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REVIEW

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In vitro generation of pancreatic β-cells for diabetes treatment. I. β-like cells derived from human pluripotent stem cells

Katarzyna Cierpka-Kmiec[1], Agata Wronska[2], Zbigniew Kmiec[2]

[1]Department of Hypertension and Diabetology, Medical University of Gdansk, Poland
[2]Department of Histology, Medical University of Gdansk, Poland

Abstract
Diabetes mellitus is a chronic disease that affects hundreds of millions of people worldwide. Type 1 diabetes (T1D) is characterized by the lack of pancreatic β-cells that had been destroyed as a result of an autoimmune response. Therefore, in patients with T1D, the replacement therapy with functional β-cells derived from extrinsic sources could be a preferable option as compared to insulin treatment. Unfortunately, successful transplantation of whole pancreata or pancreatic islets into patients with diabetes is available only to a fraction of them due to the scarcity of donors. The rapid development of cell reprogramming methods made it possible to generate large numbers of human β-like cells derived from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs). This review describes the basis of *in vitro* differentiaton protocols of β-like cells that mimic changes of the main signaling pathways during the key stages of human and murine pancreas development, which are described first. During the last 15 years it was found that there are no important differences between hESCs and hiPSCs in their differentiation capacities into β-like cells and the expression profiles of the key transcription factors. The *in vitro* produced β-like cells are immature as demonstrated by functional tests in rodents and single-cell transcriptomic and proteomic analyses. After the transplantation of the β cell progenitors into immunocompromised diabetic mice, a few weeks have to pass before the increased insulin levels in response to glucose load appear. There is a continuous progress in the development of open-type encapsulation devices which allow the vascularization of the transplanted cells and protect them against host's immune cells. The results of the first clinical trial of human partially differentiated endocrine progenitors of β cells transplanted into patients with T1D will be published in the year 2019. It is hoped that further improvements in the techniques of large-scale generation of the β-like cells derived from human pluripotent stem cells will bring us closer to their clinical application as a form of cause-directed therapy for people with diabetes. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 1–14)*

Key words: diabetes; human pancreas development; hESC; hiPSC; *in vitro* differentiation; transcription factors; β-like cells; β cell replacement

Abbreviations*:

Arx — aristaless-related homeobox; bFGF — basic fibroblast growth factor; bHLH — basic helix-loop-helix family; BMP — bone morphogenetic protein; Cdx2 — caudal-type homeobox transcription factor 2; c-Myc — v-Myc avian myelocytomatosis viral oncogene homolog; CPA — carboxypeptidase A; CXCR4 — chemokine (C-X-C motif) receptor 4; DE — definitive endoderm; DLL1 — delta-like canonical Notch ligand 1; Dpc — day post conception; EP — endocrine progenitor; EGF — epidermal growth factor; ESC — embryonic stem cell; Ex4 — exendin-4; FACS — fluorescence-activated cell sorting; FGF — fibroblast growth factor; FoxA1, -2 — forkhead box A1, -2; Gata4 — GATA-binding protein 4; Gata6 — GATA-binding protein 6; Glis — Gli similar family of transcription factors; GSIS — glucose-stimulated insulin secretion; GSK3β — glucagon synthase kinase 3β; hESC — human embryonic stem cell; Hnf4 — hepatocyte nuclear factor 4; Hnf6 — hepatocyte

Correspondence address: Prof. Zbigniew Kmiec, MD, PhD
Department of Histology, Medical University of Gdansk
Debinki 1, 80–210 Gdansk, Poland
e-mail: zkmiec@gumed.edu.pl

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nuclear factor 6, synonym — Onecut1; hPSC — human pluripotent stem cell; hiPSC — human induced pluripotent stem cell; IDE1/2 — inducers of definite endoderm 1/2; Idx1 — islet/duodenum homeobox 1, synonym of Pdx1; IGF-1 — insulin-like growth factor 1; Ins — insulin; Ipf1, insulin promoter factor 1, synonym of Pdx1; iPSC — induced pluripotent stem cell; Irx1 — iroquois homeobox protein 1; Isl1 — LIM homeobox 1; KGF — keratinocyte growth factor; Klf4 — Krüppel-like factor 4; Ldb1-Isl1 — LIM domain-binding protein 1–islet 1; MafA, -B — avian musculoaponeurotic fibrosarcoma oncogene homolog-A or -B; Mesi1 — Meis homeobox 1; Mist1- bHLH family member, a15; N0); Mnx1 — motor neuron and pancreas homeobox 1; MPC — multipotent progenitor cell; MPP — multipotent pancreatic progenitor; NeuroD1 — neurogenic differentiation factor 1; Neurog3 — neurogenin 3; Ngn3, acronym for Neurog3; Nkx2.2 — Nirenberg and Kim homeoboxfactor 2.2; Nkx6.1 — Nk6 homeobox protein 1; Oct4 — octamer binding transcription factor 4; Onecut — synonym of Hnf6; Pax4, -6 — paired box homeodomain transcription factor 4, -6; Pdx1 — pancreatic and duodenal homeobox 1 transcription factor; PKC — protein kinase C; PSC — pluripotent stem cell; Ptf1a — pancreas transcription factor 1 subunit alpha; RA — retinoic acid; Rbpj — Ras-associated protein Rap1; Rfx — regulatory factor X; Shh — sonic hedgehog; Sox9, -17 — sex-determining region Y box 9, -17; SUSD2 — Sushi domain containing 2; T1D — type 1 diabetes; Tcf1 — transcription factor 12; TGF-β — transforming growth factor β; Wnt3 — wingless-type MMTV integration site family, member 3.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that currently affects more than four hundred millions of people worldwide [1]. DM is caused by a nearly absolute lack of insulin in type 1 diabetes (T1D) patients or an insufficient release of insulin in type 2 diabetes (T2D) related to insulin resistance of peripheral tissues, mainly, but not only, due to increased body and fat mass in obese patients. T1D is usually caused by autoimmune mechanisms that result in the reduction of β cells' number in pancreatic islets. The pathogenesis of T2D is based on the overload of β cells by the resistance of the peripheral tissues, such as skeletal muscles and fat cells, to insulin's action caused by increased serum levels of free fatty acids and oxidative stress in pancreatic islet cells. Although for almost one hundred years exogenous insulin has been administered to T1D patients as a substitute of the endogenous hormone, the lifelong treatment does not affect the cause of the disease and may be associated with many complications of which episodes of hypoglycemia are most important. T2D is treated by drugs that increase the secretion of insulin, its tissue sensitivity, improve glucose disposal, or delay glucose absorption in the gastrointestinal tract. However, in many T2D patients the exhaustion of β cells eventually occurs and leads to the additional treatment with insulin. The best causative treatment for T1D would be to increase the number of functional β cells. This has been in fact achieved in a relatively small number of patients, either through transplantation of cadaver-derived whole pancreas, pancreatic islets, or infusion of isolated β cells into gastrointestinal venous system of recipients. These treatment options are available in many developed countries; however, to a limited extent only. The main limitation, similarly as with transplantation of other organs, is the scarcity of donor tissues, which makes the allotransplantation options practically unavailable to the majority of patients with DM. Moreover, the transplanted islets require lifelong immunosuppression which poses further health problems.

The continuous progress of molecular and cell biology techniques makes it possible to generate *in vitro* human functional β cells and introduce them into diabetic patients to increase endogenous insulin production in response to changes in blood glucose concentrations. In this short review we will describe the methods that have been developed not only to produce *ex vivo* β-like cells but also to efficiently protect them from an immune attack after transplantation into diabetic patients (the term 'β-like cell' is here defined as an insulin-positive cell that contains secretory granules; however, it does not imply presentation of the same functional characteristics as primary human β cell). There are two main sources for the *in vitro* generation of human β-like cells: (i) human embryonic stem cells (hESCs) and human induced pluripotent stem cells, both referred further as pluripotent stem cells (hiPSCs), and (ii) differentiated cells from various organs that can be transdifferentiated into β-like cells, mainly by genetic manipulations. It seems obvious that the obtained β-like cells should express molecular markers of normal β cells such as a set of specific transcription factors and other molecules that characterize mature β cells as a result of terminal differentiation. Moreover, after transplantation into animals with experimentally-induced diabetes or into patients with diabetes, these *ex vivo* generated β-like cells should present features of functional maturity such as rapid alterations of intracellular Ca^{2+} concentration and secretion of insulin in response to changing glucose levels.

*Since originally most of the transcription factors have been detected and described in *Drosphila* and non-human vertebrates, their abbreviations are written in small letters.

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The elucidation of the molecular background of the stages of pancreas development in mouse and humans laid solid foundations for the implementation of cell and molecular biology techniques to transform hESC, hiPSC, or normal somatic cells into functional β-like cells, processes often termed collectively as 'cell reprogramming'. This paper will provide an overview of the progress in the field of the *in vitro* β cell neogenesis from pluripotent stem cells as a potential source of insulin-secreting cells for patients with diabetes. Since the protocols for obtaining *in vitro* β-like cells from human PSCs to a great extent recapitulate the embryonic stages of pancreas development, a short review of the factors that control the development of human (and murine) pancreas, and especially β cells, will be first provided.

The embryonic development of the pancreas and its driving forces

The development of pancreas is, in general, very similar in many vertebrate species. Since the availability of early human embryos for studies is limited, the knowledge about the mechanisms of the embryonic development of pancreas in mouse (and other vertebrates) could be generally transposed to the early stages of pancreatic development in humans. The genetic manipulations in mice allowed for the identification of multiple transcription factors which in a complex but coordinated way control the development and growth of the pancreas. Many, although not all, of these transcriptions factors were shown to play a key role in human pancreatic development. Moreover, in transgenic mice it was possible to perform lineage tracing experiments that enabled to characterize the developmental history of the major cell types of endocrine and exocrine parts of the pancreas [2]. This knowledge has been used by many groups to mimic *in vitro* the developmental pathway of β cells in a step-wise differentiation of hESC or hiPSCs into insulin-secreting β cells, and also other pancreatic islets' cell types of which α, δ, ε, and PP cells secrete glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively.

In mammals pancreatic development begins in two distinct areas that later fuse in one organ. In mouse during gastrulation the flat definitive endoderm folds to form primitive gut tube in which foregut, midgut, and hindgut can be recognized. The foregut is divided into the anterior part that gives rise to the thymus, thyroid, parathyroid, and lungs, and the posterior foregut. The evaginations of the posterior foregut appear at the embryonic day 9 (E9d) as a dorsal pancreatic bud (at the junction with midgut) and as the ventral bud at E9.5d [3, 4]. Due to the gut rotation at E12–13d, the ventral and dorsal buds become adjacent and fuse with each other. There are two developmental phases during pancreas development in mouse. During the primary transition (E9.5–12.5d) there is massive proliferation of pancreatic progenitor cells forming stratified epithelium in which several micro-lumen structures develop that later fuse [3, 5–7]. At this stage few endocrine cells appear that are mainly polyhormonal, expressing both glucagon and insulin, and some monohormonal cells; however, these cells probably do not commit to mature islets [3, 8]. During the secondary transition from E13.5d to E16.5d microlumens fuse to create the characteristic tubular structures lined by pancreatic epithelium in a form of plexus that branches further into a continuous epithelial network, segregated into tip and trunk domains [7, 9, 10]. At this stage of pancreatogenesis the proliferation of endocrine progenitor cells is extensive and all five types of endocrine cells are generated [3]. The pancreatic acini originate from distal tip cells whereas the trunk cells will differentiate into ductal and endocrine cells [3, 7, 11]. The extensive proliferation of pancreatic epithelium is accompanied by the differentiation of endocrine progenitors into neurogenin 3 (Ngn3)-positive cells followed by the formation of mature endocrine cells [12–14].

In comparison to the bulk of data on early embryogenesis in model non-human species such as mouse and chick, the information about the early stages of human development, and of pancreas in particular, is limited, mainly due to the scarcity of the early human embryos ($<$ 8 weeks of gestation, G8w) available for investigations. However, similarly as in mouse, the differentiation of the human endoderm into β cells can be classified into the following pivotal stages: (i) definitive endoderm (DE), (ii) primitive gut tube, (iii) foregut endoderm, (iv) pancreatic anlagen composed of multipotent progenitor cells (MPCs) that will differentiate into the three major pancreatic cell lineages, *i.e.* exocrine, ductal, and endocrine cells, (v) endocrine progenitors, and (vi) β cells [15–18].

Pan and Brissova [16] and Jennings *et al.* [17] compared the stages of human pancreas development, its key features, and estimation of an equivalent timeline of the mouse pancreas development. At the gestational day 25–27 (G26–27d), *i.e.* at the Carnegie stages 9–10 (CS9–10), the transient contact of the notochord (and dorsal aortae) with the pre-pancreatic endoderm suppresses the expression of the sonic hedgehog (SHH) pathway. This induces folding of the

posterior foregut-derived definitive endoderm which results in the formation of the pancreatic dorsal bud at G26d that is followed by the formation of two ventral buds at G30d (CS12–13) [15, 17]. The left ventral bud gradually regresses and the right ventral bud migrates backwards at G35d (CS15) as a result of gut rotation and finally fuses with the dorsal bud at gestational weeks 6–7 (G6–7w, CS18–20) to form a single organ with the main pancreatic duct formed by the coalescence of the dorsal and ventral ducts [15]. In humans the dorsal pancreatic bud will form the major part of the head, the body, and the tail of the future pancreas whereas the right ventral bud will form the inferior part of the head and uncinate process [4]. Between G35d and G45d (CS15 and CS19, respectively) an extensive growth of the pancreatic anlagen leads to the formation of ductal branching and appearance of microlumens in the acinar cell compartment as well as extensive proliferation of multipotent progenitor cells. At G7w (CS19) tip-trunk compartmentalization becomes distinguishable: populations of tip-like cells will differentiate into exocrine acini whereas the trunk-like cells will develop into endocrine and ductal cells [16, 17].

These morphogenetic processes are initiated and controlled by transcription factors and signaling molecules that activate or suppress basic signaling pathways such as WNT, SHH, and Notch (discussed in 11, 15, 18, 19). At G30d (CS12) the expression of the crucial transcription factor PDX1 (pancreatic and duodenal homeobox 1), the earliest pancreas-specific transcription factor, can be detected in the endoderm differentiating into pancreatic anlagen [15], similarly as during mouse embryogenesis in the pancreatic endoderm [5, 6, 11]. Soon thereafter, the expression of other transcription factors: SRY (sex-determining region Y)-box 9 (SOX9), GATA4 (Gata-binding protein 4), and NKX6.1, necessary for human pancreatic development, takes place [15, 20].

Similarly as in mouse, the expression of the NEUROG3 transcription factor is a sign of the beginning of the endocrine commitment [21] that is further controlled by the expression of other transcription factors. At G7.5w (CS21) the first endocrine, insulin-positive cells appear, and at G9w glucagon–expressing α cells emerge [15, 21–23]. At G10w the delamination and endocrine cell clustering starts so that at G12-13w all types of endocrine cells are present in the developing islets [15, 16, 21, 22].

The role of numerous transcription factors that induce and control the development of the pancreas by the regulation of gene expression during the subsequent stages of pancreas development has been in depth characterized in mouse [11, 18, 19, 24]. They were found, although with few exceptions, to play a similar role during the development of human pancreas [15, 17, 25]. In mouse the differentiation of the multipotent progenitor cells into exocrine, endocrine, and ductal lineages of the adult pancreas is regulated by the coexpression of the key transcription factors such as Pdx1, Ptf1a, Foxa2, Sox9, Nkx6.1, and the presence of carboxypeptidase 1A (Cpa) [6, 11, 18, 24]. The development of the ductal/endocrine lineages from MPCs is associated with the loss of Ptf1a and maintenance of Nkx6.1 expression, whereas the development of the exocrine compartment depends on the downregulation of Nkx6.1 and continued expression of Ptf1a [26]. The differentiation of β cells is controlled by the Pdx1 transcription factor which promotes specification and maturation of MPCs into pancreatic β cells in concert with the other transcription factors such as neurogenin 3 (Ngn3), Nkx6.1, and MafA [6, 18]. Many of the transcription factors that control the development of the murine pancreas were identified during the stages of human pancreas differentiation [13, 15, 25] and are shown in Figure 1. The role of some transcription factors that are crucial for the pancreatogenesis will be shortly characterized.

In humans PDX1, also called insulin promoter factor 1 (Ipf1) or islet/duodenum homeobox 1 (Idx1), a marker of early MPCs, is detected at G30d (CS12) in a presumptive pancreatic endoderm even before pancreatic bud formation from definitive endoderm takes place [15]. PDX1 plays a pivotal role in the differentiation of all pancreatic cell types, as Pdx1 knockout mice exhibit pancreatic agenesis [27]. The role of PDX1 in the human pancreatic development was confirmed when a neonate presented lack of exocrine pancreas function and insulin-requiring hyperglycemia caused by a homozygous point deletion resulting in a frame shift [28]. Direct lineage tracing studies in mouse showed that Pdx1-expressing progenitors in the early mouse embryo give rise to all pancreatic cells types, *i.e.* ductal, exocrine, and endocrine cells, and that the progenitors for the mature pancreatic ducts separate from the endocrine/ /exocrine tissues before the E12.5 [2]. In the pancreatic endocrine cells Pdx1 is expressed mainly in the β cells where it binds to and activates the insulin promoter and increases insulin gene transcription. Larsen and Grappen-Botting [11] presented a graphical overview of the interconnectivity of the pancreatic progenitor gene regulatory network at the early stages of mouse pancreatic development, with the Pdx1, Ptf1a, Foxa2, and Sox9 transcription factors as the key nodes. A recent genome-wide analysis of the *in vitro* generated

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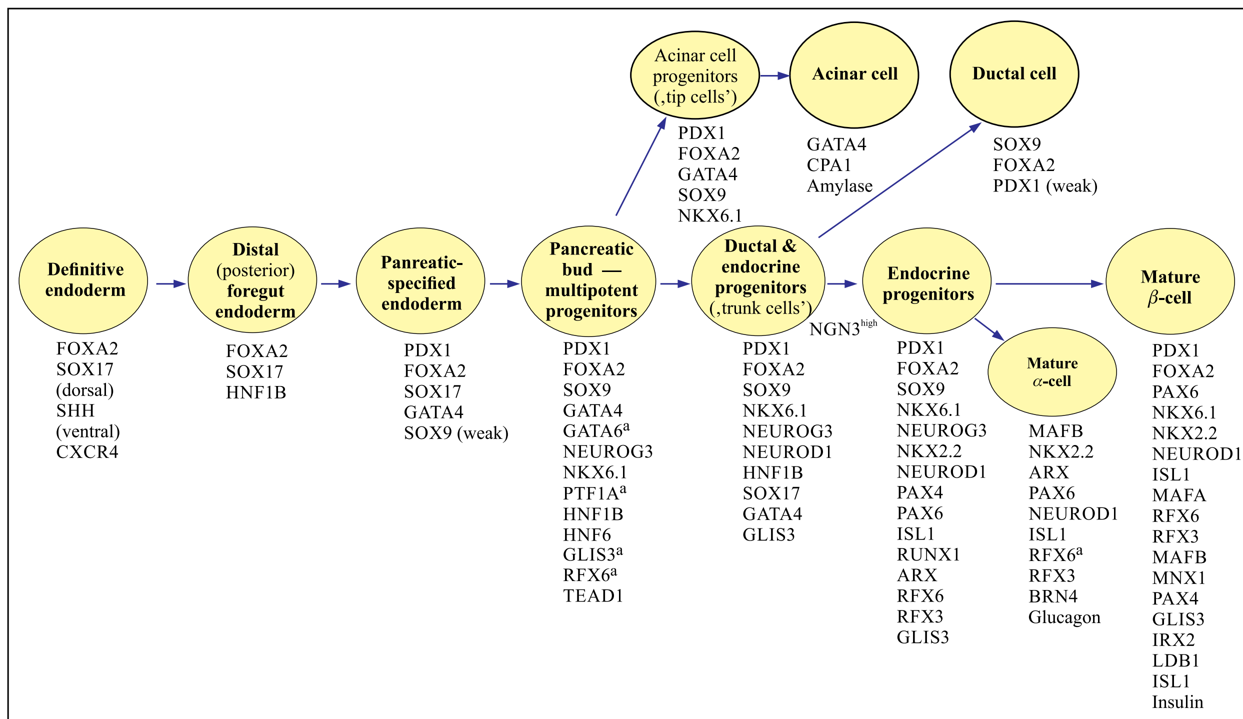

Figure 1. The expression of key transcription factors (TFs) at the sequential stages of the human definitive endoderm differentiation into pancreatic lineages of acinar, ductal, α and β cells. The capital letters denote human TFs which were first detected at the respective phases of pancreas differentiation in mouse. [a]detected as transcripts in human embryos. Abbreviations are explained past the Abstract. Based on the data summarized by Jennings *et al.* [17], Al-Khawada *et al.* [19], Lyttle *et al.* [22], Riedel *et al.* [23], Conrad *et al.* [25], and Santosa *et al.* [41].

human pancreatic progenitor cells by the Chip-seq for PDX1 revealed a total of 8088 PDX1-bound regions that map to 5664 genes. The PDX1 target regions include important pancreatic transcription factors such as PDX1 itself, RFX6, HNF1B, and MEIS1 as well as signaling molecules and factors important for β cell function [29]. Moreover, the application of the ChIP-seq technique for PDX1 disclosed several novel PDX1 target genes including RFX3, required in mouse for the differentiation and function of the β cells, and the ligand of the Notch receptor DLL1, which is important for endocrine induction [29].

The *PTF1A* (pancreas transcription factor 1 subunit alpha) gene on human chromosome 10 encodes a transcription factor with a key role in early human pancreas development and cerebellar neurogenesis [26, 30]. The transcripts of the *PTF1A* have been demonstrated during the development of human pancreas; however, in contrast to mouse, the presence of PTF1A protein has not been demonstrated in humans yet, probably due to the lack of available antibodies [17]. In mouse acinar differentiation is regulated by a set of transcription factors including Ptf1a and Mist1 [31]. Ptf1a (p48) is an important bHLH factor that forms a complex with Tcf12 and Rbpjl, which allows the expression of genes for the secretory enzymes present in the mature acini [32]. It is coexpressed with Pdx1 in both dorsal and ventral pancreatic buds from E9.0 to E9.5, and later, at E12.5, Ptf1a expression is found in growing tips of the branching epithelium to eventually end up in the acinar cells [6].

Neurogenin 3 (NEUROG3, NGN3) belongs to a family of basic helix-loop-helix (bHLH) transcription factors involved in the development of central nervous system and embryonic pancreas. Neurog3 is transiently expressed in mouse [7, 33] and human pancreatic endocrine progenitor cells [15]. In mouse, there are two phases of Ngn3 expression, *i.e.* during the first and second transition, the latter peaking at E15.5 [7]. Ngn3 is required for endocrine differentiation, as demonstrated by the complete lack of all types of pancreatic endocrine cells in Neurog3-deficient mice [12]. Ngn3 initiates the differentiation of Pdx1-expressing progenitor cells and activates the expression of additional transcription factors important for the differentiation of MPCs into endocrine cell lineages [12]. In mouse single-cell lineage tracing has shown that α and β cells originate from different Neurog3-expressing endocrine cells [12]. NGN3 expression during human pancreatic development starts from G8w with

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a peak at the end of the first trimester [15], concomitantly with the development of islets which at G12-13w contain α, β, δ, and γ cells [15, 20]. The NGN3 expression tightly correlates with the appearance of fetal insulin-containing β cells; however, it becomes undetectable after G35w [34] and is not present in mature human endocrine cells [17, 22]. Interestingly, in humans NEUROG3 may not be required for the development of the endocrine pancreas since the patients with *NGN3* mutation were born with circulating C-peptide [35]. In mouse, apart from Ngn3, Pax4 and Arx transcription factors are important for the determination of endocrine cell fate [6, 36]. NGN3 is also critical for human pancreatic endocrine development since null mutations in *NGN3* cause neonatal diabetes and block β cell differentiation from human pluripotent stem cells [37].

NKX6.1 (NK6 homeobox protein 1) and NKX2.2 transcription factors are expressed in pancreatic endoderm and endocrine precursor cells and are essential for promoting β cell specification by controlling cell lineages between endocrine and acinar cell fate. In mouse the expression of Nkx6.1 is required for the development of the β cell lineage from endocrine progenitors [38] so that Nkx6.1 is specifically expressed in adult β cells. In humans the expression of NKX6.1 follows that of PDX1 and SOX9 and takes place prior to the transient activation of NGN3 that is necessary for the differentiation of β and other islet endocrine cell types [17]. However, a recent single-cell gene expression analysis of human ESCs differentiation into β cell lineage revealed that the activation of NKX6.1 can be initiated before or after endocrine commitment (downstream of NGN3 expression) without negative effect on the generation of β-like cells *in vitro* [39].

MAFA (v-Maf musculoaponeurotic fibrosarcoma oncogene homolog A) is a key transcription factor for fetal β cell maturation. Nearly no MAFA is detected in human embryo until gestational week 21, and its expression gradually increases after birth, being limited at adult age to β cells [21]. This transcription factor binds to the enhancer/promoter region of the insulin gene RIPE3b/C1-A2 and activates insulin gene expression in response to glucose [40, 41].

In addition to the transcription factors mentioned above, there are many other which participate in the control of the maintenance and expansion of multipotent pancreas progenitors and endocrine progenitors (Fig. 1). Their function and interdependency, revealed mainly in the studies of mouse pancreas development, have been extensively reviewed by Jørgensen *et al.* [6] and other authors [5, 11, 18, 19, 41–43].

Although the development of the human pancreas generally recapitulates the stages of mouse pancreas differentiation, there are some important interspecies differences which can affect the strategies for the *in vitro* β cell differentiation, as summarized by Jennings *et al.* [17], Nair and Hebrok [44], and Balboa *et al.* [45]. For instance, the expression of the key transcription factor PDX1 necessary for the development of pancreatic progenitor cells occurs in the human endoderm later than in mouse, *i.e.* after gut closure once mesenchyme has separated notochord and aorta from dorsal foregut [15]. The expression of neurogenin 3 is biphasic during murine pancreatogenesis [7] but not in humans [15]. Other differences refer to the different timing of the expression of the transcription factors GATA4, SOX17, and NKX2.2 [6, 15]. For example, the expression of GATA4 is delayed in human pancreatic development, and SOX17, a definitive endoderm marker which exists in the presumptive human pancreatic endoderm, is lost in rodent pancreatic epithelium [15]. In the mouse, pancreatic islets are formed only close to birth (E19–21) whereas in human embryos islets appear at G12w [15]. Moreover, β cells are the first endocrine cells detected in human pancreas [15, 20, 22] in contrast to mouse, with α cells forming first [33]. In mouse there are two phases of the appearance of endocrine cells during pancreatic development, referred to as primary and secondary transition, respectively. In human embryos no early pancreatic endocrine differentiation takes place so that only one wave of endocrine differentiation takes place [3, 15, 34]. Whereas in the developing human pancreas the NKX2.2 transcription factor is not expressed in multipotent pancreatic progenitor cells [15] (Fig. 1), Nkx2.2 is widely expressed in the murine pancreatic bud until E13 when its expression becomes restricted to the Ngn3-positive cells [46]. MafB expression is lost postnatally from mouse [11] but not human [23] β cells.

In a landmark study, Jennings *et al.* [47] used laser capture, RNA amplification, and computational analysis of deep sequencing to reveal the transcriptomic programs regulating the earliest development of pancreas, liver, and biliary tree in human embryos. They found that pancreas-enriched gene expression was less conserved between human and mouse than for the liver. Apart from confirming that the dorsal pancreatic bud was enriched for components of Notch, Wnt, BMP, and FGF signaling, they discovered over 30 transcription factors which have yet not been associated with the differentiation of pancreatic endoderm [47]. Altogether, the reported differences between some stages of β cells differentiation in mouse and human (and generally between mature

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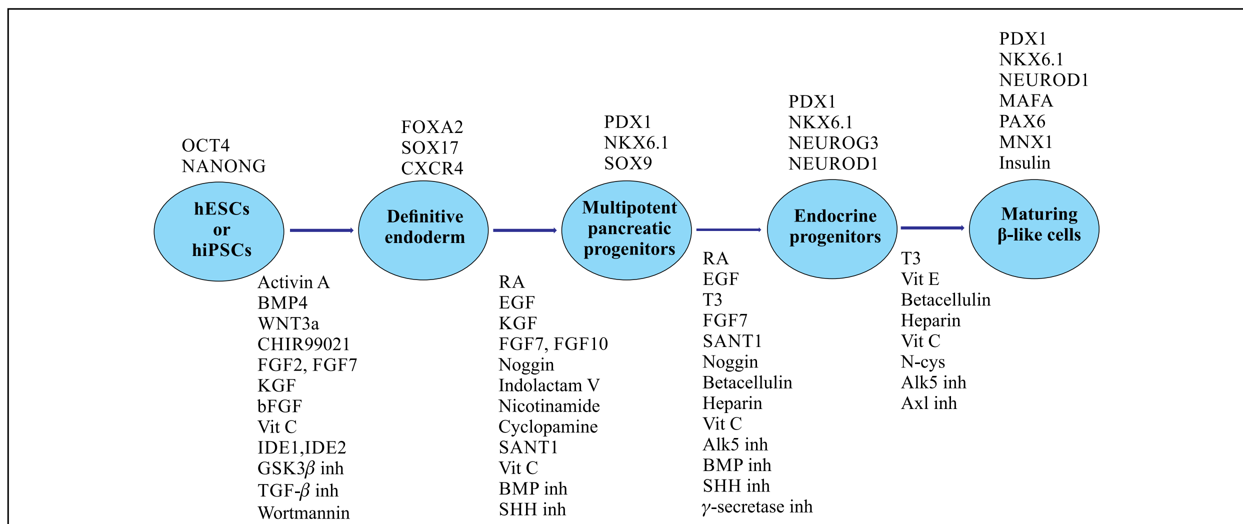

Figure 2. Main stages of an idealized, simplified protocol of the differentiation of hESCs or hiPSCs into β-like cells. The major markers of the differentiation stages are shown above and the growth factors and other molecules controlling the signaling pathways directing definitive endoderm cells' transformation into β cell lineage are shown below the presented developmental stages (based on data presented in (59–64, 67–71). Abbreviations as listed on page 1 and: ALK5iII — activin receptor-like kinase 5 inhibitor II; Axl — receptor tyrosine kinase; CHIR99021 — a selective GSK3β inhibitor; inh — inhibitor; N-cys — N-acetyl cysteine; SANT1 — Shh antagonist; T3 — triiodothyronine; wortmannin — phosphoinositide 3-kinase inhibitor.

endocrine cells in the murine and human pancreata, not mentioned in this review) suggest caution when relating findings from mice to humans. It is expected that further development of computational algorithms will allow using the transcriptomic and proteomic data derived from the studies of human embryos to facilitate stem cell research and clinical interpretation without inter-species extrapolation.

The elucidation of the chronology and hierarchy of the signaling events during mouse and human pancreas development, apart from its pure scientific importance, together with the breakthrough technologies of human embryonic stem cells' differentiation and human induced-pluripotent stem cells made it possible to generate large numbers of multipotent pancreatic progenitor cells that could be further differentiated *in vitro*, and after transplantation into immunocompromised diabetic mice develop and mature into insulin-secreting β-like cells, possibly also in humans with diabetes.

In vitro recapitulation of the developmental stages of pancreas enables the reprogramming of hESCs or human iPSCs into insulin-secreting cells

The progress in the understanding of the molecular mechanisms of pancreas development, together with the advent of techniques of human ESCs differentiation [48] and generation of mouse [49] and human [50, 51] induced-pluripotent stem cells made it possible to produce *in vitro* β-like cells with the aim of replacing dysfunctional or lacking β cells in patients with diabetes. Both types of pluripotent stem cells express unlimited proliferation capacity *in vitro* and may differentiate under appropriate culture conditions into virtually any cell type. Thus, they offer new, fascinating possibilities of future treatment of patients with diabetes by the transplantation of *in vitro* generated insulin-secreting cells. Generally, the strategies for the generation of functional β-like cells are based on mimicking *in vitro* the developmental stages of *in vivo* pancreas and β cell development by the exposition of the sequentially cultured differentiating hESCs or human iPSCs (hiPSCs) to empirically determined cocktails of various key growth factors and small molecules which temporally activate transcription factor cascades and signaling pathways specific for the phases of definitive endoderm, pancreatic endoderm, pancreatic progenitors, endocrine progenitors, and finally of β cell lineage differentiation (Fig. 2).

Currently, two basic strategies have been pursued for the production of β-like cells for cellular replacement therapy for patients with diabetes: (i) *in vitro* differentiation of hESCs or hiPSCs, and (ii) *in vitro* transdifferentiation of differentiated somatic cells, of-

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ten, but not exclusively, of endodermal origin, without reverting to the pluripotent stage through gene manipulation [52]. The second option will not be described in this short review. However, it has to be noted that the ultimate target of both approaches is the effective functioning in patients with diabetes of insulin-secreting cells able to produce adequate amounts of insulin in response to the rapid changes of blood glucose levels.

Human embryonic stem cells obtained from the inner cell mass of human blastocysts [48] have been investigated extensively as pluripotent stem cells for the *in vitro* generation of many cell types. Scientists from the laboratory of CyThera (later Novocell, Inc., and currently ViaCyte, Inc.) described a method of effective hESC differentiation into definitive endoderm (DE) cells [53]. A year later they developed the first highly replicable multi-step protocol of the differentiation of the DE cells into pancreatic endocrine cells, which was based on mimicking pancreas development through the stages of primitive gut tube, posterior foregut, pancreatic endoderm and endocrine progenitor cells. They obtained polyhormonal cells that expressed insulin and other pancreatic hormones; however, these cells did not respond to increased glucose levels by enhanced insulin secretion [54]. After improving the *in vitro* differentiation protocol, the same group showed that after transplantation of hESC-derived immature pancreatic endoderm cells (pancreatic progenitors expressing both PDX1 and NKX6.1 transcription factors) into immunodeficient mice with experimentally-induced diabetes, normoglycemia was restored after 3–4 months, which suggested that this time was needed for the *in vivo* differentiation and maturation of these cells into glucose-responsive insulin-secreting cells [55]. These early protocols of hESC differentiation presented low efficiency, lasted for many weeks, and resulted in a heterogeneous population of insulin-immunoreactive cells that were often polyhormonal [54–56]. After improving many stages of hESC differentiation into insulin-secreting cells, a few years ago the ViaCyte started the first clinical phase I/II trial involving hESCs-derived immature pancreatic progenitor cells co-expressing PDX1 and NKX6.1 (PEC-01 cells) in patients with T1D (ClinicalTrials.gov identifier: NCT02239354, trial VX-1). These cells were placed in immunoprotective capsules and implanted under the skin of the patients. Initial data from clinical evaluation of the safety, long-term tolerability and efficacy of this system is expected to be published in the middle of the year 2019. Two years ago ViaCyte initiated two other clinical trials to test a new macroencapsulation device ("open Encaptra Device") in which direct vascularization of the PEC-01 cells can take place (ClinicalTrials.gov identifiers: NCT03162926 and NCT03163511). Although the use of the differentiated ESCs bears the risk of teratoma formation as found in diabetic mice [57], so far cancers have not been reported in the T1D patients who participate in the ViaCyte trials (www.viacyte.com). In January 2019, the Center for Beta Cell Therapy in Diabetes and ViaCyte announced the start of the European clinical trial of PEC-Direct known also as VC-2, an encapsulated pancreatic progenitor cell product designed to replace lost insulin-producing β cells in patients with T1D (www.betacelltherapy.org).

The technique of de-differentiation of somatic cells by transcription factors'-based somatic cell reprogramming was first demonstrated for mouse adult fibroblasts by Takahashi and Yamanaka [49] who used retroviral transduction with Oct3/4, Sox2, c-Myc, and Klf4, and soon afterwards was used for the reprogramming of human dermal fibroblasts by retroviral transduction with POUF51 (OCT-4), SOX-2, KLF4, and MYC [50]. The lentiviral transduction of human fetal fibroblasts with OCT-4, SOX-2, NANONG, and LIN28 was sufficient for the generation of human induced pluripotent stem cells that had normal karyotypes, expressed telomerase, and maintained the developmental potential to differentiate into advanced derivatives of all three primary germ layers [51]. The use of hiPSCs which exhibit the morphology and growth properties of ESCs obviates the need for human embryos to obtain ESCs for *in vitro* differentiation programs into almost all types of somatic cells. Similarly to the differentiation of hESCs, the *in vitro* generation of β-like cells from hiPSCs is based on the sequential modulation of multiple signaling pathways controlling pancreas development, which has been best characterized in mouse [11], however, only partially in humans [15–17]. Substantial progress in the *in vitro* production of large numbers of β cell progenitors was achieved by the introduction of improved 'second generation' protocols for differentiation of human ESCs [58] or human iPSCs [59, 60] into β-like cells. The protocol described by Rezania *et al.* [58] comprised 7 stages: 1. hESCs or hiPSCs, 2. Definitive endoderm, 3. Primitive gut tube, 4. Posterior foregut, 5. Multipotent pancreatic progenitors, 6. Endocrine progenitors, 7. Immature endocrine cells, 8. β-like cells. These *in vitro* differentiation steps are promoted and controlled by various empirically-defined sets of factors known to affect many signaling pathways of the respective stages of the developing pancreas in mouse and humans [11, 17–19, 24, 26, 41–45]. There have been numerous modifications of the differentiation protocols of hESCs or hiPSCs

into β-like cells aimed at the production of large populations of relatively pure multipotent pancreatic progenitors (enriched in PDX1 and NKX6.1 cells) which can be further induced into monohormonal, insulin-secreting and glucose-responsive cells. Paglucia *et al.* [59] used large-scale floating cell culture system for the differentiation of hESCs and hiPSCs by the use of a combination of 11 small molecules to obtain β-like cells through the stages of definitive endoderm, posterior gut tube, and early pancreatic progenitor cells. After 4–5 weeks, with efficiency higher than 30%, they obtained glucose-responsive, monohormonal insulin-producing cells that showed typical ultrastructure of β cells and co-expressed key β cell markers, including high mRNA levels of the transcription factor MAFA. Two weeks after the transplantation of these partially differentiated cells into immunodeficient diabetic NRG-Akita mice, a model of diabetes [61], normalization of blood glucose level was observed [59]. Ma and Zhu [62] have graphically summarized three improved protocols [59–61] for the differentiation of hESCs/hiPSCs into functional pancreatic β-like cells. More recently, Yabe *et al.* [63] used a six-stage protocol for the differentiation of human iPSCs to pancreatic β cells using defined culture media without feeders or serum. They showed that induction of definitive endoderm by a selective glycogen synthase-kinase-3β inhibitor and spheroid formation at the final stage of differentiation are important for the generation of functional β-like cells. After the transplantation of these cells under the kidney capsule of streptozotocin-induced diabetic non-obese immunocompromised (NOD/SCID) mice, blood glucose levels gradually decreased over the next 4 weeks; however, not in all transplanted animals [63]. Memon *et al.* [64] found that dissociation of densely formed definitive endoderm cells obtained from human iPSCs and re-plating them at low density, followed by a longer period of retinoic acid (RA) and FGF10 signaling resulted in a high yield of PDX1+/NKX6.1+ pancreatic progenitors. Recently, a novel protocol for rapid and footprint-free differentiation of hESCs to endocrine cells by the lipofection technique was described by Ida *et al.* [65]. The authors introduced synthetic mRNAs (synRNAs) encoding transcription factors PDX1 and NKX6.1 by the lipofection reagent into hESCs and demonstrated that pancreatic endocrine hormones were highly expressed in cells transfected with synRNA-PDX1 and synRNA-NKX6.1 at day 13, as shown by immunohistochemical staining of insulin, glucagon, and somatostatin in various populations of the obtained cells. The hESCs were able to differentiate into pancreatic endoderm cells within 3 days, and within 7–9 days into multipotent pancreatic progenitors [65]. Since growth factors are costly components of the culture media necessary for *in vitro* cell differentiation, Kondo *et al.* [66], after screening of 1250 small molecules, found that the addition of sodium cromoglicate facilitated the differentiation of endocrine precursors from multiple hiPSC/hESC lines and substantially increased the induction rate of insulin-positive cells.

Many other groups have modified culture conditions and sets of small molecules used at the sequential stages to shorten the time of the *in vitro* differentiation of hESCs or hiPSCs into insulin-secreting β-like cells and to increase the efficiency of the procedures. The various chemical modulators of the sequential *in vitro* differentiation stages have been recently characterized [67–71].

Figure 2 shows the main stages of an idealized protocol for the differentiation of hESCs or hiPSCs into β-like cells and presents the key growth factors and other molecules that affect multiple signaling pathways at each differentiation step. These *in vitro* differentiation stages mimic the developmental events that take place during murine or human pancreatogenesis and β cell lineage development. Thus, the first stage of transforming hESCs or hiPSCs into definitive endoderm is based on mimicking Nodal and Wnt signaling pathways by activin A as well as IDE1 and IDE2, small molecules that act synergistically with activin A [72]. High concentrations of activin A mimic the proendodermal role of Nodal during *in vivo* gastrulation, whereas Wnt3a ligand, involved in endodermal patterning in mammals, was later replaced by CHIR99021 shown to be more effective [73] for the induction of definitive endoderm marked by the expression of FOXA2, SOX17 and CXCR4 (Fig. 2). The next step (for simplicity we omit here the intermediary stages of primitive gut tube and posterior foregut, as described in the original protocol by Rezania *et al.* [58]) is the formation of multipotent pancreatic progenitors (pancreatic endoderm) that is characterized by the expression of the key transcription factors PDX1 and NKX6.1. The differentiation media contain retinoic acid (RA), which activates HOX genes during *in vivo* pancreatic and liver endoderm formation, cyclopamine and SANT1 (which inhibit Shh signaling and promote pancreatic lineage development), fibroblast growth factors such as FGF7 and FGF10 (in mouse FGF10 promotes the expansion of the Pdx1-expressing endodermal cells), and Noggin, an inhibitor of BMP signaling that suppresses hepatic lineage differentiation. Moreover, activators of the protein kinase C (PKC), such as epidermal growth

factor (EGF) with nicotinamide (a poly(A-ribose) inhibitor), EGF with keratinocyte growth factor (KGF) or indolactam V (screened from over 4,000 chemicals (74)), and inhibitors of the BMP and Shh signaling (Shh inhibits the development of pancreatic endoderm in mouse) are also added to the culture media to increase the population of multipotent pancreatic progenitor cells (Fig. 2). The next stage, the generation of endocrine progenitors, is achieved in the presence of RA, EGF (required for branching morphogenesis during development of endocrine lineage in mouse pancreas [11]), triiodothyronine (T3, increases expression of neurogenin 3 necessary for β cell differentiation and maturation), betacellulin (EGF receptor ligand that maintains NKX6.1 expression [59]), heparin, vitamin C (prevents the formation of polyhormonal cells [58]), BMP receptor inhibitor (Alk5i II, activin receptor-like kinase 5 inhibitor II), and γ-secretase inhibitor (suppresses Notch signaling). The differentiation of the endocrine progenitors into β-like cells is promoted by T3, vitamin E, betacellulin, heparin, Alk5 inhibitor, as well as Axl receptor tyrosine kinase (Axl) inhibitor and N-acetyl cysteine, which induce the expression of MAFA [58, 59] (Fig. 2).

These shortly characterized multi-step protocols of the *in vitro* differentiation of hESCs or hiPSCs into β-like cells are technically complicated, costly, take usually a few weeks, and often suffer from low differentiation efficiency, which is partially caused by the genetic variability of different hESC and hiPSC lines. To shorten the long differentiation procedures, Trott *et al.* [75] developed culture conditions by using feeder-based platform (mouse embryonic fibroblasts) that support long-term self-renewal of human multipotent pancreatic progenitors which express key pancreatic transcription factors, including PDX1 and SOX9, and exhibit transcriptomes closely related to their *in vivo* counterparts. These cells can be differentiated into glucagon- and insulin-expressing cells *in vitro* and *in vivo*. This type of approach offers a convenient alternative to pluripotent cells as a source of adult cell types for diabetes treatment and modeling of the development of human pancreas.

Southard *et al.* [76] created a pluripotent cell line derived from human primary pancreatic tissue by reprogramming the cells (without knowledge of their specificity) with non-integrating vectors, and generated definitive endoderm after a four-day differentiation protocol. The selected SR1423 cell line was differentiated according to a simplified protocol to generate after 4 weeks populations with more than 60% of insulin-expressing cells that secreted insulin in response to glucose and reversed diabetes in rodents. The authors suggest that after banking following the guidelines of Good Manufacturing Practice, the SR1423 is a candidate cell line for the production of insulin-secreting cells useful for the treatment of insulin-dependent diabetes.

Since the protocols of hESC or hiPSCs differentiation into β cell lineage mimic the *in vivo* endocrine pancreas development, the expression of transcriptions factors or surface markers at the corresponding *in vitro* stages has been used to validate the applied protocols. Fluorescence-activated cell sorting (FACS) with specific cell surface markers has been used for the isolation and further characterization of the human fetal pancreatic cells as well as for the characterization of *in vitro* differentiating hESCs/hiPSCs cells. The examples of surface markers used for FACS-sorting of pancreatic progenitor cells include CD142 (tissue factor, a marker of pancreatic endodermal cells that also labels additional cell types) [77], CD24 (a marker for PDX1-expressing pancreatic progenitors derived from hESCs) [78], and GP2. GP2, a glycoprotein present in pancreatic secretory granules, expressed together with the key transcription factors NKX6.1 and PTF1α, was shown to be a cell surface marker of human multipotent pancreatic progenitors [79–80]. Ramond *et al.* [81] investigated human fetal pancreatic epithelial cells by FACS analysis using a combination of the cell surface markers GP2, ECAD (also known as CDH1, *i.e.* cadherin 1), CD142, and SUSD2 (Sushi domain containing 2, a marker used to enrich NEUROG3-positive cell population from hPSC-derived pancreatic cells and the human fetal pancreas [82]). They identified distinct endocrine populations at different stages of their development (pancreatic progenitors, endocrine progenitors, and endocrine cells) and showed that a subset of the GP2-positive cells undergoes endocrine differentiation by down-regulating GP2 and CD142 and up-regulating the expression of NEUROG3, a marker of endocrine lineage differentiation [81]. The same group improved the purity of the cell populations isolated from three human fetal pancreata at G9w by applying an additional surface marker, CD133 (prominin 1, often expressed on adult stem cells), to exclude ductal cells, along with using granularity to distinguish endocrine cells [83]. The transcriptional profiling of the isolated cell populations at the single-cell level using qPCR showed that, at a single time point, different steps of the endocrine differentiation can be identified. Moreover, this approach enabled to benchmark the pancreatic cell types produced *in vitro* from one hESCs and two hiPSCs cell lines using an established endocrine-biased differentiation protocol [83]. Fur-

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ther studies of single-cell transcriptomes of mouse and human progenitors at different steps of pancreas development will allow to capture intermediate stages of differentiation, increase the efficacy of the *in vitro* generation of β cell progenitors, and discover factors that will improve the functionality of the β-like cells.

Validation of the β-like cells generated *in vitro*

Although the β-like cells show the morphological and some of the functional characteristics of the *bona fide* adult β cells, they must be regarded as immature β cells. The comparison of single cell transcriptomes and proteomes of β-like cells generated from hiPSCs with those of mature β cells revealed the lack of some molecules present in the *bona fide* β cells [84]. Numerous tests have been applied to characterize the phenotype of the *in vitro* generated β-like cells. They include the expression of specific surface molecules to perform FACS sorting, immunohistochemistry to detect insulin or C peptide, marker gene analysis by real-time PCR, the presence of potassium channels Kir6.1 and Kir6.2 and the ATP-binding cassette channel Sur1 which are necessary for insulin secretion [85]. Coupled to GSIS (glucose-stimulated insulin secretion) assessment, the presence of these proteins should be used in order to establish optimal β-like cells' response to glucose. The functional assays include testing of the extent of glucose-stimulated insulin secretion in terms of its amount and stimulation index (*i.e.* the ratio of insulin secretion levels between low- and high-glucose concentrations), fluctuations of Ca^{2+} intracellular levels, or secretion of C-peptide. Initially, the hESC-derived β-like cells presented suppressed glucose-stimulated insulin secretion [56], and in response to a rapid increase of glucose concentration they did not reproduce the high amplitudes of fast changes of Ca^{2+} concentrations that are a hallmark of the mature β cells isolated from human pancreatic islets [84, 85].

Concluding remarks

The ultimate target of *in vitro* produced β-like cells is their phenotypic and functional similarity to the authentic β cells of human pancreas. Despite the continuous improvements in the differentiation protocols of human ESCs or hIPSCs for scalable *in vitro* generation of β-like cells, the time is not yet ready for their use as an alternative diabetes treatment. It has been shown that there are differences between different hESCs and hiESCs in their transcriptomic profiles and efficacy of reprogramming into β-like cell progenitors [86]. Moreover, the β-like cells rarely present pure populations since they are often contaminated by other endocrine cell types and are sometimes polyhormonal. Thus, reproducibility and standardization of the *in vitro* differentiation processes present essential requirements that have to be clearly defined for the acceptance of the β-like cells for future therapies by health regulatory authorities. There are important ethical concerns associated with the use of embryonic cells from destroyed human embryos. Also the use of human induced pluripotent stem cell lines carries the risk of transmitting possible disease hereditary traits from a donor.

It has been demonstrated that *in vitro* generation of β-like cells is possible also with iPSCs derived from patients with T1D and T2D [87–89] and maturity-onset diabetes of the young [90–92]. It is hoped that generation of hiPSCs derived *e.g.* from fibroblasts of patients with diabetes [93] will improve our understanding of the underlying genetic abnormalities, provide targets for high-throughput screening of thousands of molecules as possible therapeutics, and offer personalized treatment by autologous transplantation, since the allogenic transplantation of hPSC-derived β-like cells is likely to induce an immune response. The advancements in the engineering of encapsulation devices that allow for the contact of the transplanted cells with the invading blood vessels but protect them from contact with the immune cells provide hope that these obstacles could be overcome in the near future [94–97]. Obviously, we need more clinically relevant information from the ongoing clinical trials of the diabetes treatment with hESC- or hiPSC-derived β-like cells regarding the functionality of the transplanted cells and immune reactivity of the recipients. Since all these important problems are also relevant for the β-like cells obtained from *in vitro* direct transdifferentiation of differentiated somatic cells, they will be discussed in our next review paper.

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