth, islets undergo changes in proliferation rates, nutrient metabolism and maturation [46, 47]. Expression patterns of *MAFA* during the first decade of life increase significantly but remain heterogenous in adults [48]. Wang et al. profiled juvenile islets from a 19-month-old female and a 2 year old male using the Fluidigm C1 microfluidic system and found a partially completed differentiation program in both sets of juvenile islets [17]. Two significant gene expression patterns were observed, a reduced adult endocrine gene signature and a mis-expression of adult α and β cell gene signatures in juvenile β and α cells, respectively. In addition, genes expressed by juvenile cells resembled those expressed by diseased islets from Type 2 diabetes patients.

5. Human Pluripotent Stem Cell (hPSC)-Derived Islets

Multiple stem cell-derived tissue cell types have been characterized by scRNA-seq including endothelial cells [49], neurons [50], retinal cells [51] and cerebral [52] and kidney organoids [53]. Major SC-Islet differentiation protocols, published in 2014, have now reached efficiencies ready for clinical translation [2]. Implementation of scRNA-seq analysis can improve differentiation efficiencies, define cell therapy composition both pre- and post-transplant and ultimately improve clinical efficacy. In clinical allogeneic islet transplantation, flow cytometric and immunocytochemical staining and quantification analyses have been used to determine pre-transplant islet composition. These protein detection methods rely on a predetermined knowledge of cell markers. Islets derived from stem cells are less defined and vary based on the differentiation protocol used and the starting pluripotent stem cell line [54]. Based on this, unbiased cell type identification and discovery is best suited for scRNA-seq-based transcriptomic analyses.

The various differentiation protocols for converting pluripotent stem cells into SC-Islets have been previously reviewed by Harb et al. [55] and Millman et al. [56]. Islets can be produced from hPSCs with six stage [57] or seven stage [58] differentiation protocols or through a combination of both protocols [59, 60]. Protocols range between 21 and 35 days and generate between 30% and 60% SC-β cells. Off-target cells include non-islet cells that are either endocrine or non-endocrine cell types [54]. Four studies have sequenced stem cell derived islets from both embryonic and induced pluripotent stem cells across various stages of differentiation (Stage 3 to Stage 7 – pancreatic progenitor to islet cells) [45, 54, 59, 61]. Previous studies have sequenced cells prior to Stage 3 pancreatic progenitors, including pluripotent stem cells (Stage 0) [62, 63] and definitive endoderm stage cells (Stage 1) [64, 65]. This section will discuss the analyses performed on Stage 6 or Stage 7 cells generated from the three studies that have sequenced hPSC-derived islets or islet progenitors [54, 59, 61].

Functional analysis of stem cell-derived β cells revealed a stable phenotype in culture based on in vitro glucose-triggered insulin release assays [54, 57, 58, 66-68]. Unlike what has been observed with adult human islets, there were no signs of β cell loss or conversion into other islet cell types with prolonged time in culture [54, 69, 70]. In vitro derived β cell gene expression profiles are similar overall to human β cells [68], but can vary in gene expression levels, e.g. *HADH* [44, 54, 68].

In Krentz, et al. 4,462 Stage 6 day 1 (S6d1) cells, generated from the hES cell line CyT49 (Rezania protocol) passed QC for scRNA-seq analysis [61]. The S6d1 cells sequenced by Krentz formed 9 clusters that were classified into 5 cell types: endocrine (Endo), endocrine progenitors (EP) and off-targets including duct, liver, and an unknown cell type. The unknown cell population is enriched in genes such as *CXCL14, CA3, CRABP2, S100A11, ARHGAP29, NR2F2, TFAP2B, PDGFC*, etc. [61]. Endocrine cells, which made up 74.3% of the total population, expressed *INS, GCG* or *SST*, while some EP cells also expressed *INS*. Endocrine progenitors clustered into 3 groups (EP1, EP2 and EP3) and do not all give rise to islet cells. The EP1 cluster is the largest cluster and expresses NEUROG3. The EP2 cluster contains cells that express genes associated with serotonin production (*FEV, DDC* and *TPH1*) which Veres et al. ultimately found give rise to enterochromaffin-like (EC) cells. In the Ramond et al. study hPSC-derived progenitors, equivalent of Population B, were discovered to mis-expresses both *RFX6* and *CDX2* [44]. Expression of CDX2 indicates a mixed

pancreas-duodenum fate which could later give rise to intestinal cells [71]. The EP3 population of cells express genes such as GAST, NKX6-3, ONECUT3, PCSK1, DDC, etc.

In Balboa et al., 2,171 Stage 7 cells were sequenced using inDrops and passed quality control for subsequent analyses [59]. Cells were differentiated from hiPSCs derived from patients with a monogenic form of diabetes (*INS* gene mutations C96R and C109Y). These mutations impair β cell development and result in neonatal diabetes. Balboa et al. used scRNA-seq to study the transcriptomes of cells differentiated from C96R mutant and CRISPR/Cas9-corrected hiPSCs. Transcriptomic analysis unveiled activation of stress response pathways reported in β cells by other scRNA-seq studies [24]. The β cells harboring the *INS* C96R mutation suffered from increased ER-stress and impaired cellular proliferation which was also present in the mutant progenitors. Compared to corrected cells, mutant cells displayed upregulated UPR-related genes: *MANF, HSPA5, HSPA8, HSP90B1, PDIA6*, and ER associated degradation pathway components (*HM13, HERPUD1, SEC61B*, and *SDF2L1*). Downregulated genes included mitochondrial respiratory chain subunits (*MT-CO1*, *MT-CO2*), *PAX6, RFX6*, and the mTOR regulator *LAMTOR5*. Stage 7 differentiated cells clustered into four different cell populations, expressing markers of β cells, endocrine progenitor cells, α cells, and proliferating α cells (Table 4). The progenitor cell population express enterochromaffin cell markers including DDC, FEV, TPH1, LMX1A.

Table 4 Top genes differentially expressed between by SC-Islets in Balboa et al [59].

β cells	Progenitor cells	α cells	Proliferating
NEFM	DDC	CLU	RRM2
DLK1	FEV	ARFGEF3	TOP2A
ERO1B	TPH1	SERPINI1	CENPF
PCSK1	CBLN1	SERPINA1	PRC1
SCD	DNAJC12	ARX	TUBA1B
PLXNA2	RETREG1	ALDH1A1	HIST1H4C
NEFL	COL5A2	DPP4	PCLAF
IGDCC3	IGFBP5	ITGB1	KPNA2
SPEG	S100A11	VIM	HBD
ENTPD3	ANKS1B	IGFBP2	TUBB
PDX1	SYT13	IRX2	TUBBP1
CCBE1	PRPS2	BTG1	H2AFZ
PRUNE2	ZNF703	ATP1B1	TUBB4B
KIF5C	GOLM1	NKAIN4	SMC4
BASP1	STC1	LY6H	HMGN2
CALB2	MME	MGST1	STMN1
SVIP	RAB3B	SLC50A1	MAD2L1
TUBA1A	NKX6-1	APCDD1	RRM1
C1QL1	LMX1A	FXYD3	TMEM106C
CADM1	EVL	LOXL4	AURKA
MAP1B	CCNY	SLC7A8	CKS1B
CHRNA3	NEUROD1	PTGER3	CKAP2

In Veres et al., >100,000 individual cells were sampled throughout the course of differentiation, of which 57,782 were Stage 6 cells (S6d1-S6d36). Cells derived from both hiPSCs and the HUES8 hES cell line using the Pagliuca et al. protocol were sequenced using inDrops. Roughly twice as many Stage 6 SC-Islet cells (57,782) from 11 differentiation batches were sequenced in this study alone, in comparison to the 33,271 cells sequenced from 73 cadaveric human donor derived islets over 4 years (2015-2019). Unlike less common cadaveric islet sources, SC-Islets can be produced in an unlimited supply. Non-endocrine cells consist of several cell types including duct-like cells (*CFTR, MUC1, CDX2*), acinar-like cells (*CPA1*), and mesenchymal-like cells (*VIM, SPARC*). The Hippo pathway and parts of the WNT, EGF, Notch, and Hedgehog signaling pathways remain active in several of these populations. Proper suppression of the Hippo pathway is necessary for endocrine induction as shown recently in embryonic explants and Stage 5 hPSC-derived progenitor cells [72].

An off-target cell population identified by all groups is marked by the serotonin-producing machinery and resembles intestinal enterochromaffin (EC) cells [54, 59, 61]. The emergence of this off-target cell population is observed across the 3 differentiation protocols and is marked by *FEV* and *TPH1* expression. Tryptophan hydroxylase (TPH1) is an essential enzyme for serotonin synthesis [73] and the serotonergic transcription factor, FEV has been implicated in both mouse [74] and human [44] β cell development. Several fate specifying transcription factors are shared between enteroendocrine and β cell development such as *FEV, ARX, NKX2.2* and *PAX4* [75-77]. Improper expression and timing of these factors in hPSC-islet differentiation protocols, leads to the emergence of a mixed islet endocrine and enteroendocrine cell population [54, 78]. The Balboa study identified progenitor cells that express EC cell markers such as *FEV* and *MNX1* [59]. In both the Veres and Krentz studies, the serotonin-producing EC cells express *TPH1, DDC, FEV, CXCL12, CXCL14* and *PTHLH*. The role of these cells or their secreted products on SC-β cell function or survival are unknown. Interestingly, in adult human islets, the pancreatic polypeptide-producing γ cells also express *FEV, TPH1*, and *SLC6A4,* a serotonin reuptake transporter [19].

Removal of non-endocrine progenitor cells from the CHGA+ endocrine fraction is possible by re-aggregation or FACS-based enrichment of β cells [54, 67, 68, 79]. Enrichment of stem cell-derived β cells can lead to improved in vitro function [68]. Recent bulk RNA-seq analysis of FACS purified hPSC-derived β cells identified functional and maturation relevant markers which increased relative to pre-enrichment, mixed cell population levels [68]. Gene sets representative of oxidative phosphorylation, protein secretion, ribosomal constituents and various metabolic pathways such as the TCA cycle were also up-regulated [68]. In Veres et al., single cell analysis of stem cell-derived islets identified a surface marker, also expressed by human β cells [80], that could be used for immunomagnetic microbead-based enrichment of β cells. The laminin-binding integrin alpha 1 (ITGA1, also called CD49A) is expressed mainly by SC-β cells, less so by SC-EC cells, and could be used to produce purified SC-β cell aggregates with enhanced in vitro function [54, 81].

6. Summary and Outlook

Single cell RNA sequencing technology has been applied to study developmental trajectories in myogenesis, hematopoiesis, neurogenesis and now islet development. Transcriptomic data obtained from adult human islets has produced a comprehensive catalog of islet cell types, subtypes, functionally stressed and ultimately diseased cell states [17-21, 24, 25]. Activation of stress response pathways in β cells can impact insulin production, proliferative capacity and

disease predisposition [28]. Single cell analyses have also been used to study islet development in utero and in vitro from pluripotent stem cells [44, 54, 59, 61]. Transcriptomic comparisons of the endocrine specification process in human fetal pancreas at 9 weeks of development with in vitro pluripotent stem cells have revealed several similarities and differences [44]. A key difference is the mis-expression of *CDX2* in stem cell-derived islet differentiation protocols which is absent from human pancreas development. Transcriptomic characterization of human islets and SC-Islets has revealed surface markers useful for elimination of unwanted off-target cells such as enterochromaffin-like cells and enrichment of CD49A-positive β cells [54]. Additional information generated from single cell technologies include spatial information, epigenetic and proteomic data sets. Combining these with transcriptomic data will lead to a better understanding of islet cell functional states and pave the way for efficient in vitro production of β cells from stem cells.

Acknowledgments

The authors thank Adrian Veres for excellent feedback.

Author Contributions

C.X., G.H. wrote the manuscript. C.X., Y.P., and L.Y. provided input to the content, reviewed and edited the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

References

1. International Diabetes Federation. IDF diabetes atlas eighth edition 2017. [cited 2019 Jun 28]. Available from: https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html
2. Senior PA, Pettus JH. Stem cell therapies for Type 1 diabetes: Current status and proposed road map to guide successful clinical trials. Diabet Med. 2019; 36: 297-307.
3. Regev A, Teichmann SA, Lander ES, Amit I, Benoist C, Birney E, et al. The human cell atlas. Elife. 2017; 6: e27041.
4. Stegle O, Teichmann SA, Marioni JC. Computational and analytical challenges in single-cell transcriptomics. Nat Rev Genet. 2015; 16: 133-145.
5. Pollen AA, Nowakowski TJ, Shuga J, Wang X, Leyrat AA, Lui JH, et al. Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. Nat Biotechnol. 2014; 32: 1053-1058.
6. Klein AM, Mazutis L, Akartuna I, Tallapragada N, Veres A, Li V, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell. 2015; 161: 1187-1201.
7. Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, et al. Highly Parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell. 2015; 161: 1202-1214.
8. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, et al. mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods. 2009; 6: 377-382.
9. Svensson V, Vento-Tormo R, Teichmann SA. Exponential scaling of single-cell RNA-seq in the

past decade. Nat Protoc. 2018; 13: 599-604.
10. Tritschler S, Theis FJ, Lickert H, Böttcher A. Systematic single-cell analysis provides new insights into heterogeneity and plasticity of the pancreas. Mol Metab. 2017; 6: 974-990.
11. Ståhl PL, Salmén F, Vickovic S, Lundmark A, Navarro JF, Magnusson J, et al. Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. Science. 2016; 353: 78-82.
12. Saelens W, Cannoodt R, Todorov H, Saeys Y. A comparison of single-cell trajectory inference methods. Nat Biotechnol. 2019; 37: 547-554.
13. Haghverdi L, Lun ATL, Morgan MD, Marioni JC. Batch effects in single-cell RNA-sequencing data are corrected by matching mutual nearest neighbors. Nat Biotechnol. 2018; 36: 421-427.
14. Fadista J, Vikman P, Laakso EO, Mollet IG, Esguerra J Lou, Taneera J, et al. Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. Proc Natl Acad Sci USA. 2014; 111: 13924-13929.
15. Nica AC, Ongen H, Irminger J-C, Bosco D, Berney T, Antonarakis SE, et al. Cell-type, allelic, and genetic signatures in the human pancreatic beta cell transcriptome. Genome Res. 2013; 23: 1554-1562.
16. Li J, Klughammer J, Farlik M, Penz T, Spittler A, Barbieux C, et al. Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. EMBO Rep. 2016; 17: 178-187.
17. Wang YJ, Schug J, Won K-J, Liu C, Naji A, Avrahami D, et al. Single-cell transcriptomics of the human endocrine pancreas. Diabetes. 2016; 65: 3028-3038.
18. Muraro MJ, Dharmadhikari G, Grün D, Groen N, Dielen T, Jansen E, et al. A Single-cell transcriptome atlas of the human pancreas. Cell Syst. 2016; 3: 385-394.
19. Lawlor N, George J, Bolisetty M, Kursawe R, Sun L, Sivakamasundari V, et al. Single-cell transcriptomes identify human islet cell signatures and reveal cell-type-specific expression changes in type 2 diabetes. Genome Res. 2017; 27: 208-222.
20. Segerstolpe Å, Palasantza A, Eliasson P, Andersson E-M, Andréasson A-C, Sun X, et al. Single-cell transcriptome profiling of human pancreatic islets in health and type 2 diabetes. Cell Metab. 2016; 24: 593-607.
21. Xin Y, Kim J, Okamoto H, Ni M, Wei Y, Adler C, et al. RNA sequencing of single human islet cells reveals type 2 diabetes genes. Cell Metab. 2016; 24: 608-615.
22. Guillaumet-Adkins A, Rodríguez-Esteban G, Mereu E, Mendez-Lago M, Jaitin DA, Villanueva A, et al. Single-cell transcriptome conservation in cryopreserved cells and tissues. Genome Biol. 2017; 18: 45.
23. Manning Fox JE, Lyon J, Dai XQ, Wright RC, Hayward J, van de Bunt M, et al. Human islet function following 20 years of cryogenic biobanking. Diabetologia. 2015; 58: 1503-1512.
24. Baron M, Veres A, Wolock SL, Faust AL, Gaujoux R, Vetere A, et al. A single-cell transcriptomic map of the human and mouse pancreas reveals inter- and intra-cell population structure. Cell Syst. 2016; 3: 346-360.
25. Dorajoo R, Ali Y, Tay VSY, Kang J, Samydurai S, Liu J, et al. Single-cell transcriptomics of East-Asian pancreatic islets cells. Sci Rep. 2017; 7: 5024.
26. Dominguez Gutierrez G, Kim J, Lee A-H, Tong J, Niu J, Gray SM, et al. Gene signature of the human pancreatic ε cell. Endocrinology. 2018; 159: 4023-4032.
27. Dominguez Gutierrez G, Xin Y, Okamoto H, Kim J, Lee A-H, Ni M, et al. Gene signature of

proliferating human pancreatic α Cells. Endocrinology. 2018; 159: 3177-3186.
28. Xin Y, Dominguez Gutierrez G, Okamoto H, Kim J, Lee A-H, Adler C, et al. Pseudotime ordering of single human β-cells reveals states of insulin production and unfolded protein response. Diabetes. 2018; 67: 1783-1794.
29. Tyler SR, Rotti PG, Sun X, Yi Y, Xie W, Winter MC, et al. PyMINEr finds gene and autocrine-paracrine networks from human islet scRNA-seq. Cell Rep. 2019; 26: 1951-1964.
30. Dorrell C, Schug J, Canaday PS, Russ HA, Tarlow BD, Grompe MT, et al. Human islets contain four distinct subtypes of β cells. Nat Commun. 2016; 7: 11756.
31. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M. Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (pfk-2/fbpase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes. 2004; 53: 1020-1029.
32. Tudzarova-Trajkovska S, Butler PC. (12) International application published under the Patent Cooperation Treaty (PCT) (19) World Intellectual Property Organization International Bureau (10) International publication number (43) International publication date. 2017.
33. Saunders DC, Brissova M, Phillips N, Shrestha S, Walker JT, Aramandla R, et al. Ectonucleoside triphosphate diphosphohydrolase-3 antibody targets adult human pancreatic β cells for in vitro and in vivo analysis. Cell Metab. 2019; 29: 745-754.
34. Pipeleers D. The biosociology of pancreatic B cells. Diabetologia. 1987; 30: 277-291.
35. Gutierrez GD, Gromada J, Sussel L. Heterogeneity of the pancreatic beta cell. Front Genet. 2017; 8: 22.
36. Lam CJ, Cox AR, Jacobson DR, Rankin MM, Kushner JA. Highly proliferative α-cell–related islet endocrine cells in human pancreata. Diabetes. 2018; 67: 674-686.
37. Zhang H, Ackermann AM, Gusarova GA, Lowe D, Feng X, Kopsombut UG, et al. The FoxM1 transcription factor is required to maintain pancreatic beta-cell mass. Mol Endocrinol. 2006; 20: 1853-1866.
38. Chen H, Gu X, Su I, Bottino R, Contreras JL, Tarakhovsky A, et al. Polycomb protein Ezh2 regulates pancreatic -cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev. 2009; 23: 975-985.
39. Butler AE, Dhawan S, Hoang J, Cory M, Zeng K, Fritsch H, et al. β-cell deficit in obese type 2 diabetes, a minor role of β-cell dedifferentiation and degranulation. J Clin Endocrinol Metab. 2016; 101: 523-532.
40. Yoon KH, Ko SH, Cho JH, Lee JM, Ahn YB, Song KH, et al. Selective β-cell loss and α-cell expansion in patients with type 2 diabetes mellitus in Korea. J Clin Endocrinol Metab. 2003; 88: 2300-2308.
41. Costes S, Gurlo T, Rivera JF, Butler PC. UCHL1 deficiency exacerbates human islet amyloid polypeptide toxicity in β-cells. Autophagy. 2014; 10: 1004-1014.
42. Brackeva B, De Punt V, Kramer G, Costa O, Verhaeghen K, Stangé G, et al. Potential of UCHL1 as biomarker for destruction of pancreatic beta cells. J Proteomics. 2015; 117: 156-167.
43. Piper K, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, et al. Beta cell differentiation during early human pancreas development. J Endocrinol. 2004; 181: 11-23.
44. Ramond C, Beydag-Tasöz BS, Azad A, van de Bunt M, Petersen MBK, Beer NL, et al. Understanding human fetal pancreas development using subpopulation sorting, RNA sequencing and single-cell profiling. Development. 2018; 145: dev165480.

45. Ramond C, Glaser N, Berthault C, Ameri J, Kirkegaard JS, Hansson M, et al. Reconstructing human pancreatic differentiation by mapping specific cell populations during development. Elife. 2017; 6: e27564.
46. Shih HP, Wang A, Sander M. Pancreas organogenesis: From lineage determination to morphogenesis. Annu Rev Cell Dev Biol. 2013; 29: 81-105.
47. Bouwens L. Islet morphogenesis and stem cell markers. Cell Biochem Biophys. 2004; 40: 81-88.
48. Arda HE, Li L, Tsai J, Torre EA, Rosli Y, Peiris H, et al. Age-dependent pancreatic gene regulation reveals mechanisms governing human β cell function. Cell Metab. 2016; 23: 909-920.
49. Paik DT, Tian L, Lee J, Sayed N, Chen IY, Rhee S, et al. Large-scale single-cell rna-seq reveals molecular signatures of heterogeneous populations of human induced pluripotent stem cell-derived endothelial cells. Circ Res. 2018; 123: 443-450.
50. Bardy C, van den Hurk M, Kakaradov B, Erwin JA, Jaeger BN, Hernandez R V, et al. Predicting the functional states of human iPSC-derived neurons with single-cell RNA-seq and electrophysiology. Mol Psychiatry. 2016; 21: 1573-1588.
51. Collin J, Queen R, Zerti D, Dorgau B, Hussain R, Coxhead J, et al. Deconstructing retinal organoids: single cell rna-seq reveals the cellular components of human pluripotent stem cell-derived retina. Stem Cells. 2019; 37: 593-598.
52. Camp JG, Badsha F, Florio M, Kanton S, Gerber T, Wilsch-Bräuninger M, et al. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. Proc Natl Acad Sci. 2015; 112: 201520760.
53. Wilson PC, Humphreys BD. Kidney and organoid single-cell transcriptomics: The end of the beginning. Pediatr Nephrol. 2019. doi: 10.1007/s00467-018-4177-y.
54. Veres A, Faust AL, Bushnell HL, Engquist EN, Kenty JH-R, Harb G, et al. Charting cellular identity during human in vitro β-cell differentiation. Nature. 2019; 569: 368-373.
55. Harb G, Poh Y-C, Pagliuca F. Stem cell-derived insulin-producing β cells to treat diabetes. Curr Transplant Reports. 2017; 4: 202-210.
56. Millman JR, Pagliuca FW. Autologous pluripotent stem cell-derived β-like cells for diabetes cellular therapy. Diabetes. 2017; 66: 1111-1120.
57. Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic β cells in vitro. Cell. 2014; 159: 428-439.
58. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol. 2014; 32: 1121-1133.
59. Balboa D, Saarimäki-Vire J, Borshagovski D, Survila M, Lindholm P, Galli E, et al. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. Elife. 2018; 7: e38519.
60. Saarimäki-Vire J, Balboa D, Russell MA, Saarikettu J, Kinnunen M, Keskitalo S, et al. An activating STAT3 mutation causes neonatal diabetes through premature induction of pancreatic differentiation. Cell Rep. 2017; 19: 281-294.
61. Krentz NAJ, Lee MYY, Xu EE, Sproul SLJ, Maslova A, Sasaki S, et al. Single-cell transcriptome profiling of mouse and hESC-derived pancreatic progenitors. Stem cell reports. 2018; 11: 1551-1564.

62. Han X, Chen H, Huang D, Chen H, Fei L, Cheng C, et al. Mapping human pluripotent stem cell differentiation pathways using high throughput single-cell RNA-sequencing. Genome Biol. 2018; 19: 47.
63. Yan L, Yang M, Guo H, Yang L, Wu J, Li R, et al. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol. 2013; 20: 1131-1139.
64. Chu L-F, Leng N, Zhang J, Hou Z, Mamott D, Vereide DT, et al. Single-cell RNA-seq reveals novel regulators of human embryonic stem cell differentiation to definitive endoderm. Genome Biol. 2016; 17: 173.
65. Lu J, Baccei A, Lummertz da Rocha E, Guillermier C, McManus S, Finney LA, et al. Single-cell RNA sequencing reveals metallothionein heterogeneity during hESC differentiation to definitive endoderm. Stem Cell Res. 2018; 28: 48-55.
66. Saxena P, Heng BC, Bai P, Folcher M, Zulewski H, Fussenegger M. A programmable synthetic lineage-control network that differentiates human IPSCs into glucose-sensitive insulin-secreting beta-like cells. Nat Commun. 2016; 7: 11247.
67. Velazco-Cruz L, Song J, Maxwell KG, Goedegebuure MM, Augsornworawat P, Hogrebe NJ, et al. Acquisition of dynamic function in human stem cell-derived β cells. Stem cell reports. 2019; 12: 351-365.
68. Nair GG, Liu JS, Russ HA, Tran S, Saxton MS, Chen R, et al. Recapitulating endocrine cell clustering in culture promotes maturation of human stem-cell-derived β cells. Nat Cell Biol. 2019; 21: 263-274.
69. Spijker HS, Ravelli RBG, Mommaas-Kienhuis AM, van Apeldoorn AA, Engelse MA, Zaldumbide A, et al. Conversion of mature human β-cells into glucagon-producing α-cells. Diabetes. 2013; 62: 2471-2480.
70. Russ HA, Bar Y, Ravassard P, Efrat S. In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes. 2008; 57: 1575-1583.
71. Guo R-J, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. Cancer Biol Ther. 2004; 3: 593-601.
72. Mamidi A, Prawiro C, Seymour PA, de Lichtenberg KH, Jackson A, Serup P, et al. Mechanosignalling via integrins directs fate decisions of pancreatic progenitors. Nature. 2018; 564: 114-118.
73. Kim H, Kim YG, Choi W, Moon JH, Hwang I, Kim K, et al. Generation of a highly efficient and tissue-specific tryptophan hydroxylase 1 knockout mouse model. Sci Rep. 2018; 8: 17642.
74. Ohta Y, Kosaka Y, Kishimoto N, Wang J, Smith SB, Honig G, et al. Convergence of the insulin and serotonin programs in the pancreatic -cell. Diabetes. 2011; 60: 3208-3216.
75. Ryu GR, Lee E, Kim JJ, Moon S-D, Ko S-H, Ahn Y-B, et al. Comparison of enteroendocrine cells and pancreatic β-cells using gene expression profiling and insulin gene methylation. PLoS One. 2018; 13: e0206401.
76. Beucher A, Gjernes E, Collin C, Courtney M, Meunier A, Collombat P, et al. The homeodomain-containing transcription factors Arx and Pax4 control enteroendocrine subtype specification in mice. PLoS One. 2012; 7: e36449.
77. Desai S, Loomis Z, Pugh-Bernard A, Schrunk J, Doyle MJ, Minic A, et al. Nkx2.2 regulates cell fate choice in the enteroendocrine cell lineages of the intestine. Dev Biol. 2008; 313: 58-66.
78. Petersen MBK, Azad A, Ingvorsen C, Hess K, Hansson M, Grapin-Botton A, et al. Single-Cell gene expression analysis of a human esc model of pancreatic endocrine development reveals

different paths to β-cell differentiation. Stem Cell Reports. 2017; 9: 1246-1261.
79. Agulnick AD, Ambruzs DM, Moorman MA, Bhoumik A, Cesario RM, Payne JK, et al. Insulin-producing endocrine cells differentiated in vitro from human embryonic stem cells function in macroencapsulation devices in vivo. Stem Cells Transl Med. 2015; 4: 1214-1222.
80. Kaido T, Yebra M, Cirulli V, Montgomery AM. Regulation of human β-cell adhesion, motility, and insulin secretion by collagen iv and its receptor $\alpha_1\beta_1$. J Biol Chem. 2004; 279: 53762-53769.
81. Kfir MolakandovNeta LAVONAvital BECKMichel RevelOfer ELHANANIYoav SoenMichael WalkerArik Hasson. US20180369290A1 - methods for differentiating and purifying pancreatic endocrine cells - Google Patents.