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REVIEW

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A perfect islet: reviewing recent protocol developments and proposing strategies for stem cell derived functional pancreatic islets

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Abstract

The search for an effective cell replacement therapy for diabetes has driven the development of "perfect" pancreatic islets from human pluripotent stem cells (hPSCs). These hPSC-derived pancreatic islet-like β cells can overcome the limitations for disease modelling, drug development and transplantation therapies in diabetes. Nevertheless, challenges remain in generating fully functional and mature β cells from hPSCs. This review underscores the significant efforts made by researchers to optimize various differentiation protocols aimed at enhancing the efficiency and quality of hPSC-derived pancreatic islets and proposes methods for their improvement. By emulating the natural developmental processes of pancreatic embryogenesis, specific growth factors, signaling molecules and culture conditions are employed to guide hPSCs towards the formation of mature β cells capable of secreting insulin in response to glucose. However, the efficiency of these protocols varies greatly among different human embryonic stem cell (hESC) and induced pluripotent stem cell (hiPSC) lines. This variability poses a particular challenge for generating patient-specific β cells. Despite recent advancements, the ultimate goal remains to develop a highly efficient directed differentiation protocol that is applicable across all genetic backgrounds of hPSCs. Although progress has been made, further research is required to optimize the protocols and characterization methods that could ensure the safety and efficacy of hPSC-derived pancreatic islets before they can be utilized in clinical settings.

Keywords Pancreatic islet, Stem cells, Human induced pluripotency, Differentiation protocols, Mature B cells

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Introduction

Diabetes mellitus (DM) is a highly common chronic metabolic disorder, primarily characterized by hyperglycemia, affecting millions of the population worldwide with an exponential projected growth from 463 million in 2019 to 700 million in 2045 [1]. Type 1 DM (T1D) and Type 2 DM (T2D) are the most prevalent forms of diabetes observed in clinical practice [1]. In T1D, pancreatic islet β cells undergo autoimmune destruction leading to a lack of insulin production. For that reason, optimal treatment of TID is vital, and lack of proper management can result in severe complications. In the case of T2D, there is insufficient insulin production due to β cell loss through apoptosis, together with impaired insulin sensitivity in peripheral tissues resulting in chronic secondary complications [2]. The current treatment strategies for DM include the continuous routine administration of exogenous insulin which is the only economical mode of treatment available for T1D, and also a common mainstay treatment for T2D alongside pharmaceutical agents and bariatric surgery [2]. Pancreatic islet transplantation, a standout treatment option particularly used in the management of T1D, has been shown to have clinical benefits, providing sustained glucose-sensitive insulin production together with a significantly reduced need for exogenous insulin [3]. However, given the scarcity of obtaining cadaveric donor islets and the life-long dependence on immunosuppressive medications, islet transplantation is viewed as an impractical option for clinical implementation. Therefore, an unmet need exists for effective alternative therapies that offer long-term relapse-free disease remission with minimum adverse effects [4].

Emerging reports highlight the use of stem cell-derived pancreatic β cells (SC-β cells) and islets (SC-islets) as a promising alternative to overcome the shortage of donor islets [5, 6]. Over the past few decades, many investigators have focused on generating protocols that delineate the differentiation of functional pancreatic β cells from a range of hPSCs. Upon cell clustering into islet-like structures and transplantation for further maturation in vivo, these cells have proven to be a potential therapeutic option for diabetes despite significant ethical and technical obstacles whilst offering an unlimited source of insulin-secreting β cells for cell replacement therapies and drug discovery. From this standpoint, this review discusses the overview of developments in islet differentiation protocols and characterization methods for enhancing and tracking the functionality of hPSC-derived pancreatic islet-like β cells and also delineates the present challenges whilst proposing strategies for their improvement.

The healthy pancreas

Embryology and development of human pancreas

Over the past several decades, the understanding of crucial steps in the embryonic development of the mouse pancreas has significantly improved whereas information about human pancreas development remains limited due to the lack of access to human fetal pancreatic tissue (>22 week's gestation) [7]. Here, we summarized what is currently known about human pancreas organogenesis and the key signaling events involved. The human pancreas is a composite glandular organ mostly comprised of exocrine cells (99%) and the endocrine portion forms the remaining 1%. These endocrine cells are arranged as discrete "Islets of Langerhans" which are composed of β cell (54%) followed by alpha (α) (35%), delta (11%), a low percentage of pancreatic polypeptide cells, and very few epsilon cells [2]. The hormonal secretions, particularly insulin from β cells and glucagon from α cells, facilitates glucose homeostasis in the body.

Embryologically, early human pancreas specification begins from the foregut-midgut boundary, which forms from the anterior endoderm invagination (anterial intestinal portal) during Carnegie Stage 10 (CS10). During early CS13, the first signs of budding of the dorsal pancreas from the foregut endoderm begins [8]. The dorsal bud further forms the pancreatic tail, neck and a portion of the pancreatic head whilst the ventral bud goes on to form the uncinate process and part of the pancreatic head [9]. The process of epithelial branching and polarity has not been elucidated fully during human pancreas development. In week 7, the two pancreatic buds orientate themselves for fusion by rotating 90 degrees clockwise. The ventral bud is present caudally while the dorsal bud is more cranial, in line with pancreatic organ structure. Errors in this morphogenic process can result in two well-documented malformations: pancreas divisum and annular pancreas [6].

For the rest of the embryonic period, the human pancreas undergoes a large expansion of proliferative progenitor cells. The difference in pancreatic progenitor cells becomes noticeable from CS19. The potential of human pancreatic progenitors from the end of embryogenesis/early fetal period was well demonstrated by Scharfmann and colleagues. They transplanted the whole human embryonic pancreas under the kidney capsule of adult SCID mice and observed the development of pancreas with all cell lineages (both endocrine and exocrine cells) [10]. Furthermore, they used this murine in vivo incubation model to generate a successfully functional human β cell line [10, 11].

Major regulators of human pancreas development and islet differentiation

PDX1

Also known as insulin promoter factor 1, PDX1 is a homeodomain transcription factor that orchestrates multiple steps, namely proliferation, growth and differentiation of pancreas [12]. Expression of PDX1 in the primordial endoderm is stimulated by Fibroblast growth factor 2 (FGF2) and Activin β2 secreted by the notochord [13]. PDX1 plays a critical role in the primary transition, as it is required for initiating the formation of both the ventral and dorsal pancreatic primordia and branching of the pancreatic buds [12]. Mutation of the PDX1 gene potentially leads to multiple pancreatic dysfunctions and failure of proper pancreatic development. Homozygous mutation of the PDX1 gene causes pancreatic agenesis and a heterozygous mutation leads to MODY4 (maturity-onset diabetes of the young 4) [14]. During the secondary transition, PDX1 is required for the subsequent differentiation of both the endocrine and exocrine lineages. In mature β cells, depletion and/or reduction of PDX1 induces glucose intolerance, which suggests the critical role of PDX1 in maintaining β cell function, as PDX1 acts as a transcription factor for genes that code for proteins required for β cell function such as INS and GLUT2 [15]. This highlights the significance of PDX1 as the cornerstone of pancreatic organogenesis and the regulator of β cell maturation and identity.

NEUROG3/NGN3

The basic helix-loop-helix transcription factor neurogenin3 (ngn3) initiates the endocrine program during mouse pancreatic organogenesis by activating the expression of downstream transcription factors that are responsible for maturation, and lineage specification of endocrine precursor [16]. These factors include NK6 Homeobox 1 (Nkx6.1) [17], Neurogenic differentiation 1 (NeuroD1) [18] and Paired Box 4 (Pax4) [19]. In contrast to mouse studies, all reported patients with biallelic mutations in NEUROG3 have functional endocrine cells despite suffering from enteric anendocrinosis due to a lack of intestinal enteroendocrine cells [20]. However, in a study conducted using human embryonic stem cells, complete knockout of the NEUROG3 gene completely blocked the endocrine differentiation pathway whereas a 75–90% knockdown of the NEUROG3 gene caused a reduction, but not a complete loss, of endocrine cells [21]. Interestingly, a study using the CUT&RUN technique to map NEUROG3 occupancy in hiPSC-derived pancreatic endocrine progenitors revealed the interaction of NEUROG3 with many important pancreatic islet transcription factor (TF) genes and its possible transcriptional regulation of islet cell differentiation. Additionally, the study also showed the interaction of NEUROG3 with multiple genes that are associated with several key steps of the insulin secretion pathway and revealed the increased overlapping of NEUROG3 binding regions with a series of T2D-associated single nucleotide polymorphisms (SNPs) [22].

NEUROD1

The transcription factor NeuroD1, binds and activate promoters via conserved DNA sequence elements called E-boxes. NeuroD1 is one of the downstream transcription factor of Neurog3 responsible for implementing the endocrine differentiation program in both human and mice [23]. NeuroD1 plays a vital role in maintaining the mature phenotype of pancreatic β cells and studies revealed that disruption of NeuroD1 during pancreatic development causes neonatal diabetes [24]. Also, mutations in the NEUROD1 gene in humans result in the development of Maturity-onset diabetes of the young-type 6 (MODY6) [25]. Studies on rat reported that NeuroD1 forms a heterodimer with the bHLH protein E47 that regulates the transcription of the insulin and glucagon genes [26, 27]. The NeuroD1/E47 ratio in the heterodimer pool dictates whether the insulin or glucagon gene is expressed [26]. In addition to the roles NeuroD1 plays in the development of β cells, it also plays a key role in the maintenance of insulin secretion in β cells. Several mechanisms have been proposed to explain how NeuroD1 maintains insulin secretion, including the transcriptional activation of mouse sulfonylurea receptor-1 (Sur1) via associating with NeuroD1 in a tissue specific manner [28]. Another mechanism might be the repression of somatostatin expression which is an inhibitor of insulin production and secretion [29]. A recent study emphasized that deficiency of NeuroD1 significantly altered the H3K27me3 histone modification pattern in the promoter regions of differentially expressed genes, that further resulted in overall deregulation of endocrine cell differentiation and their functional properties [24]. Overall, the regulatory network of NeuroD1 plays a crucial role in pancreatic endocrine lineage commitment and differentiation.

NKX6.1

The homeobox protein NKX6.1 is a transcription factor that plays an indispensable role during early and late stages of pancreatic development. The role of NKX6.1 in pancreas development is two-fold. Firstly, it plays an indirect role in directing the multipotent pancreatic progenitor cells towards an endocrine fate through a cross-antagonism mechanism with Ptf1a, the main transcription factor involved in determining exocrine fate [30, 31]. Induction of NKX6.1 in multipotent pancreatic progenitor cells reduces the expression of Ptf1a and therefore blocks the exocrine differentiation pathway

[31]. Secondly, NKX6.1 expression in multipotent pancreatic progenitor cells is crucial for the commitment of these cells to form functional glucose responsive monohormonal β cells [32]. Notably, induction of NKX6.1 after the multipotent pancreatic cell stage results in the formation of non-functional polyhormonal β cells illustrating the importance of NKX6.1 expression during the multipotent pancreatic cell stage [32]. In order to understand the plasticity of human pancreatic islet cells, such as the ability to dedifferentiate and transdifferentiate, Fujita et al. conducted a study to evaluate the proportion of cells that are NKX6.1 positive and ARX negative. They demonstrated that the expression ratios of transcription factors NKX6.1 (NKX6.1+/GCG+) and ARX (arista less-related homeobox) (ARX−/GCG+) in glucagon-secreting α cells closely correlated with the reduction of β cell volume in human pancreas in 34 patients. This study further signifies the role of NKX6.1 in the trans-differentiation of α cells to insulin-releasing β cells [33].

NKX2.2

NKX2.2, a member of the NK2 class of homeodomain transcription factors, is a conserved master regulatory transcription factor for the differentiation of pancreatic islet cells and for the functional maintenance of monohormonal β cells in both human and mice islets [34]. As organogenesis proceeds, NKX2.2 expression becomes progressively restricted to β cells, plus a subset of α and pancreatic polypeptide-secreting cells [35]. Molecular analysis has revealed that both NKX2.2 and NeuroD1 are critical regulators involved in β cell formation and its functional maturation. NKX2.2 co-ordinately activates NeuroD1 with Ngn3 in endocrine progenitor cells and also maintains the expression of NeuroD1. In mice, knocking out Nkx2.2 gene expression completely obliterates insulin-producing β cells and reduces the number of glucagon-producing α cells and pancreatic polypeptide (PP)-secreting cells with an increased generation of ghrelin-secreting epsilon cells [36]. In a homozygosity analysis of transcription factor mutations in patients with neonatal diabetes, patients with homozygous NKX2.2 mutations had severe diabetes without any defects in pancreatic exocrine function [37]. Furthermore, in a recent case report, NKX2.2 mutation resulted in a syndrome of diabetes with undetectable c-peptide, indicating the absence of insulin-producing β cells and a paradoxical increase in ghrelin followed by an oral glucose tolerance test. Hence, suggesting NKX2.2 plays a similar role in both human and murine pancreas development [38].

PAX4

The Paired Box (PAX) genes encode a family of highly conserved transcription factors that play vital roles in regulating embryonic cell patterning, proliferation and differentiation [39]. A transient expression of PAX4 was observed in pancreatic tissues and was found to be colocalized with other endocrine markers, namely Ngn3, Nkx2.2, Islet1 and Pax6. Pax4 also associates with insulin and glucagon further signifying its role in the formation of β and α cells [40]. The expression of Pax4 in pancreatic progenitor cells was determined following the expression of the endocrine fate transcription factor Neurog3 in mouse embryos. As an early precursor of pancreatic endocrine cells, Neurog3 is responsible for the expression of major transcription factors, such as Pax4 and Arx, that direct differentiation of endocrine progenitor cell fate, respectively [41]. Furthermore, studies have shown that forced expression of PAX4 in α cells during embryogenesis can induce reprogramming of α cells to β cells through a mechanism possibly involving ARX gene suppression, suggesting that embryonic α cells might be used as a cell pool to bolster the number of β cells [42]. Additionally, the interaction of Pax4 with Nkx2.2 is essential to initiate the differentiation of pancreatic β cells and a loss of Pax4 significantly downregulates the expression of Pdx1, the homeobox gene HB9 and INS in β cell precursors [40].

MAFA

Musculoaponeurotic fibrosarcoma (MAF) bZIP transcription factor A (MAFA), is a large basic leucine zipper (bZIP) transcription factor (TF), first identified as trans-acting factor binding to the RIPE3b/C1 element of the insulin promoter [43]. MAFA has an essential role in the differentiation and glucose responsiveness of adult β cells. Several studies suggest that the expression of MAFA is time sensitive and a premature expression in pancreatic endocrine cells inhibits the expression of hormone-releasing cells [44]. During pancreatic development, MAFA expression is visible only after the specification of INS^{+} β cells that are expressing another Maf factor, MAFB. After birth, these insulin positive cells exhibit MAFA expression but stop MAFB expression and gain glucose responsiveness. The ectopic expression of Mafa, Pdx1 and Neurod1 (or Ngn3) has the potential to convert adult liver or pancreatic acinar cells into mature functional β cells [45, 46]. Urocortin3 (UCN3) is another marker associated with the acquisition of functional maturity in β cells though are nevertheless not a necessary marker for β cell maturation. This discovery significantly improved hPSC-derived pancreatic islet β cell protocols by overcoming the major challenge associated with production of immature β-cells lacking appropriate glucose-stimulated insulin secretion (GSIS).

RFX-6

The regulatory factor X-box binding transcription factor (RFX) is a winged helix transcription factor that targets the X-box, a conserved cis-regulatory element [47]. Of the eight RFX family members, RFX-6 is specifically expressed in the pancreas of both mice and humans, where it contributes to embryonic pancreatic development and endocrine cell differentiation. A homozygous mutation in the RFX-6 gene causes Mitchell-Riley syndrome (MRS), marked by severe neonatal hyperglycemia, a hypoplastic pancreas and intestinal atresia. In contrast, a heterozygous mutation in this gene is associated with a milder form of maturity-onset diabetes of the young (MODY) [48]. Beyond its developmental role, recent research has identified RFX-6 in adult human beta cells, where it influences insulin gene expression and secretion [47]. Interestingly, during the differentiation of induced pluripotent stem cells (iPSCs) into pancreatic progenitor cells, deletion of RFX-6 does not impact the expression of PDX-1 and NKX-6.1 but significantly reduces the expression of other critical genes for endocrine development, such as PAX6, NEUROD1, ARX, MAFB and CHGA [48]. RFX-6 knock out cells show persistent expression of SOX9 and NEUROG3, leading to increased apoptosis and alterations in endocrine lineage specification [49].

MAFB

MAF BZIP Transcription Factor B (MAFB), part of the large Maf transcription factor family, is expressed in both developing and mature human β-cells [50]. In mice, however, MafB is found in both α- and β-cells during embryonic and neonatal stages but later becomes restricted to islet α-cells. MafB appears in the pancreatic epithelium as early as embryonic day 10.5, preceding the expression of MafA [51]. Studies indicate MafB's role in α- and β-cell differentiation, shown by reduced numbers of these cells in MafB-deficient mice [52]. Notably, MafB supports adult β-cell function under pathological conditions in mice and is re-expressed in insulin-secreting cells if MafA is absent [53]. Research on MAFB knockout in human pluripotent stem cells (hPSCs) further underscores its importance in β-cell development and function. Although MAFB knockout cells progress normally through the progenitor stage, endocrine cell development is significantly affected, with an increase in somatostatin- and pancreatic polypeptide-producing cells and a reduction in insulin- and glucagon-producing cells [54]. Unlike other transcription factors involved in β-cell development, MAFB has not been associated with diabetes susceptibility, as its significant role is primarily in the later stages of hormone-producing cell development within the islets.

PAX-6

The paired and homeodomain containing transcription factor is a transcription factor featuring two DNA-binding domains—a paired box and a homeobox—along with a proline-serine-threonine (PST)-rich transactivation domain at its C-terminus [55, 56]. In the developing mouse pancreas, Pax6 appears as early as embryonic day 9.0 in both dorsal and ventral pancreatic buds and is later confined to endocrine cells throughout development and adulthood. Targeted disruption of Pax-6 in mice is lethal and in these mice the pancreas lacks α-cells and shows a significant reduction in β-, δ-, and γ-cells [55, 57]. Furthermore, conditional knock out of Pax6 in the mouse model results in a diabetic phenotype, underscoring the critical function of Pax6 in controlling glucose homeostasis and endocrine cell function [57]. Pax6 plays a critical role in insulin production and overall β-cell activity by directly binding to and activating the promoters of the genes for insulin, Pdx1, MafA and PC1/3 [58, 59]. Moreover, PAX6 regulates GSIS in β-cells by modulating both proximal and distal signaling pathways, underscoring PAX6's role in the β-cell dysfunction seen inT2D [60].

GLIS3

GLI similar protein 3 (GLIS3) is a member of the GLIS subfamily of Krüppel-like zinc finger transcription factors [61]. In mice, it is expressed at the early notochord stage, with mRNA detected at embryonic day 11.5 (e11.5) and protein expression beginning at e13.5 [61, 62]. Further, Glis3 expression is maintained in bipotent progenitor cells, which differentiate into either endocrine progenitors or the ductal lineage; in coordination with Sox9, Glis3 directly regulates Ngn3 expression to facilitate the differentiation of these progenitors into the proendocrine lineage [63, 64]. As endocrine lineage determination progresses, GLIS3 expression becomes restricted to β-cells and pancreatic polypeptide (PP) cells, highlighting its critical role in β-cell development and function. In β-cells, GLIS3 can serve as an activator or repressor of various genes involved in insulin secretion, including Nkx6.1, MafA, Ins1/2 and ChgA [62, 65], and plays a significant role in regulating beta cell mass maintenance [66]. In humans, mutations in GLIS3 are associated with neonatal diabetes and genome wide association studies (GWAS) have linked GLIS3 to Type 1 and Type 2 diabetes [67, 68]. Notably, GLIS3 knockdown studies in adult β-cells, cultured human islets, and hESC-derived β-cells show a correlation between GLIS3 and apoptosis [66, 69].

Insight into the major signalling events associated with pancreatic islet formation

TGF-β signaling

The transforming growth factor β (TGF-β) superfamily of growth and differentiation factors in mammals is encoded by 33 genes which consist of three main TGF-β isoforms (TGF-β 1, 2 and 3), activin, nodal, bone morphogenic proteins (BMPs) and various growth and differentiation factors [70]. Signal transmission of the TGF- β superfamily occurs via binding of a TGF-β superfamily ligands to type I and type II transmembrane serine/threonine kinase receptors [70]. Upon binding of a ligand to these receptors, type II receptors recruit and phosphorylate type I receptors which, in turn, activates downstream Smads which are responsible for controlling the expression of genes regulated by the TGF-β superfamily [71]. During the primary transition, the TGF-β ligand, activin, secreted by the notochord, acts on the endoderm to increase the expression of the pancreatic fate determinator, PDX1, thereby committing the pre-pancreatic endoderm to a pancreatic fate [72]. In vitro 3D collagen gels mediated culture of pancreatic rudiments from mouse embryos and their exposure to activin/TGF-β1 significantly influenced the development of endocrine cells, particularly β cells and PP cells [73]. During the secondary transition, TGF-β signaling is thought to inhibit the endocrine differentiation pathway [74]. It plays a similar role in human pancreas development as it does in human pluripotent cells, as the inhibition of the TGF-β signaling pathway by adding the small molecule ALK4/5/7 inhibitor SB431542 (SB) immediately following PDX1 induction resulted in the promotion of endocrine differentiation [75]. Nodal, a member of the TGFβ family is likely to have an indistinguishable difference with Activin mediated signaling for the specification of the primary body axis and the development of endoderm [76]. They both act through the same downstream signaling effectors except with the special requirement of a co-receptor, EGF-CFC (small cysteine-rich extracellular proteins), for the Nodal activity [77]. Furthermore, the EGF-CFC co-receptors are blocked by soluble inhibitors of the Lefty subclass of TGFβ factors through a negative feedback mechanism for this pathway [78].

Bone morphogenic proteins (BMPs), the largest class of signaling molecules belonging to the TGF-β superfamily, have multiple contrasting, stage dependent roles in pancreatic development. During the primary transition, BMP4 produced by the lateral mesodermal plate promotes reprogramming of endodermal cells for commitment to a hepatic rather than a pancreatic cell fate [79]. Inhibition of BMP4 is required for the commitment of endodermal cells to a pancreatic fate [79]. However, BMP 4 expression in pancreatic progenitor cells promotes their expansion via increased expression of the Inhibitor of DNA binding 2 (Id2) transcription factor [80]. Also, the addition of BMP4 to the proliferation media during the derivation of pancreatic progenitors from hESCs significantly promotes the early derivation of $PDX1^{+}NKX6.1^{+}$ pancreatic progenitors and thereby contributes to the development of an efficient pancreatic differentiation protocol [81]. During the secondary transition, BMP4 inhibits endocrine differentiation via inactivation of the transcription factor NeuroD1 [80]. Intriguingly, that the inhibition of TGF-β signaling by BMP7 induces adult human pancreatic exocrine cell differentiation toward an endocrine insulin-producing β cell fate is evidence of crosstalk between 2 superfamily ligands and represents a safer and easy alternative to genetic reprogramming [82].

Hedgehog signaling

Hedgehog signaling molecules are secreted proteins that elicit concentration dependent responses via binding to their target receptors [83]. Intriguingly, Shh the well investigated hedgehog gene expression is excluded from pancreatic epithelium throughout embryogenesis, suggesting that its function is inhibitive for pancreas development. The notochord-derived signals inhibit the expression of Shh in the dorsal endoderm region thereby creating a sharp molecular boundary between the stomach/duodenum epithelium and pancreatic tissue which further permits pancreas development [72]. The elevated levels of Shh impair the pancreatic islet formation by significantly reducing the expression of PDX1 and INS and this was rescued after co-culturing with notochord [84]. In addition, the role of FGF signaling in regulating the Hedgehog pathway during pancreas formation has also been reported. At low concentration, FGF2 inhibits the expression of Shh in pancreatic endoderm and upregulates Shh at higher concentration [85].

Retinoic acid (RA)signaling

RA, a metabolite of vitamin A, plays an essential role in the generation of pancreatic progenitor cells that form the dorsal bud of the pancreas [86]. Caudal type homeobox 4 (Cdx4), a protein expressed in the posterior dorsal endoderm, blocks the action of RA; this limits the induction of pancreatic fate by RA to the dorsal portion of the pancreas [86]. Targeted deletion of the retinaldehyde dehydrogenase 2 (Raldh2) gene, which encodes the enzyme required to synthesize RA in mouse embryo, resulted in failure to develop a dorsal pancreatic bud and pancreatic agenesis [87]. During the later stage of pancreatic development, it negatively regulates the differentiation of pancreatic progenitors and the differentiation of endocrine cells [88]. Based on these studies, researchers have utilized exogenous RA stimulation in a dose-dependent manner (high to low) for initial pancreas specification in hPSC to β cell differentiation protocols.

WNT/β-catenin signaling

The canonical Wingless-related integration site (WNT)/β-catenin signaling pathway controls a myriad of developmental processes [89]. During the primary transition, activation of the WNT/β-catenin signaling pathway in the endoderm blocks the differentiation of endodermal cells into pancreatic progenitor cells by downregulating the expression of PDX1 [90]. Later, during the secondary transition, activation of the WNT/β-catenin pathway blocks endocrine differentiation, possibly by a mechanism involving the suppression of NGN3 [91]. In contrast, activation of the WNT5A/JNK pathway during the endocrine progenitor stage was reported to be both a necessary and sufficient signal for inducing β cell differentiation [92]. Fugino et al. reported that murine pancreatic islets incubated with both Wnt3a and Wnt5a conditioned media demonstrated markedly improved GSIS that was abolished by genetic ablation of the low-density lipoprotein receptor-related protein 5 (LRP5) coreceptor [93]. Aly et al. incubated human islets with a conditioned medium from L-cells that constitutively express Wnt3a, R-spondin-3 and Noggin supplemented with RhoA/Rock inhibitors and found a 20-fold increase in the number of Ki67-expressing β-cells whilst maintaining the β-cell phenotype and insulin secretion [94].

Notch signaling

Notch signaling plays a critical role during the various developmental stages of human pancreas [95, 96]. During lateral inhibition, a Ngn3 positive cell that has committed to an endocrine fate expresses the Notch ligand Delta on its surface. Notch ligand Delta activates the Notch receptor on neighbouring cells and finally suppresses the expression of the pro-endocrine transcription factor Ngn3 and results in the inhibition of endocrine fate determination [96]. By contrast, studies have revealed that inhibition of the Notch signaling pathway causes premature differentiation of the multipotent progenitor cells (MPCs) into endocrine cells and the inactivated Notch pathway promotes acinar cell differentiation [97, 98]. Also, studies have demonstrated that Notch signaling functions as a negative regulator of the pro-endocrine factor Ngn3, and the inhibition of the Notch pathway significantly enhances the induction of Ngn3 and further upregulates the formation of INS $^{+}$ β cells [98, 99].

Insulin signaling

The binding and activation of Insulin (INS) to the INSRs (Insulin receptors) activates various signaling pathways, mainly metabolic or mitogenic pathways. The PI3K (phosphatidylinositol 3-kinase) and Cbl/Cbl-associated protein (CAP) pathways mostly mediate the metabolic effects of insulin, while the MAPK (mitogen-activated protein kinase) pathway is involved in mitogenesis and cell growth [100]. During the early developmental stages of the pancreas, insulin signaling acts as a negative feedback signal in regulating pancreatic progenitor cell differentiation. Interestingly, there is evidence that supports INS signaling (PI3K/Akt) and Notch signaling cross talk in pancreatic progenitor cells, especially due to the strong phenotypic similarity between the loss of these signaling pathways [101, 102]. It is evident that insulin signaling in pancreatic progenitor cells maintains a positive circuit between PI3K/Akt and Notch signaling that inhibits endocrine cell differentiation and prevents the premature differentiation of islet β cells.

Taken together, all these findings suggest that pancreatic organogenesis and the formation of islet cells are enhanced by these mentioned key transcription factors and signaling mechanisms, either independently or collaboratively, to achieve the regulatory functions at different time-points. A schematic overview of the summarized key signaling events modulating the efficient differentiation of hPSC derived mature pancreatic islet cells in conjunction with natural pancreatic developmental process is shown in Figure 1. Understanding the crucial pancreatic developmental cues and the expression profiles of these key lineage-specific transcription factors provides a basis for in vitro β cell differentiation from pluripotent stem cells. Multiple protocols have been developed over the past several decades and the successful generation of mono hormonal insulin producing β cells was achieved after the publication of the Rezania et al., 2014 paper highlighting the reversal of diabetes with insulin producing cells derived in vitro from hPSCs [103]. Building on this significant breakthrough, we have classified the protocols into 'Before 2014' and 'After 2014' sections. An overview of all major published hPSC derived pancreatic islet like β cell protocols, organized by specific stages and their publication years, is presented here and in Figure 2.

Comparison of published hPSC derived pancreatic B like cell differentiation protocols

Stage 1: definitive endoderm

The induction of definitive endoderm is the first stage of SC-β cell differentiation. As consistently demonstrated by majority of the protocols, the use of a TGFβ superfamily member to activate nodal signaling and a GSK3 inhibitor to activate canonical WNT/β-catenin pathway are the crucial steps for the effective induction of definitive endoderm [104–109]. This involves a combination of activin A, an analog to the molecule Nodal, to trigger the TGF-β pathway and CHIR99021, a GSK3β inhibitor [5, 104]. WNT3a has been used along with activin A to activate the canonical Wnt signaling [106]. Growth differentiation factor 8 (GDF8), another TGFβ superfamily member, has also alternatively been used to induce the

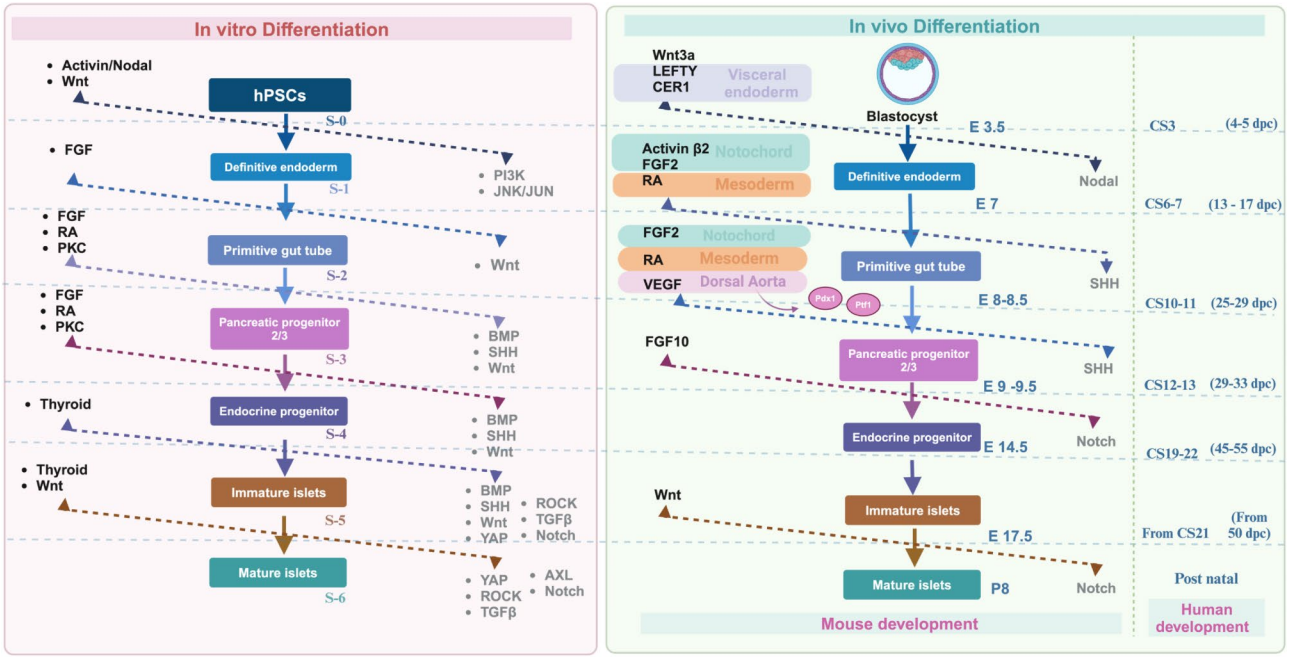

Fig. 1 Schematic outline of key signaling events regulating (upregulating and downregulating) the efficient differentiation of hPSC derived mature pancreatic islet cells (Left). The reported signaling pathways that are upregulated and downregulated during in vivo mouse pancreatic development are shown along with the corresponding time scale for human pancreas development (Right). Readers are requested to refer to DEVELOPMENTAL DYNAMICS 240:530–565, 2011 and Nature Reviews Drug Discovery volume 20:920–940, 2021 for a detailed diagrammatic model of mouse pancreatic organogenesis Activin/Nodal are growth factors belongs to TGFβ superfamily; LEFTY, Right-left determination factor; CER1, Cerberus; Wnt, Wingless; TGFβ, Transforming Growth Factor β; FGF, Fibroblast Growth Factor; PI3K, Phosphoinositide 3-kinase; JNK/JUN, Jun N-terminal kinase; RA, Retinoic Acid; PKC, Protein kinase C; BMP, bone morphogenic protein; SHH, sonic hedgehog; YAP, yes-associated protein 1; ROCK, Rho-associated protein kinase; Notch, Neurogenic locus notch homolog protein 1; AXL, Tyrosine-protein kinase receptor UFO; E, Embryonic days; dpc, days post coitus; P, postnatal; CS, Carnegie stage; EGF, epidermal growth factor; FGF, fibroblast growth factor

formation of definitive endoderm [103]. The efficiency of definitive endoderm formation is evaluated by the percentages of key markers such as SOX17, FOXA2 and CXCR4. Rezania et al. used a GSK3β inhibitor in place of the previously described activin-activated WNT3A pathway to induce the differentiation of definitive endoderm in stage 1 [103]. The addition of basic fibroblast growth factor (FGF2), in combination with Activin A and CHIR99021, significantly improves the percentage of $SOX17^+/FOXA2^+$ cells [110]. By screening a library of 330 monoclonal antibodies (mAbs) directed against the detection of definitive endoderm (DE) subpopulations in H9 -hESC cells revealed that, in the $CD177^+$ subpopulation of DE, generated β-like cells have improved maturation and function when compared to the $CD275^+$ subset of cells [111].

Stage 2: primitive gut tube

During primitive gut tube morphogenesis, the pancreatic endoderm comes closer to the mesodermal cell types including the notochord and they act as the major signaling source that controls the fate of pancreas development [112]. Further studies have shown that the signaling molecules released from the notochord, which is located adjacent to the dorsal endoderm, lead to the inhibition of the previously-activated TGF-β pathway, effected by ceasing the use of activin A/nodal, alongside sonic hedgehog (shh) downregulation [13]. Instead, the early growth response gene 1 (EGR1) is expressed through exposure to a range of FGFs including FGF2, 7 or 10 that activates FGF receptor 2b [5, 104, 105]. Studies have identified the significance of FGF2 released from the notochord in repressing the expression of SHH in the adjacent pre-pancreatic endoderm and thereby promoting the global patterning of pancreatic endoderm [72]. The addition of ALK4,5,7 signaling inhibitors like TGF-β RI Kinase Inhibitor IV, along with keratinocyte growth factor (KGF), proved to be an effective method in generating pancreatic progenitor cells in the next stage [107, 113].

Stage 3/4: pancreatic progenitors

During stage 3, posterior foregut cells are developed to specifically yield cells with pancreatic or hepatic tissue fate. This commitment of the posterior foregut to a pancreatic lineage begins with the expression of the key transcription factor, PDX1, along with enhanced levels of SRY-Box Transcription Factor 9 (SOX9) and hepatocyte nuclear factor-1 beta (HNF1B) [13]. RA plays a key role

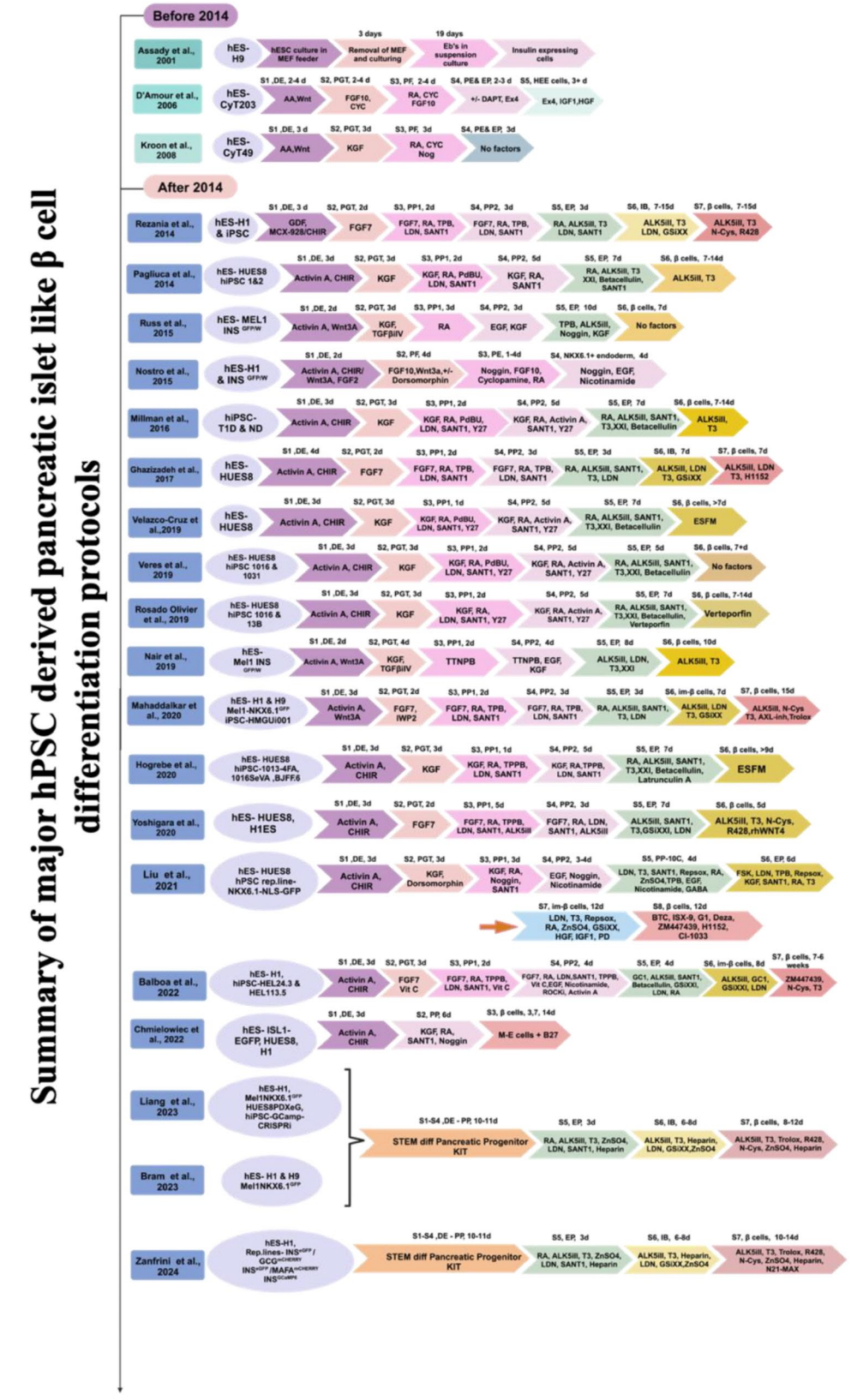

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Fig. 2 An overall summary of major hPSC derived pancreatic islet like beta cell differentiation protocols in sequential steps that recapitulate in vivo development. We classified the protocols into 'Before 2014' and 'After 2014' sections based on the significant breakthrough of generating mono hormonal insulin producing β cells after 2014. An overview of all major protocols, organized by their specific stages, time duration, important growth factors and small molecules that were added at each stage and their corresponding year of publication, is presented here

S1-S7, Stages from 1–7; hES, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; DE, Definitive Endoderm; PGT, Primitive Gut Tube; PP1/2, Pancreatic Progenitor1/2; PE, Pancreatic Endocrine; EP, Endocrine progenitors; im.β cells, immatured β cells; T1D, Type 1 Diabetes; ND, Non diabetic

in PDX1 induction; however, an increased expression of RA receptors is associated with pancreatic exocrine cells [114]. In addition, the inhibition of BMP and FGF10 promotes the development of pancreatic endoderm [115]. In lieu of using Noggin to inhibit BMP, Rezania et al. preferred the small molecule, LDN, along with a PKC activator molecule, TATA-binding protein (TPB), and FGF7 [103]. Recently, glycoprotein 2 (GP2) was identified as a cell surface marker specific to pancreatic progenitor cells and those cells expressing GP2 eventually developed into islet-like β cells both in vivo and in vitro [116]. In the Nostro protocol, a combination of NOGGIN, EGF and Nicotinamide treatment generated a NKX6.1$^+$ progenitor population from different hPSC cell line-derived pancreatic endoderms [105]. Hogrebe et al. demonstrated that polymerized cytoskeleton delays the increase of the endocrine specification factor Neurog3 expression in pancreatic progenitors, and also hinders the ability of cells to differentiate into INS$^+$ β cells later on. They addressed this inhibition using well-timed cytoskeletal depolymerization with latrunculin A, allowing for the robust production of SC-β cells in planar culture [106].

Stage 5: pancreatic endocrine cells

To induce pancreatic endocrine formation from these progenitors, inhibition of SHH, Notch, BMP and TGF β are required for the specification and maturation of endocrine cells from the PDX1 progenitor population [117]. Most of the protocols used the gamma-secretase inhibitors DAPT, XXI, and Dibenzazepine (YO-01027) for the expression of endocrine lineage markers [103, 116, 118]. The addition of Activin receptor-like kinase (ALK5i, a TGFb receptor inhibitor) inhibitor and exposure to the thyroid hormone, Triiodothyronine (T3) at stage 5 induces the expression of pancreatic hormones and specific markers [103].

The transition of cells from planar culture to an air-liquid interface during stage 5 significantly enhanced the expression of key transcription factors such as NKX6.1 and PDX1, along with the expression of endocrine precursor markers like NGN3, NKX2.2 and NEUROD1 [103]. Treatment with Alk5i at stage 5 had a positive effect on the specification of β-like cells. Following the generation of enhanced pancreatic progenitor cells using another inhibitor of SHH signaling, namely SANT1, Pagliuca et al. introduced a chemical cocktail with variety of factors including T3, XXI (a gamma-secretase inhibitor), heparin, Alk5iII and betacellulin (an EGF family member) to trigger endocrine formation and subsequent β cell specification [119]. A later study by Nostro et al. emphasized the significance of the length of treatment and confirmed that a shorter duration of treatment with RA, FGF10, inhibitors of the BMP and hedgehog signaling pathways was favorable for the NKX6.1$^+$ population whilst a longer duration is more conducive to the development of polyhormonal cells [105]. To induce NEUROG3 expression in PDX1$^+$ /NKX6.1$^+$ progenitors, Russ et al. employed BMP and ALK inhibition for 5 days for the efficient generation of INS$^+$/NKX6.1$^+$ β like cells [109].

Stage 6: immature islet formation

Following the generation of endocrine precursors, the latter stages focus upon honing in to the mono-hormonal function of each endocrine cell type within the islets, with a specific focus on β cell maturation [119]. This begins with the formation of immature islet clusters during stage 6, which requires a three-dimensional arrangement of cells either as suspension clusters or as aggregates on transwells [106]. Endocrine progenitor cells with NEUROD1, ISL1, NKX2.2, and PAX6, together with PDX1 and NKX6.1 expression, exhibited a higher proliferation rate and enhanced basal level of insulin secretion and are thus considered to be immature β cells until the postnatal period [120, 121]. Postnatal acquisition of β cell functional maturity begins with the expression of insulin transcription and secretion-related genes, including MAFA, NEUROD1, PAX6, PCSK1/3, ABCC8, SLC30A8, GCK and GLUT1, that enables the β cells to both trigger and amplify their insulin signaling and GSIS, the hallmarks of mature β cells [122].

A combination of small molecular factors including ALK5iII, T3, gamma-secretase inhibitor XX (GSiXX) and LDN treatment induced the expression of all major markers of β cell maturation, and concurrently inhibited the expression of PTF1A, a marker of exocrine lineage. The addition of Alk5iII together with thyroid hormone T3, increases the insulin expression in NKX6.1$^+$ endocrine cells and also generates mature SC-β cells expressing PDX1, NKX6.1, c-peptide and INS [105]. Nonetheless, these generated SC-β cells are transcriptionally and functionally immature β cells when compared to primary β cells.

Stage 7: islet maturation

MAFA is a key gene involved in regulating the expression of multiple genes associated with β cell maturation. Rezania et al. introduced R428, an inhibitor of tyrosine kinase receptor AXL, together with the N-acetyl cysteine enhanced the expression of MAFA [103]. The stage 7 media containing the chemical cocktail of R428, N-Cys, ALK5iII and T3 induced a 16-fold induction of MAFA and the cells were functional and capable of reversing diabetes when transplanted into immunodeficient mice [103]. By contrast, Balboa et al. omitted the ALK5 inhibitor and included an antiproliferative aurora kinase inhibitor, ZM447439 along with T3 and N-cys and observed a stable number of mono-hormonal β cells despite physiological and transcriptomic heterogeneity [5].

ROCK inhibitor H1152 treatment during stage7 promotes the functional maturation of β cells, with an increased expression of β cell maturity markers MAFA and UCN3 [123]. The iPSC-derived human islet-like organoids were stimulated with a non-canonical Wnt signaling molecule, WNT4, which fosters the metabolic maturation of β cells and promotes robust GSIS [124]. Also, the removal of Alk5iII (that is used in many protocols) at the final stage together with resizing the cellular cluster size were able to generate mature β cells that successfully achieve first and second phase dynamic insulin secretion [125]. Another protocol used an extended culture of 3D pancreatic progenitor clusters with a cocktail of ten chemicals to generate functional glucose responsive β cells [126]. Nevertheless, despite exhibiting key β cell maturity markers and dynamic GSIS assays, these SC-β cells failed to match the glucose responsiveness and transcriptomic profile of primary cadaveric islets [126].

Culture methods and conditions – 2D versus 3D

2D culture methods, like planar culture, offer a simpler and more straightforward approach to differentiate β cells. 2D culture simplifies the differentiation protocol by eliminating more complicated and expensive 3D culture methods and is amenable for conducting small drug screening experiments, protocol optimization for different cell lines and in generating large batches of differentiated cells for cell therapy. Conversely, 3D culture methods provide a more complex and physiologically relevant environment for β-cell differentiation. The cells can arrange either as suspension clusters or as aggregates on Transwells to form pancreatic islet-like spheroids, that mimic the natural architecture of pancreatic islets.

Most of the previously established protocols successfully generated glucose responsive SC-β cells via utilizing a 3D arrangement of pancreatic progenitor cells. The Hogrebe group optimized their protocol using traditional planar differentiation methods and were able to efficiently stabilize glucose levels of mice with severe diabetes within two weeks post-transplantation [127]. Their protocol demonstrates that, with minimal requirements such as a standard Matrigel-coated 6 or 24-well plate and with differentiation media simply added in the proper order, the potential for high scalability and rapid production of functional SC-β cells could be expedited [106]. Balboa's group focused upon achieving metabolic maturation of β cells and further divided stage 7 to systematically assess suspension characteristics and the impact of the stage on functional maturation [5]. They established an optimized protocol to generate human SC-islets that display glucose responsive insulin release along with multifaceted analysis including functional assays, cell physiology analyses, metabolic tracing experiments and single cell RNA transcriptomic analysis throughout 6 weeks of in vitro maturation and 6 months of mouse engraftment. Intriguingly, their study has illustrated the profound variation in SC-islet cytoarchitecture during stage7 from core-mantle organization to intermingled clusters of INS + and GCG + cells along with an 80% reduction in Ki-67 + INS + proliferative marker expression [5].

Braam et al. demonstrated the generation of aggregates of hPSC-derived pancreatic progenitors using a commercially available kit into near uniform spheroids and further differentiated the progenitors towards insulin expressing, glucose responsive cells using a static suspension culture platform [108]. The efficient and reproducible derivation of pancreatic progenitors using STEMdiff™ Pancreatic Progenitor Kit is a 14-day differentiation protocol in 2D monolayer culture. The pancreatic progenitors expressing PDX1, NKX6.1, and SOX9 markers from 2D culture were aggregated into spheroid 3D suspension culture, with an AggreWell™ platform (STEMCELL Technologies, Canada). However, that the GSIS response of these hPSC-derived uniform islets is still lower than primary human islets, suggesting that future efforts should focus on optimizing the protocol to improve their functionality [108].

Although SC-β cells can be generated successfully using multiple differentiation protocols, they produce other cells alongside β cells. Veres et al. provided a resource for the future development of SC-β cell differentiation protocols by using single-cell RNA sequencing experiments to comprehensively characterize the cells that are formed during SC-β cell differentiation [6]. They grew hPSC cells in 3D culture and followed a 6-step differentiation protocol for generating SC-β cells [6]. Moreover, they identified CD49a as a surface marker of SC-β cells and generated successfully pure SC-β cell clusters via a magnetic microbead sorting method. Nonetheless, there was still a lower secretion magnitude versus primary cadaveric islets [6].

Millman et al. for the first time generated β like cells by differentiating hiPSCs from patients with type 1 diabetes and identified no detectable differences when compared to SC-β cells derived from non-diabetic individuals [114]. After undergoing planar differentiation to pancreatic progenitors, they continued the differentiation in suspension culture to generate functional glucose responsive β cells [114]. Nair et al. improved the differentiation protocol by introducing a cell culture condition to recapitulate the critical events that occur during pancreatic islet organogenesis and β cell maturation [125]. In order to reduce the stresses that cells experienced during 3D shaking culture and to reduce cell loss and improve viability of endocrine clusters, Liang et al. developed a 96 well based static 3D culture system for generating insulin secreting islet-like clusters from endocrine cells [110]. Fantuzzi et al. performed an in-depth characterization of hiPSC-derived pancreatic β cells by used a rotating suspension or static microwells, the two different 3D culture systems, for the last 3 stages of the differentiation process [128]. They also compared suspension aggregates to microwell aggregates and observed 5–10% more INS^+ cells and less GCG + cells, with increased reproducibility between iPSC cell lines, in microwell aggregates, concluding that microwells offer a more effective and user-friendly platform [128].

Interestingly, a recent study established an exciting feeder-free and Matrigel-free system in planar culture for the long-term expansion and cryopreservation of pancreatic progenitors in a defined medium on fibronectin [129]. This development in hESC differentiation to cells is through the long-term expansion of pancreatic endoderm (ePE) cells in 2D culture with a defined substrate, fibronectin, a polymer identified through polymer screening that enables the accumulation of $NKX6.1^+$ cells over time, and facilitated the production of hormone-producing β like cells. Moreover, RNA seq data in two cell lines revealed that the percentage of NKX6.1 expression was reduced during expansion of these ePE cells [129]. To overcome this limitation of progenitor expansion, Luka et al. modulated certain signaling pathways, such as stimulation of specific mitogenic pathways, RA signaling inhibition, TGFβ and Wnt signaling pathway suppression, which enabled the expansion of pancreatic progenitors over many passages [130]. Such advancements in the protocol further allows the banking of pancreatic progenitor cells from diverse hPSC cell lines which are crucial for studying the pathophysiology of diabetes, personalized medicine and cell replacement therapy.

A recent report highlighted the role of exosomal miRNAs in iPSC-based differentiation to insulin producing cells in vitro [131]. The β cell markers NKX6.1, MAFA and Insulin were markedly upregulated in iPSC cells cultured with exosomes isolated from MIN6 cells on day 7. miRNA seq analysis of MIN6 released exosomes revealed that four exosomal miRNAs, namely miR-706, miR-709, miR-466c-5p and miR-423-5p, actively modulate the differentiation of iPSCs to β like cells. Notwithstanding, the underlying mechanism of miRNA in regulating the differentiation is obscure. These results further confirm the effectiveness and feasibility of obtaining insulin producing cells using exosomes in 2-D culture, that can avoid the biosafety concerns associated with DNA-based reprogramming techniques and thereby making iPSCs more suitable for clinical trials [131].

Intriguingly, another study has shown the generation of GINS (Gastric insulin secreting) organoids from hGSCs (human Gastric Stem Cells) by transgene activation of NPM factors (NGN3, PDX1 and MAFA). Lentiviral integration of the Ngn3ER fusion gene (Ngn3 and oestrogen receptor) was incorporated into the hGSCs and differentiation was initiated by 4OH-tamoxifen treatment for 2 days followed by lentiviral integration of a Pdx1-mafa co-expression cassette. After 4 days of 2D culturing, generated GINS cells were aggregated into spherical organoids and further maintained for 4 weeks which resulted in efficient glucose responsive organoids and restored glucose homeostasis for over 100 days in diabetic mice after transplantation [132]. Hence, this is a promising approach, via the ectopic expression of NPM factors for production of autologous cell therapies to treat diabetes. In vitro reprogramming studies have shown that, after adult hGSCs, α-cells and γ-cells showed the second largest proportion of cells that can produce insulin (~80%) after transplantation [133]. The sole inactivation of the *Arx* gene in pancreatic α-cells resulted in the cells displaying a β-cell phenotype and enabled reversal of chemically induced diabetes in mice [134]. Moreover, human α-cells have been reprogrammed in vitro using PDX1 plus MAFA overexpression into insulin secreting cells with reduced immunogenicity, hence showing the higher reprogramming capacity of α-cells into insulin releasing β like islet cells [135].

Evaluation of protocol advancements

The characterization of hiPSC-derived β cells have advanced considerably in the field of stem cell research. The methods used to characterize hiPSCs and the stages of β cell differentiation are designed to evaluate and validate the outcomes at different stages of differentiation, the functionality of the generated β cells/islets and/or the efficiency of the protocol used. The conventional tests used to detect the presence of the markers of a given stage qualitatively or semi-quantitatively are RT-PCR, immunostaining and western blot [136]. qRT-PCR is the simplest way to track the success of a differentiation protocol via quantifying the large changes in gene expression when cells progress through various stages. In the case

of planar culture, cells can be fixed and immunostained directly on the plate for specific biomarkers of various differentiation stages and images can be captured with confocal or wide-field fluorescence microscope [106].

Flow cytometry is most widely used to perform the qualitative and quantitative analysis of expressed markers at the end of a desired stage during differentiation. The poly hormonal and non-endocrine cell populations generated during the directed differentiation protocols can be easily identified with flow cytometry approaches. The key assay that indicates functionality of the generated islets/ β cells is GSIS, using either the static or perifusion method, where cells/islets are challenged with low and high glucose and/or KCl solution and ELISA is used to detect the amount of c-peptide and insulin secreted [5, 106]. Some groups have transplanted endocrine progenitors or mature β cells in vivo into a diabetic mouse model and observed the function of transplanted cells using a glucose challenge [107, 109, 113]. Transmission electron microscopy (TEM) analysis has shown the presence of INS/GCG secretory granules whilst TEM analysis of mitochondrial structure has been studied to determine the maturity of the cells [105, 109]. Electrophysiology, to determine membrane potential of SC-β cells, changes in Ca^{2+}, K^{+}-mediated depolarization and exocytosis on stimulation with different small molecules has also shed light on the ability of the SC-β cells/islets to produce and release insulin granules appropriately upon stimulation. The calcium signalling assay using the dye Fura-2-acetoxymethyl ester (FURA2) and measuring signals using Total Internal Reflection Fluorescence (TIRF) microscopy is also indicative of the functionality of SC- β cells [5, 104, 113].

Transcriptomic analysis, such as single-cell RNA sequencing (scRNA-seq) and computational analysis profiles enable the characterization of various cell types based on gene expression in an unbiased manner without prior knowledge of the cell type [137]. Intriguingly, transcriptomic profiling performed at various differentiation stages identified glycoprotein 2 (Gp2) as a potential marker for pancreatic progenitor cells having the potential to differentiate into endocrine and exocrine cells as more SC-β like cells were generated from Gp2 + pancreatic progenitors [138, 139]. Single cell RNA analysis offers deeper insights into the heterogenous cell population, their development and the signaling pathways involved and indicates the transcriptional dynamics of a given cell population [5]. Studies have identified through scRNA sequencing three different endocrine populations, SC-β, -α, and -EC cells, along with a small population of Sst + Hhex + Isl1 + cells, plus a population of Sox9 + non-endocrine cells arising during the directed differentiation of SC-islets [6]. Single-nucleus multi-omic sequencing was used to study the chromatin accessibility and the transcriptomic profiles and gene expression profiles of enterochromaffin-like cells and SC-β cells versus primary islets [140]. High resolution sequencing time course analysis has been used to study the dynamics of gene expression during the different stages and stage transitions [6]. Assay for Transposase-Accessible Chromatin (ATAC) analysis to study chromatin accessibility across the genome aids in understanding of the epigenomic pancreatic progenitor signatures that affect stage-specific gene expression in cells generated by different protocols in vitro versus fetal development to confirm the efficiency of the protocol in recapitulating pancreatic development [137].

Proposing strategies for developing "a perfect islet"

Even though great strides have been made in SC-β cell differentiation methods over the past two decades, there are still several concerns, namely low differentiation efficiency, increased polyhormonal cell ratio and limited functional maturity of β cells. Intriguingly, recent reports have indicated the importance of leveraging exosomes and their cargoes as potential biomarkers for evaluating the risk of developing T1DM [141]. The analysis of MIN6 derived exosomal miRNA sequencing revealed that four exosomal miRNAs, namely miR-706, miR-709, miR-466c-5p and miR-423-5p, with significantly higher differential expression between the exosomes and parental cells, are involved in actively modulating the differentiation of iPSCs towards functional β cells. The underlying mechanisms of how these exosomes communicate through specific miRNAs is still obscure [131]. Emerging studies have reported the role of dysregulated ER stress and its associated signaling pathways in β cell dysfunction promoting diabetes pathogenesis [142]. A comparative analysis of the morphological features and stress response in hPSCs with other somatic cells revealed the elevated vulnerability of hPSCs to ER stress due to the lower expression of ER chaperone proteins like the binding immunoglobulin protein, BiP [143]. Li et al. reported the effect of imeglimin, a novel antidiabetic agent on protecting both human islets and hPSC-derived pancreatic pseudoislets against ER stress-induced apoptosis, thus illustrating a promising strategy to improve differentiation protocols for the generation of functionally mature pancreatic β-like cells by preventing their apoptosis both in vitro and in vivo [144]. Using both genetic and nutritional approaches, Jaafar et al. elucidated the molecular mechanisms driving both metabolic and transcriptional remodeling of pancreatic β cells during the neonatal period. They demonstrated that it is the switch from the nutrient sensor target of rapamycin (mTORC1) to the energy sensor 5′-adenosine monophosphate–activated protein kinase (AMPK) dependent

cellular signaling that is responsible for the critical insulin-responsive phenotype of mature β cells which is essential for postnatal life [145].

The beneficial role of inhibiting Poly (ADP-ribose) polymerase-1 (PARP1), a fundamental DNA repair enzyme, in human islet differentiation and maturation has recently been explored. The depletion of PARP1 by RNAi or the potent PARP1 inhibitor, PJ34, in combination with Activin A promoted human pancreatic progenitor PANC1 cell differentiation to mature insulin producing β cells via augmenting p38 MAPK phosphorylation and Neurogenin-3 re-activation [146]. Single cell transcriptomics data revealed β cell trans-differentiation into α-like GCG expressing cells following the inactivation of Taf4, a subunit of the transcription factor TFIID, further emphasized the essential role of Taf4 in normal β cell function. Introducing small molecules that can upregulate the expression of Taf4 can significantly improve the directed differentiation protocols for generating mature β like islet cells [147]. These recent studies indicate that incorporating these new concepts into current differentiation protocols can successfully generate functional pancreatic islets from hPSCs (Figure 3).

Clinical trials of hPSC derived islets and existing challenges

Advancements in technology have enabled human clinical trials using either stem cell-derived beta cells or pancreatic endoderm (PE) cells. In 2014, ViaCyte (ViaCyte Inc., San Diego, CA, USA) launched the first human clinical trial with PE cells derived from a human embryonic stem cell (hESC) line (clinicaltrials.gov: NCT02239354). These cells were microencapsulated in the immune-protective Encaptra device and implanted subcutaneously in immunocompetent patients. Although the treatment was safe and well tolerated, the trial was discontinued due to poor graft survival [148, 149]. To address this issue, ViaCyte improved the encapsulation device to enhance vascularization and cell survival, leading to a Phase II clinical trial in 2017 (clinicaltrials.gov: NCT03163511) [150].

More recently, ViaCyte and CRISPR Therapeutics initiated a trial using genetically modified beta cells designed to evade the immune system. Additionally, Vertex Pharmaceuticals began a clinical trial in 2021 with VX-880, a fully differentiated stem cell-derived pancreatic islet cell product, transplanted into Type 1 diabetes (T1D) patients alongside immunosuppressive therapy. In the treatment of Type 2 diabetes (T2D), autologous E-islets derived from human endoderm stem cells have successfully improved islet function [151]. A Phase I clinical trial (ChiCTR2300072200) has also shown success in treating T1D patients using autologous chemically induced pluripotent stem cell (CiPSC)-derived islet cells implanted beneath the abdominal anterior rectus sheath [152]. This successfully showed sustained insulin independence and stable glycemic control after 1 year of transplantation. However, an autologous islet transplantation still requires the use of life long immunosuppressants, indicating that immunosuppression may still be necessary due to autoimmune response, and further studies will be required in this context.

One of the major challenges of islet transplantation is the continuous usage of immunosuppressive drugs resulting in increased risk of infection, autoreactivity and malignancies. Strategies to protect SC-islet grafts using immunomodulating biomaterials or genetically engineered hypo-immune SC-islets (universal donor) could protect the SC-islets from immune attack. Advancements in encapsulation technology especially using chemically modified alginate biomaterials (triazole-thiomorpholine dioxide alginate and CXCL12-containing sodium alginate) have significantly improved biocompatibility and long-term performance of hPSC-derived β cell islet transplants in immunocompetent mice without any immunosuppression [153, 154].

Intriguingly, studies have shown that genetic engineering of the islet like organoids overexpressing immune checkpoint molecule programmed death 1 ligand 1 (PD-L1/CD274) prevented immune rejection when transplanted into non-obese diabetic (NOD) severe combined immunodeficient (scid) gamma (NSG) and diabetic immunocompetent C57BL/6J mice [124]. Another group showed that deletion of all classical HLAs with the exception of HLA-A2 and HLA-E/F/G maintained the SC-islets when transplanted into NSG mice [155]. These findings show promise for the possibility of generating immune evasive functionally mature human islets from hPSCs in vitro that can overcome autoimmune rejection without immunosuppressants in T1D patients [156]. Several remaining challenges to be addressed are production of off-target cell types like enterochromaffin cells using current differentiation methodologies and to improve insulin release from cells [157]. Therefore, improving the function of SC-islets and improving the immunomodulating biomaterial-based islet organoids with efficient nutrition and oxygen supply could significantly reduce graft rejection as well as cell production costs.

Concluding remarks

Studies to date suggest that SC-β cells are a promising option for the treatment of diabetes, offering robust functionality and durability against stress and immunogenic responses. However, more work is required to generate fully functional mature SC- β cells with a glucose response profile comparable to that of primary adult β cells. Presently, most of the differentiation protocols have focused mainly on functionality based on GSIS assay and diabetes reversal after transplantation of SC-islets into

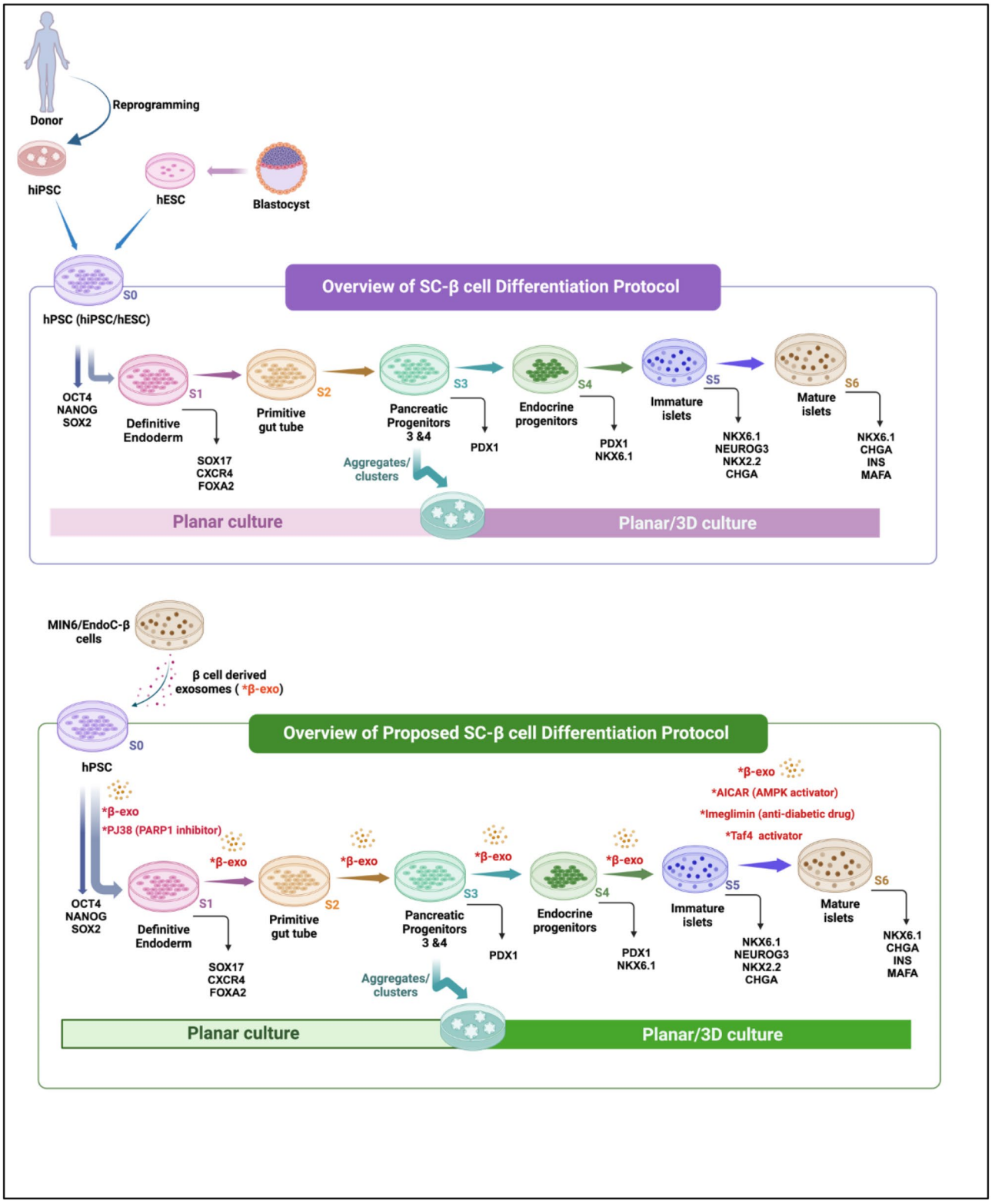

Fig. 3 Proposed SC-β cell differentiation protocol in comparison with current six stage differentiation protocol. The introduction of new factors and small molecules that could improve the generation of functional pancreatic islet like β cells from hPSCs are shown in red colour (asterisk mark) with their respective stages of the differentiation protocol

mouse models, whilst only a few studies have reported the complete characterization of differentiated cell populations. However, a universal protocol that could be employed for all types of stem cells would eliminate the need for expensive and time-consuming optimization protocols for generating SC-β cells [158]. Better understanding of mTORC1, a critical nutrient sensitive kinase essential for postnatal pancreatic β cell maturation could significantly improve differentiation protocols in the future. Additional research is required to understand the process by which exosomal miRNAs actively modulate the differentiation of iPSCs towards functional mature β-like cells. Furthermore, modulating the ER homeostasis pathway and preventing β cell apoptosis by introducing specific small molecular regulators like imeglimin might be a promising strategy to improve differentiation protocols. These ongoing endeavours will enhance our understanding of the biology of human pancreatic β cells and contribute to the efforts to generate a "a perfect islet" that can be employed in the investigation of the pathophysiology and treatment of diabetes mellitus.

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SS, SK: Conceptualization, writing original draft, reviewing & editing. LA: Writing original draft & editing. SS, LA, TJ, JMA, AM, OA, SK and MN researched the data, Writing- review & editing. SK, AEB, JMA and MN: Supervision, Writing–review & editing.

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Declarations

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Competing interests
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Use of AI
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