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REVIEW

Human pluripotent stem cell-derived β cells: Truly immature islet β cells for type 1 diabetes therapy?

Helen Jiang, Fang-Xu Jiang

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Abstract

A century has passed since the Nobel Prize winning discovery of insulin, which still remains the mainstay treatment for type 1 diabetes mellitus (T1DM) to this day. True to the words of its discoverer Sir Frederick Banting, "insulin is not a cure for diabetes, it is a treatment", millions of people with T1DM are dependent on daily insulin medications for life. Clinical donor islet transplantation has proven that T1DM is curable, however due to profound shortages of donor islets, it is not a mainstream treatment option for T1DM. Human pluripotent stem cell derived insulin-secreting cells, pervasively known as stem cell-derived β cells (SC-β cells), are a promising alternative source and have the potential to become a T1DM treatment through cell replacement therapy. Here we briefly review how islet β cells develop and mature *in vivo* and several types of reported SC-β cells produced using different *ex vivo* protocols in the last decade. Although some markers of maturation were expressed and glucose stimulated insulin secretion was shown, the SC-β cells have not been directly compared to their *in vivo* counterparts, generally have limited glucose response, and are not yet fully matured. Due to the presence of extra-pancreatic insulin-expressing cells, and ethical and technological issues, further clarification of the true nature of these SC-β cells is required.

Key Words: Human pluripotent stem cells; Stem cell-derived β cells; Islet β cells; Type 1 diabetes mellitus; Cell replacement therapy

Core Tip: Diabetes mellitus (DM) is a metabolic condition of absolute or relative deficiency in insulin. Since the discovery of insulin 100 years ago, there has been slow progress in the treatment of type 1 DM (T1DM) in clinical practice. In the scientific community however, there is much interest and progression in the research of human pluripotent stem cell derived insulin producing β-like cells, pervasively known as stem cell-derived β cells (SC-β cells). If they are determined to be genuine, scalable and functionally matured, SC-β cells have the potential to cure T1DM through cell replacement therapy.

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INTRODUCTION

In this coronavirus disease 2019 pandemic era, there is a silent growing epidemic of significant public health burden with tremendous social and economic costs. This growing epidemic is not an infectious disease, but a chronic non-communicating metabolic disease - it is the epidemic of diabetes mellitus (DM). There was an estimated 537 million adults with DM globally in 2021[1], with the prevalence increasing each year due to the rising incidence of type 2 DM (T2DM) worldwide[2]. DM is a metabolic disorder characterised by a disruption in glucose homeostasis leading to hyperglycaemia, and broadly consists of 2 main types: T1DM and T2DM. T1DM is the absolute deficiency of insulin due to the autoimmune destruction of insulin-secreting β cells in the islets of Langerhans of the pancreas, and is usually first diagnosed in children and young adults. T2DM is the relative deficiency of insulin function due to insulin resistance in peripheral tissues, and sometimes with reduced insulin secretion due to dysfunctional or dedifferentiated β cells, usually occurring in adults.

Hyperglycaemia in T1DM and T2DM can cause various microvascular complications such as diabetic retinopathy and blindness, nephropathy and kidney failure requiring dialysis, as well as peripheral neuropathy and infected foot ulcers that lead to amputations. It can also cause macrovascular complications such as peripheral artery disease, coronary artery disease and stroke. These complications lead to significant morbidity and mortality, as well as substantial associated health and social costs[3,4].

INSULIN IS NOT A CURE FOR DIABETES

These were Sir Frederick Banting's words to the world during his Nobel Lecture for his 1923 Nobel Prize winning discovery of insulin. Subsequent discoveries on primary insulin sequences and radioimmune assay for insulin and other peptide hormones were also awarded the Nobel Prizes (Figure 1)[5]. A century later, unfortunately there is still no cure for DM, and life-long insulin replacement remains the mainstay of treatment for T1DM and controlling high blood sugar levels with antihyperglycaemic agents in most T2DM individuals. The continuous blood glucose monitoring and insulin pump known as artificial pancreas or bionic pancreas still presents the risk of developing complications, though reduced, because this and other current treatments cannot achieve physiological glucose homeostasis in patients[6,7]. These treatments themselves are also not without risks. Insulin as well as some oral anti-hyperglycaemics, such as sulfonylureas and glinides, are associated with the risk of hypoglycaemia which can lead to seizures, coma and even death[8,9]. Thus, there is a critical need for more effective and curative treatments to reduce the global burden of this disease.

The landmark proof-of-concept has demonstrated over the last 2 decades that clinical transplantation of donated human islets are able to restore β-cell function and achieve insulin independence immediately with improvement in glycaemic control and avoid the risk of hypoglycaemia episodes[10-13]. However, a large amount of approximately 340-750 million islet cells are required for successful transplantation in a patient of 68 kg weight[11,14]. Thus, the widespread application of donor islet transplantation is severely limited by the insufficient supply of human organ donor pancreases[15,16]. In addition to supply issues, another challenge to this treatment option is the prevention of transplant rejection, immune destruction and cell death of the transplanted islet cells[17]. To address the donor shortage issue, alternative scalable insulin-secreting tissues must be identified and developed. Due to their ability for theoretically infinite self-renewal and differentiation into all cell types in the body, human pluripotent stem cells (hPSCs) hold great promise for generating surrogate insulin-secreting cells *ex vivo*, pervasively known as stem cell-derived β cells (SC-β cells)[18-20] or SC-islets[21] in the literature. In order to help understand the true nature of these SC-β cells, we briefly introduce how islet β cells develop *in vivo*.

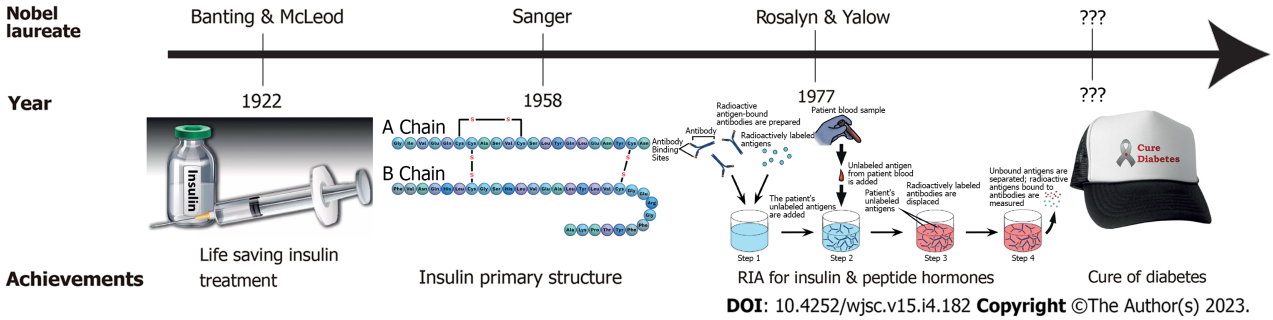

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Figure 1 Nobel prizes awarded for the endeavour towards curing diabetes. Insulin is not a cure for diabetes. Three Nobel prizes have been awarded in this endeavour[5].

IN VIVO DEVELOPMENT OF ISLET BETA CELLS

The pancreas is derived from the embryonic endoderm, one of the three germ layers, which is formed during gastrulation of embryogenesis. In addition to the pancreas, the definitive endoderm also gives rise to the liver, lung, thymus and other organs of the respiratory and digestive tracts[22]. The endoderm located in the foregut region gives rise to the dorsal and ventral buds of the pancreas which rotate to form one organ, then pancreatic epithelium is induced and expands, from which endocrine progenitors arise. The endocrine progenitors then differentiate into the β cells that secrete the hormone insulin, α cells that secrete the hormone glucagon, δ cells that secrete the hormone somatostatin, ε cells that secrete the hormone ghrelin, and PP cells that secrete the hormone pancreatic polypeptide. The pancreatic endocrine cells start to organize into clusters forming islets before birth, and the Islets of Langerhans become fully formed at around 2-3 wk after birth[22]. Human islets are made up of 40%-60% β cells and 30% α cells[22]. The adult pancreas is made up of exocrine cells that secrete digestive tract enzymes, duct cells that make up the ductal tree to transport digestive enzymes and islet cells that secrete hormones into the bloodstream for glucose homeostasis[23].

Mechanistically, the pancreatic islets are initiated by the transient expression of a high level of the transcription factor neurogenin-3 (NGN3)[24]. NGN3 is important in committing all pancreatic endocrine cell types, the deficiency of which leads to the absence of pancreatic endocrine cells[25,26]. The molecular mechanisms for the development of each pancreatic endocrine cells are not completely defined, however it is suggested that insulin-producing β cells are differentiated from the pancreatic progenitors that express transcription factor genes pancreatic and duodenal homeobox 1 (PDX1) and NK6 homeobox 1 (NKX6-1), and then turn on NGN3[27,28]. There are several β cell transcription factors, including PDX1, NKX6-1 and MAF BZIP transcription factor A (MAFA), which play a critical role in activating insulin transcription and regulating insulin secretion[29-31].

PDX1 is a homeodomain transcription factor homogenously expressed in the early pancreatic bud and its expression persists into mature β cells; the absence of PDX1 leads to agenesis of the pancreas [32]. NKX6-1 and the helix-loop-helix transcription factor Beta2/NeuroD determine islet cell differentiation during embryogenesis, and maintain specific islet cell hormone expression in adults[32]. Knockout of mouse *Nkx6-1* gene leads to a significant inhibition in the formation of β cells[33]. NeuroD is initially expressed in pancreatic epithelium during development, before being expressed in $NGN3^+$ endocrine progenitors, and finally exclusively expressed in β cells after birth. The absence of Beta2/NeuroD leads to reduced mouse endocrine cells, in particular β cells, increased apoptosis and arrestment in islet morphology[25,34]. Beta2/NeuroD is also a critical transcriptional activator of the insulin gene[35,36].

β-cell maturation including maturation of other clinically important cell types is a postnatal development process. For example maturation of mouse and human β cells takes place approximately 3 wk[37] and 26-44 wk after birth[38,39] respectively. The maturation process is controlled by transcription factors and exhibited in maturing at the gene, protein, subcellular, intercellular and metabolic levels.

ISLET BETA CELL MATURATION REGULATED BY TRANSCRIPTION FACTORS

Following β-cell specific NeuroD deletion, the mice developed glucose intolerance and the islets displayed features of foetal/neonatal β cells such as overexpression of glycolytic genes, lactate dehydrogenase (LDHA), Neuropeptide Y, and higher basal insulin secretion and oxygen consumption due to the reliance on oxidative metabolism of glucose[40-46]. That is, the glucose metabolic profile of mouse β cells without NeuroD was equivalent to immature β cells. The mutations of NeuroD cause maturity onset diabetes of the young[22,33]. NeuroD is also critical for maintaining a matured functional state of

islet β cells[40]. These data suggest that NeuroD regulates islet β-cell maturation, though its postnatal dynamic expression profile is not available. Thus, identification of Beta2/NeuroD activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

MafA is another transcription factor being demonstrated to regulate the maturation of islet cell organisation, β cell mass and β cell function from 3 wk of age in mice using the gene targeting strategy [47]. MafA expression reaches their adult levels at 3 mo in rats[48] coinciding with the obtaining of mature glucose stimulated insulin secretion (GSIS). Aguayo-Mazzucato *et al*[49] were the first to demonstrate that MAFA overexpression and the thyroid hormone triiodothyronine (T3) treatment are able to increase human foetal islet-like clusters, insulin secretion at 16.8 mmol/L glucose and proinsulin-to-insulin processing. Chromatin immunoprecipitation experiment showed binding of thyroid receptors to MafA promoter, thereby confirming that T3 directly regulates the expression of MafA[50]. The thyroid hormone receptor is also demonstrated to be expressed on human mature islets [51], though its postnatal development profile is unknown.

Furthermore, a recent study shows that the expression of the orphan nuclear transcription factor estrogen-related receptor gamma (ERRγ) is a hallmark of mature β cells[52]. ERRs consist of three paralogs in mammals, namely ERRα (NR3B1 or Esrrα), ERRβ (NR3B2 or Essrβ) and ERRγ (NR3B3 or Essrγ). ERRγ is progressively upregulated in mouse islets from 2 to 6 wk of age (5-fold higher in adults compared to neonatal β cells) and ERRγ transcriptional network promotes mitochondrial oxidative metabolism in mouse β cells, required for functional maturation of β cells and glucose homeostasis[52]. Mice with β cell-specific ERRγ deletion failed to develop a mature GSIS. With the developmentally deleted β cell-specific ERRγ knockout mouse islets, RNA sequencing (RNA-seq) revealed that the expression of 4189 genes were altered, with almost equal numbers of genes down- and up-regulated (2008 and 2182 genes respectively). Gene ontology analysis revealed that ERRγ-regulated genes are associated with processes critical for β cell function including ATP biosynthesis, cation transport, oxidative phosphorylation, electron transport and secretion[52]. However, data is not available on postnatal developmental expression of ERRs in human islet cells, which will have to be addressed in the near future. Identification of ERRγ activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

Expression of the *Sine Oculis* family of homeodomain transcription factors SIX2 and SIX3 increased with age in the human pancreatic islet β cells[53]. SIX2 and SIX3 are localised to the nucleus of adult human β cells but not detected in juvenile (under 9 years of age) β cells[53]. Using gain-of-function experiments in human β cell line, the EndoC-bH1 cells or primary juvenile human islets, evidence has demonstrated that expression of SIX2 or SIX3 were sufficient to enhance cardinal functions of human β cells[53]. Identification of SIX2 and SIX3 activators may therefore help generate matured hPSC-derived insulin-secreting cells *ex vivo*.

METABOLIC MATURATION OF ISLET BETA CELLS

Although rat islets acquired GSIS by postnatal day 21, a mature GSIS was only achieved by 3 mo[54], coinciding with the time when insulin dynamics reaches their adult levels[48]. The metabolic maturation is underscored by genes of important metabolic players in β cells such as glucose transporter 2, glucokinase, glucagon-like peptide-1 receptor and prehormone convertase 1 (encoded by *Pcsk1*) that were expressed from very low levels at postnatal day 2 to higher levels with increased age[48]. Similarly, the metabolic maturation is also underscored by genes transcribing for malate dehydrogenase, glycerol-3-phosphate dehydrogenase, glutamate oxaloacetate transaminase, pyruvate carboxylase and carnitine palmitoyl transferase 2 from much lower levels at neonatal postnatal day 2 to high levels at day 28[55]. In the same period, genes encoding proliferation regulators in β cell genes encoding platelet-derived growth factor receptor A, platelet-derived growth factor receptor B, platelet-derived growth factor B and fibroblast growth factor (FGF) receptor 1 are progressively downregulated. Mature β cells tend to have lower levels of LDHA and glycolytic genes, as it is downregulated by NeuroD from embryonic to adult islets, which then appears to possess the ability to correspond glucose metabolism with insulin secretion[56-58].

The molecular mechanism of mature β-cell secretion is well understood. The higher blood glucose levels stimulate its active transportation into the β cell cytoplasm, increasing the ATP/ADP ratio through glycolysis and oxidative metabolism and triggering the depolarisation of the β cell membrane and opening the voltage-gated Ca^{2+} channel. The AMP-activated protein kinase (AMPK) is a highly conserved sensor of intracellular adenosine nucleotide levels that is activated even with modest decreases in ATP production resulting in relative increases in AMP or ADP. In response, AMPK promotes catabolic pathways to generate more ATP, and inhibits anabolic pathways. The increase in cytoplasmic Ca^{2+} triggers the fusion of insulin granules with the plasma membrane for exocytosis of insulin contents. Coordinating with other islet cells, mature β cells produce insulin in adequate amounts and timeliness to maintain plasma glucose within a narrow concentration range[39,59]. Thus, β cell function is critical for mature GSIS. Destruction and failure of islet β cells will lead to T1DM and T2DM, respectively (Figure 2).

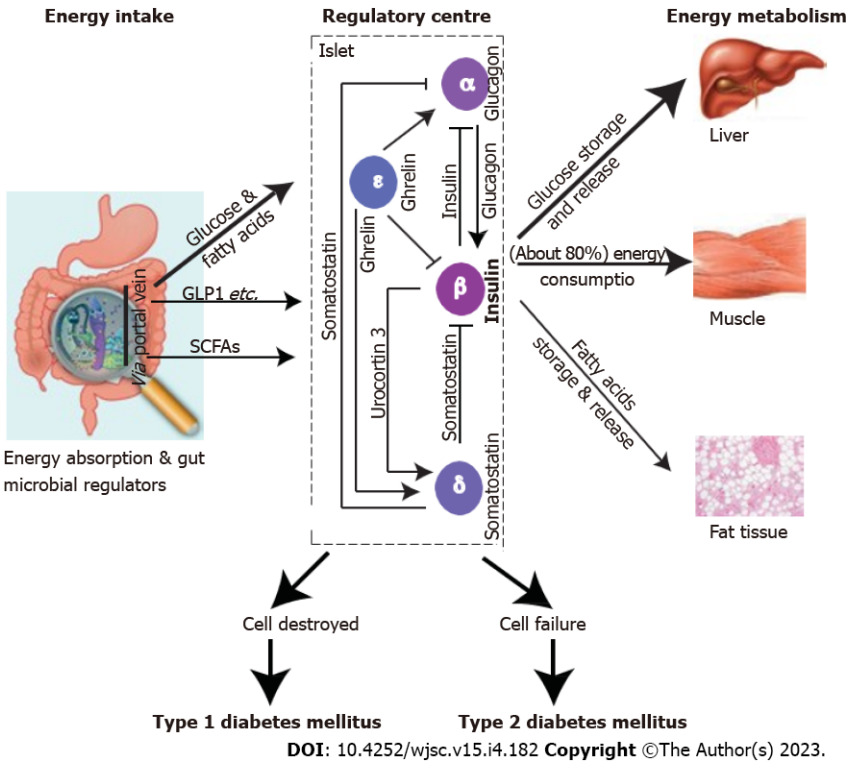

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Figure 2 Mature islets are a regulatory centre for glucose homeostasis. A simplified graphic representation of how mature pancreatic islets regulates glucose homeostasis. Skeletal muscle is the largest organ in the body (45%-55% body mass) and consumes about 80% insulin. GLP1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids.

MATURATION MARKERS OF ISLET BETA CELLS

To help with the characterization of whether hPSC-derived insulin-secreting cells *ex vivo* are matured, we briefly summarize maturation markers for *in vivo* islet β cells. Over the last decade, several potential markers for maturation of immature islet β cells were discovered. Blum *et al*[60] were the first to demonstrate that functional islet β cell maturation is marked by expression of the corticotropin-releasing factor family peptide urocortin 3 (UCN3), along with an increased glucose threshold.

Mature rat β cells expressed significantly higher levels of the gap junction connexion 36 gene (*Cx36*, also known as *Gjd2*) compared to neonatal immature counterparts, corresponding to a significantly higher membrane density of gap junctions and greater intercellular exchange of ethidium bromide[61]. Human mature islets predominantly express CX36 at mRNA and protein levels with β cell membrane harboring detectable levels of CX36 gap junction proteins[62]. Though the developmental profile of human islet CX36 is unknown, we speculate that the dynamic pattern of CX36 expression from human neonatal to mature β cells is similar to that in rats and CX36 is a potential maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

Our group recently showed that claudin 4 is the only tight junction molecule family member highly upregulated in the postnatal mouse islets and global deletion of this gene affects mature GSIS in a sex difference manner[63]. Thus, claudin 4 may also be a maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

THE DIFFERENTIATION OF SC-BETA CELLS *EX VIVO*

The advent of hPSC provided an important opportunity to overcome major challenges of clinical islet transplantation therapy through its accessibility, theoretically unlimited self-renewability and the boundless potential to generate an alternative source of donor insulin-secreting cells *ex vivo*[64,65]. The generated insulin-secreting cells can also be used for disease modelling and pharmaceutical drug testing to help establish therapeutics that improve cell function, survival and proliferation. Insulin-secreting cells differentiated from hPSCs that include human embryonic stem cells (hESC) and induced hPSCs (ihPSC) are ubiquitously termed as SC-β cells[18-20] in the literature. hESCs are generated from the inner cell mass of human blastocysts and have the infinite ability to proliferate as undifferentiated cells or differentiate into cells of all ectoderm, mesoderm or endoderm lineages[66,67].

Over recent years, there have been various protocols developed of *ex vivo* differentiation of SC-β cells [18-20] and SC-islets[18,21]. Thus far, hPSCs have been differentiated towards SC-β cells through a step-wise manner emulating *in vivo* pancreatic embryonic development[68-72]. The differentiation of hPSCs towards SC-β cells have been achieved with the application of growth factors, proteins or molecules to modulate signaling pathways to progress through each stage of pancreatic development, and is usually measured by expression of a couple of key transcription factors or C-peptide[22]. hPSCs (characterised by expression of Oct4) are first differentiated into definite endoderm cells expressing FOXA2 and SOX17 through application of a mix containing Wnt, activin A, inducer of definite endoderm, wortmannin, and sodium butyrate[22]. Then application of FGF10 and FGF7 differentiates the definite endoderm into gut tube endoderm expressing HNF1B and HNF4A[22]. The differentiation mixture containing retinoic acid, noggin KAAD-cyclopamine, FGF, and indolactum V leads to differentiation into pancreatic progenitors expressing PDX1 and HNF6, which further differentiates into endocrine progenitors (NKX6-1, NGN3, NKX2-2, PTF1A), and finally into β-cells (characterised by presence of C-peptide and insulin)[22]. We here summarize several representative protocols used to generate SC-β cells and SC-islets (Table 1).

Pagliuca *et al*[73]'s differentiation protocol was the first using specific and cocktail of inducing factors to differentiate hPSCs sequentially through 6 stages into SC-β cells (Table 1). At stages 5 and 6, there is however significant heterogeneity in the final population containing SC-β and SC-α cells, as well as SC-endocrine cells (resembling enterochromaffin cells) and non-endocrine cells (*e.g.*, exocrine cells such as pancreatic acinar, mesenchymal and ductal cells)[18]. Nevertheless, these cells appear to be stable, maintaining their identity as evidenced by their global transcriptional profiles during stage 6 cultures. At this stage, they also express the maturation marker SIX2 but several other β cell markers of maturity are not expressed such as UCN3, MAFA and SIX3[18].

The Velazco-Cruz *et al*[19]'s protocol was built upon and modified Pagliuca *et al*[73]'s protocol, and demonstrated that the SC-β cells had improved insulin secretion and greater gene expression of β cell markers compared to the cells generated with Pagliuca *et al*[73]'s protocol, but still much less than the average human islet (Table 1). Follow-up studies with the addition of differentiation factors or changes to the differentiation processes were unfortunately unsuccessful in producing more functional SC-β cells equal to human islet β cells[74-76].

In Balboa *et al*[20]'s protocol, the SC-islets had similar cytoarchitecture and functional insulin secretion pattern to islet β cells, though with immature glucose-induced mitochondrial respiration and instead retained pyruvate sensitivity - thus the SC-islets were not completely similar to functional adult islets (Table 1). Balboa *et al*[20]'s SC-β cells showed heterogeneous mature β cell marker expression, required further maturation *in vivo* after transplantation, showed upregulated expression of CHGB and MAFA after 6 mo, and did not express adult β cell factors RBP4 and SIX3[20].

Nevertheless, studies indicate that several current pancreatic progenitor differentiation protocols promote precocious endocrine commitment; ultimately resulting in the generation of non-functional polyhormonal cells[74]. The efficiency of differentiation decreases with each step, and at the final step there are very small amounts of SC-β cells that have a low insulin content, co-express insulin and glucagon, and usually respond poorly to glucose stimulation[22,70]. It was also found that these SC-β cells have little to no expression of maturation genes including MAFA and G6PC2[18,73,77]. Following transplantation, the amount of insulin secreted by SC-β cells rises[73,77,78] and the previously low or non-expressed genes of islet β cells such as MAFA, G6PC2, MNX1 and INS increases[79].

Cell purification steps will increase the safety of, and ability to upscale the manufacture of β cells. However, there are difficulties in including this step in large-scale manufacturing processes for production of reproducible PSC-derived cellular products with less variability in composition and function[80]. Several cell surface markers have been used to purify different developmental stages of PSC-derived cells[80]. Markers used include CD177 for anterior definitive endoderm cells[81], CD142, CD24 and glycoprotein 2 for pancreatic progenitors[82-85], CD49a for SC-β cells[18], and CD9 for negative selection of SC-β cells[86]. Monoclonal antibody against extracellular domain of claudin 4 might help enrich matured SC-β cells differentiated *ex vivo*.

Finally, a few maturation factors have proven useful in maturing SC-β cells *ex vivo*. For example, T3 enhanced the MAFA expression in the SC-β cells, and increased insulin content and insulin secretion at 16.8 mmol/L glucose[49]. Using an adenoviral ERRγ vector, overexpression of ERRγ increased glucose-stimulated C-peptide secretion in hPSC-derived insulin-secreting cells, thus may promote their functional maturation[52]. Therefore, identification of molecules that activate NEUROD, ERRγ, SIX2 and SIX3 will be important. Application of the activators individually or in combination may indeed promote functional maturation of genuine SC-β cells.

CURRENT *EX VIVO* SYSTEMS ARE DISTINCT FROM *IN VIVO* ISLET DEVELOPMENT NICHES

The current PSC differentiation protocols for insulin-secreting cells are mostly bulk cultures and consist of cocktails of inducing factors, which are generally based on accumulative knowledge generated from using the animal model systems. In these bulk cultures, there are cells types in the targeted lineage as

Table 1 A summary of several differentiation protocols for generating stem cell-derived β cells *ex vivo*

Differentiation protocol	Marker expression	Characterization	Ref.
The stem cell-derived β cell protocol is a 6-stage differentiation protocol using specific inducing factors for each stage (11 factors) to produce SC-islets from hPSCs, in a 3D suspension-based cell culture system (4-5 wk)	Pancreas progenitor marker: PDX1 (about 90% at stage 3). Endocrine markers: C-peptide, CHGA and NKX6-1. SC-β cell markers: INS, NKX6-1, ISL1, and others	Immunofluorescence. Ultrastructure. Insulin packaging into secretory granules. qRT-PCR. Static and dynamic GSIS. Functional test. Glucose-responsive Ca^{2+} flux, KCl depolarisation, ameliorate hyperglycaemia in diabetic mice. scRNA-seq. Purified SC-β cells with anti-CD49a	Pagliuca *et al*[73], 2014; Veres *et al* [18], 2019
Optimised 7 stage *in vitro* differentiation protocol of serial culture steps using factors such as vitamin C, ALK5 inhibitor, TGF-β receptor inhibitors, thyroid hormone (T3), R428 (AXL inhibitor), N-acetyl cysteine, Notch and BMP signalling inhibitors	Pancreas progenitor marker: PDX1 (about 90% at stage 3), NKX6-1. Endocrine markers: C-peptide, CHGA, NEUROD1, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, ISL1, MAFB. Mature β cell markers: MAFA, ABCC8, IAPP, HOPX, NEFM, SIX2, G6PC2	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Functional test in diabetic mice. Flow cytometry. Immunohistochemistry. Diabetes reversal within 40 d in mice. Metabolic analysis. Perifusion assay. Calcium imaging.	Rezania *et al*[78], 2014
Scalable 3D suspension culture system based on previous methods [101,102], with the addition of retinoic acid, cyclopamine (SHH inhibitor), Noggin (BMP inhibitor), then treatment with epidermal growth factor, KGF and Noggin (EKN). Followed by exposure to a cocktail of factors *e.g.*, TBP, ALK inhibitor, Noggin, TANK	Pancreas progenitor marker: PDX1 and NKX6-1 (90%). Endocrine markers: C-peptide, NEUROG3, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, NKX2-2, PDX1. Mature β cell markers: MAFA, MAFB, PDX1, NKX6.1, NXK2.2, ISL1, PAX6, NEUROD1, and CHGA	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Insulin biosynthesis and glucose metabolism, blood glucose reduction but not reversal of diabetes in mice. Flow cytometry. Western blot analysis. Statistical analysis	Russ *et al*[74], 2015
Six-stage differentiation strategy modulating TGF-β signaling by modulating Alk5i exposure, combined with controlling cell cluster size and use of enriched serum-free media culture	Pancreas progenitor marker: PDX1. Endocrine markers: CHGA (96%), C-peptide (73%), NKX6-1. SC-β cell markers: INS, CHGA, NKX2-2, PDX1, NKX6-1, MAFB, GCK, and GLUT1	Immunofluorescence. qRT-PCR. Static and dynamic GSIS. Flow cytometry. Light microscopy. Glucose responsive, first- and second-phase insulin release, improved glucose tolerance in mice. Western blot. Perifusion assay. Glucose tolerance test	Velazco-Cruz *et al* [19], 2019
Optimised differentiation protocol combining previous protocols. Changes made: Differentiation of hPSCs in adherent conditions until pancreatic progenitor stage. Then optimised with nicotinamide, epidermal growth factor, activin A and a ROCK inhibitor; a microwell aggregation step; and a final maturation step in suspension culture	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide. SC-β cell markers: INS. Mature β cell markers: INS, G6PC2, SIX2, GLIS3, RBP4, SIX3. HOPX, UCN3, IAPP, CPE and FXYD2 upregulated post engraftment. CHGB and MAFA upregulated 6 mo post-engraftment. B cell differentiation: SIX2, HOPX, ZBTB20. Insulin secretion genes: PCSK1, CPE, CHGB, ABCC8, FXYD2, GABRA2	Immunohistochemistry. Flow cytometry. Perifusion assay. Respirometry. Transmission electron microscopy. Electrophysiology. Exocytosis imaging. $[Ca^{2+}]_i$ imaging. $[cAMP]_m$ imaging. Metabolite tracing analysis. Ratiometric analysis. Transplantation study. scRNA-seq transcriptomic profiling. Glucose responsive biphasic insulin secretion. Glucose tolerance test	Balboa *et al*[20], 2022
Differentiation protocol using hCiPSC-islets by optimising pancreatic progenitor to β cells fate commitment by modulating signaling pathways and reconstructing islet spatial structure through 3D cell aggregates of posterior foregut-committed cells and combination of ISX9 and Wnt-C59 at stage 5	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide, CHGA and NKX6-1. β cell markers: PDX1, NKX6.1 and NKX2.2. Mature β cell markers: MAFA, UCN3	Immunofluorescence. qRT-PCR. Transmission electron microscopy. Static and dynamic GSIS. Glucose-stimulated calcium flux assay. Flow cytometry. scRNAseq. Glucose responsive biphasic insulin secretion, decrease HbA1c, restore endogenous C-peptide secretion. Glucose tolerance tests. Preclinical diabetic non-human primate transplantation study. Fasting blood glucose levels. Glycated HbA1c. scRNA-seq. Teratoma assay. Karyotype analysis. Calcium imaging. Cryo-electron microscopy. ELISA	Du *et al*[21], 2022

PDX1: Pancreatic and duodenal homeobox 1; SC-islets: Stem cell-derived islets; hPSC: Human pluripotent stem cells; C-peptide: Connecting peptide; CHGA: Chromogranin A; NKX6-1: NK6 homeobox 1; qRT-PCR: Quantitative real time-polymerase chain reaction; GSIS: Glucose-stimulated insulin secretion; SC-β cell: Stem cell-derived β cell; INS: Insulin gene; ISL1: ISL LIM homeobox 1; Ca^{2+}: Calcium; KCl: Potassium chloride; scRNA-seq: Single-cell RNA sequencing; anti-CD49a: CD49a (Integrin alpha 1) antibody; TGF-β receptor inhibitor/ALK5i: Transforming growth factor β-receptor I/activin receptor-like kinase 5 inhibitor; R428 (AXL inhibitor): A selective small-molecule inhibitor of AXL (bemcentinib, BGB324); BMP: Bone morphogenetic protein; NEUROD1: Neurogenic differentiation 1; NKX2-2: NK2 homeobox 2; MAFA: MAF BZIP transcription factor A; MAFB: MAF BZIP transcription factor B; ABCC8: ATP Binding Cassette Subfamily C Member 8; IAPP: Islet amyloid polypeptide; HOPX: HOP homeobox; NEFM: Neurofilament medium chain; SIX2: SIX homeobox 2; G6PC2: Glucose-6-phosphatase catalytic subunit 2; KGF: (FGF7) Keratinocyte growth factor; TBP: TATA-binding protein; TANK: TRAF family member associated NFKB activator; PAX6: Paired box 6; GCK: Glucokinase; GLUT1: Glucose transporter 1; ROCK inhibitor: Rho-kinase inhibitor; GLIS3: GLI-similar 3/zinc finger 3; RBP4: Retinol binding protein 4; SIX3: SIX homeobox 3; UCN3: Urocortin 3; CPE: Carboxypeptidase E; FXYD2: FXYD domain containing ion transport regulator 2; cAMP: Cyclic adenosine monophosphate; ZBTB20: Zinc finger and BTB domain containing 20; PCSK1: Proprotein convertase subtilisin/kexin type 1; GABRA2: Gamma-aminobutyric acid type A receptor subunit alpha-2; hCiPSC: Human chemically induced pluripotent stem cells; ISX9: Isoxazole-9; Wnt-C59: Nanomolar inhibitor of mammalian PORCN acyltransferase activity and blocks activation of all evaluated human Wnts; HbA1c: Hemoglobin A1c; ELISA: Enzyme-linked immunosorbent assay.

well as unwanted lineages. One or two *in vivo* biomarkers are selected based on *in vivo* islet lineage development to characterise targeted cells at different differentiated stages *ex vivo*. However, these *in vivo* biomarkers should not be extrapolated as biomarkers for the *ex vivo* differentiation conditions because of clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation (Figure 3). In other words, we do not yet fully understand the full regulatory program, or the molecular details of the 3D microenvironment niche for specific islet lineage development *in vivo* to guide the specific differentiation of hPSCs into insulin-secreting cells *ex vivo*.

EXTRAPANCREAS INSULIN-SECRETING CELLS

Perhaps the research community have also forgotten the fact that in our body, extra-pancreas insulin-secreting cells exist, which may complicate the efforts of generating genuine SC-β cells. Subverted to general knowledge, approximately a quarter of human foetal enteroendocrine K/L cells were recently shown to express high levels of insulin and other β cell genes including the transcription factor PDX1, by using samples of foetal and neonatal human small intestines derived from the endoderm during development[87]. Notably, the expression of UCN3 in the human foetal enteroendocrine K/L cells was higher than in foetal human pancreatic β cells[87]. These results were confirmed with single molecule fluorescence *in-situ* hybridisation of insulin mRNA combined with immunofluorescent antibody staining of the insulin protein[87]. Secondly, thymocytes that are derived from the foregut, adjacent to which gives rise to the pancreas, normally produce insulin to induce self-tolerance and protect the body from the autoimmune destruction of pancreatic insulin-secreting β cells[88]. Lastly, though the central nervous system is an ectoderm-derived organ, the neuronal progenitors derived from adult hippocampus and the olfactory bulb were demonstrated to undergo insulin biosynthesis[89]. Human *INS* mRNA expression is also detected in the hippocampus, amygdala and temporal lobe in addition to the olfactory bulb, cerebellar and pontine regions[90]. A historical account of the extrapancreas insulin-secreting cells is referred to in a recent review article[88]. These data suggest that it is possible that the current reported SC-β cells contain a varied percentage of non-pancreatic insulin-secreting cells. Future studies are required to increase the percentage of genuine insulin-secreting β-like cells in the *ex vivo* systems.

SC-BETA CELLS TRANSPLANTED INTO NON-HUMAN PRIMATES

To further test their functions, the chemically induced SC-islets were recently intraportally transplanted into immunosuppressed adult diabetic rhesus macaques[21]. Three months after the SC-islet transplantation, all four macaques reportedly had improvements in diabetic symptoms, glycaemic control, fasting blood sugar levels, hemoglobin A1c (HbA1c), and reduced exogenous insulin requirements[21]. However, after 5-6 mo, two of the macaques developed graft failure (the other two macaques died of immunosuppression-related complications)[21]. Autopsy conducted on the macaques found no evidence of teratoma or tumorigenesis, but levels of β cells had fallen. The authors concluded that the immunosuppression regimen used was not appropriate in preventing immune attack against the grafts[21]. Whether the short-term improvements in diabetic rhesus macaques are related to the immaturity of grafted SC-islets and/or the presence of non-pancreatic insulin-secreting cells needs to be determined in the future.

SC-BETA CELLS IN CLINICAL TRIALS AS A T1DM THERAPY

The first hPSC-derived, differentiated cell replacement T1DM therapy product named VX-880 was approved by the United States Food and Drug Administration for phase 1/2 clinical trials in March 2021. The VX-880 are SC-islets for T1DM patients with certain indications; that is, impaired hypoglycaemic awareness and severe hypoglycaemia[91]. The preliminary outcomes of the clinical trials were presented in June 2022 at the American Diabetes Association 82nd Scientific Sessions by Vertex, a United States Pharmaceutics company[91]. A half-dose of VX-880 in two patients was able to achieve glucose-responsive insulin secretion, significantly improve time-in-range (the amount of time that blood glucose level is measured to be within target blood sugar range 70-180 mg/dL or 3.9-10 mmol/L), reduce exogenous insulin requirements and improved HbA1c[91]. VX-880 was also well tolerated although with some largely mild or moderate adverse reactions[91]. For example, patient 1 showed blood glucose time-in-range change from 40.1% on 34.0 units per day of exogenous insulin at baseline to 99.9% and insulin independence at day 270 onwards. Patient 2 showed blood glucose time-in-range change from 35.9% on 25.9 units per day of exogenous insulin at baseline to 51.9% with a 30% reduction in exogenous insulin use at day 150[91]. Whereas these results are very promising, VX-880 requires a

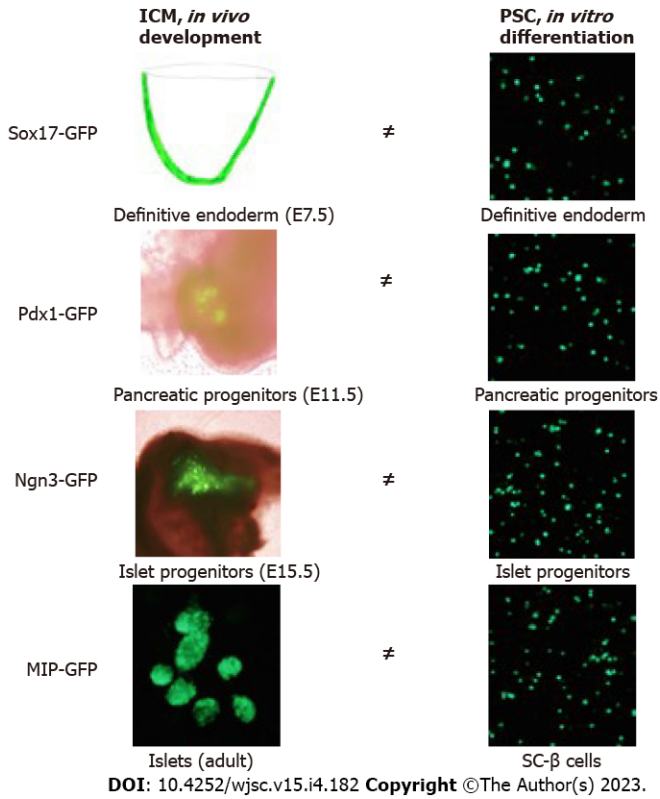

Figure 3 There are clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation of islet lineages. Details of lineage tracing mouse lines can be found in articles[103,104]. ICM: Inner cell mass; PSC: Pluripotent stem cell; Sox17: SRY-box transcription factor 17; GFP: Green fluorescent protein; PDX1: Pancreatic and duodenal homeobox 1, also known as insulin promoter factor 1; NGN3: Neurogenin-3; MIP: Mouse insulin 1 promoter; SC-β cells: Stem cell-derived β cells.

lengthy *in vivo* maturation period for blood sugar control (in patient 1) in contrast to donated islets retrieved from deceased persons, which achieved immediate insulin independence after transplantation into recipients[10-13]. The lengthy *in vivo* maturation period of grafted VX880 is a strong independent indicator that these SC-β cells are immature. It is also premature to claim the VX-880 SC-β cells are all genuine immature counterparts of islet β cells, as the duration and longevity of insulin independence was not yet available at the time of writing this article.

CONCLUSION

Immaturity of PSC-derived cells is a general obstacle, not only in the case of SC-β cells and SC-islets, but also other clinically important cell types[92]. Maturation biology is the final frontier in stem cell biology, of which our knowledge is still in its infancy. As summarised in Table 1, multiple hPSC differentiation protocols have been used in different laboratories. Consequently, off-target differentiation and aberrant differentiation from these protocols are more likely unavoidable, resulting in only a low frequency of genuine SC-β cells. Furthermore, the stage-specific differentiation factors selected may direct non-specific spatiotemporal differentiation, thus resulting in multiple cell types of the endodermal germ layer and even neuronal lineage origins. This may result in some differentiating cells along unwanted pathways and give rise to extrapancreas insulin-secreting cells. On the other hand, unwanted or off-target differentiated cellular products have accumulated in the bulk culture protocols and not been excluded for subsequent differentiation steps, which further increases the possibility of compromising the characterization through use of one or two developmental markers of *in vivo* cellular lineages. Finally, in addition to the above, there are still other challenges in this exciting field of research, such as ensuring SC-β cell survival post-transplantation given the highly vascularised islets are susceptible to ischaemic injury and loss of cell mass[93,94]. Developing methods that evade autoimmune attack in T1DM patients without the use of lifelong immunosuppression would be valuable[95].

Stage-specific *in vivo* pancreatic and islet lineage cell types would provide ideal positive controls for the *ex vivo* hPSC-derived insulin-secreting cells. Nevertheless, the human ethics issues and lack of human embryonic and foetal pancreatic tissues available prevent such reliable and precise comparison to be made between the islet lineage cells and the PSC-derived cells. However, future efforts should be made to resolve these issues. Similarly, it would be wise not to solely concentrate on undertaking

human β cell differentiation and maturation studies from hPSCs. Instead, investigating β cell differentiation and maturation from model animals will be invaluable and will facilitate the realisation of a curative stem cell therapy for people with T1DM.

In order to minimise confusion between the *ex vivo* differentiated insulin-secreting cells and islet β cells, our laboratory proposed a 4-criterial post-genomic concept for naming "β cells" a few years ago [96]. Recently, Kaestner *et al*[97] described many islet biologists/scientists much like the "Parable of the Blind Men and the Elephant" in terming "β cells". This appears to be the case in respect to claims made about SC-β cells without proper positive controls of corresponding *in vivo* islet lineage cells. Kaestner *et al*[97] further proposed six salient features of normal, fully functional mature β cells and made a recommendation to not name PSC-derived insulin-producing cells as "β cells", but conservatively as insulin-producing cells, insulin$^+$ cells or β-like cells, when there is no clear evidence that the six features of *in vivo* β cells are met.

The degree of single-cell RNA-seq (scRNA-seq) data similarity between the SC-β cells and donated islet β cells remains largely unclear. First, all scRNA-seq datasets of SC-β cells lacked a direct positive control from *in vivo* pancreatic and islet lineage cells. Second, most current scRNA-seq methods provide a high throughput but sacrifice full transcript coverage and sensitivity[98]. Third, as barcodes/inducers are introduced by the template switching of reverse transcriptase, strand invasion becomes problematic through systematic bias, namely biases from the introduction of artefacts. Fourth, loss of cDNA synthesis and bias in cDNA amplification leads to severe quantitative errors of these scRNA-seq methods[99]. Fifth, the current scRNA-seq methods suffer from impaired mRNA accounting. However, molecular spikes have significantly improved single cell mRNA accounting[100], adoption of the molecular spike method and further improvements may help address the above issues. As such, genuine SC-β cells will eventually become available as donor cells for establishing curative therapies for people suffering from T1DM in the not too distant future.

FOOTNOTES

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