Emerging routes to the generation of functional β-cells for diabetes mellitus cell therapy

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Abstract | Diabetes mellitus, which affects more than 463 million people globally, is caused by the autoimmune ablation or functional loss of insulin-producing β-cells, and prevalence is projected to continue rising over the next decades. Generating β-cells to mitigate the aberrant glucose homeostasis manifested in the disease has remained elusive. Substantial advances have been made in producing mature β-cells from human pluripotent stem cells that respond appropriately to dynamic changes in glucose concentrations in vitro and rapidly function in vivo following transplantation in mice. Other potential avenues to produce functional β-cells include: transdifferentiation of closely related cell types (for example, other pancreatic islet cells such as α-cells, or other cells derived from endoderm); the engineering of non-β-cells that are capable of modulating blood sugar; and the construction of synthetic 'cells' or particles mimicking functional aspects of β-cells. This Review focuses on the current status of generating β-cells via these diverse routes, highlighting the unique advantages and challenges of each approach. Given the remarkable progress in this field, scalable bioengineering processes are also discussed for the realization of the therapeutic potential of derived β-cells.

Artificial pancreas
A mechanical device devoid of cells that integrates glucose sensors with insulin pumps to dispense insulin as needed with minimal input from the patient.

More than 463 million people are affected by diabetes mellitus globally, which is projected to rise to 700 million by 2045 (REF.[1]). Diabetes mellitus therefore constitutes a global epidemic and is a pressing and growing health problem. The disease not only affects the quality of life of patients and their families but also exerts a tremendous burden on health-care systems across the world. For example, the economic burden in the US alone was estimated at $327 billion in 2017, up from $245 billion in 2012 (REF.[2]).

Diabetes mellitus is a chronic condition characterized by abnormal glucose metabolism due to insufficient production of the pancreatic hormone insulin. Patients are classified into two main categories: type 1 diabetes mellitus (T1DM) is mediated by an autoimmune destruction of insulin-producing β-cells, whereas type 2 diabetes mellitus (T2DM) ensues when β-cells are unable to meet the increased physiological demand for insulin. Currently, there is no cure for diabetes mellitus and exogenous insulin administration is essential for the treatment of all patients with T1DM and those with late stage T2DM.

Although life saving, the current method of insulin delivery via subcutaneous injection does not mimic the fine temporal glucose control provided by the endogenous insulin-producing β-cells and other islet cells of the pancreas. Large population studies have shown that tight glucose control is essential to prevent not just hypoglycaemia that can result in coma and death[3], but also long-term microvascular and macrovascular complications stemming from hyperglycaemia[4–6]. Technological advances, such as continuous glucose monitoring and the artificial pancreas, have improved patient outcomes[7,8], yet these tools still fall short in providing optimal long-term glycaemic control as measured via HbA_{1C}[8–10]. Results from a multicentre trial indicated that the percentage of time that blood glucose is maintained within the target glycaemic range remains suboptimal, even after adoption of automated methods of insulin delivery in patients with T1DM[11]. Acceptance of these technological advances is also fairly low, as patients are hesitant to wear several bulky devices such as sensors, pumps and monitors, all at once. Thus, although recombinant insulin provides a life-saving short-term solution, therapies resulting in permanent reconstitution of physiological blood glucose homeostasis are highly desirable in the long-term.

We propose that a central component of therapies for diabetes mellitus should be the restoration of the missing and/or dysfunctional pancreatic β-cells, the underlying cause of both T1DM and T2DM. The remarkable success of islet transplantation, which leads to insulin

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Key points

- Recent advances in human stem cell differentiation protocols enable the generation of mature β-cells with dynamic insulin secretion and metabolic properties akin to primary human β-cells.
- In addition to β-cells, other hormone-expressing islet cell types are generated under current differentiation protocols.
- The unlimited source provided by stem cell-derived β-cells and islet clusters would address the current scarcity in cadaveric donor tissues for islet transplantation, and sophisticated gene-editing tools could be used to cloak them against immune attack.
- Transdifferentiation of endogenous non-β-cells to insulin-producing cells could be exploited as an alternative strategy to increase the number of functional β-cell equivalents.
- Bioreactors are emerging as technologies for enabling diabetes mellitus cell therapies; these platforms allow precise control of critical cultivation factors for optimized large-scale stem cell differentiation towards functional islet cells.

independence in patients with T1DM for several years[12], furnishes the proof-of-principle for cell replacement approaches; however, its widespread application is currently impractical given the scarcity of donor tissue. These results have fuelled efforts to generate functional β-cells, either by inducing endogenous regeneration, or via differentiation of human pluripotent stem cells (hPSCs). In addition to their therapeutic potential, functional β-cells could serve as valuable tools for advancing the still limited knowledge of human β-cell biology.

Here, we review current efforts to generate functional human β-cells from diverse sources. First, we define the key features of a functional β-cell that dictate its identity and highlight differences between β-cells from neonates, children and adults. Next, we discuss several promising avenues to generate functional β-cells. The main focus of this section is on advances in the area of directed differentiation of hPSCs into β-cells; however, other approaches are highlighted as well, including the transdifferentiation of various somatic cells into insulin-producing cells and the engineering of synthetic cells that mimic the functions of β-cells. Finally, we summarize the challenges associated with the mass production of β-cells from hPSCs in scalable bioreactors, the applicability of quality-by-design concepts and downstream processing required to generate the final 'transplantable product'.

What constitutes a functional β-cell?

Islets of Langerhans are specialized micro-organs residing in the pancreas that are responsible for tightly regulating blood sugar levels through the coordinated release of hormones. The β-cells in islets rapidly sense increases in blood glucose concentrations after a meal and release appropriate amounts of insulin, thereby enabling sugar uptake by the liver and peripheral tissues. Upon the lowering of blood glucose levels, mature β-cells respond by terminating insulin secretion[13]. By contrast, islet-resident α-cells release glucagon that stimulates hepatic gluconeogenesis and glycogenolysis, thus raising blood glucose levels and preventing dangerous hypoglycaemia. Inhibition of insulin and glucagon secretion is mediated via negative feedback by a third islet hormone, somatostatin, released by adjacent δ-cells[14]. These finely tuned processes together maintain an average blood glucose concentration at <5.6 mM in a healthy adult.

The machinery in β-cells is primed to sense extracellular glucose and to rapidly secrete insulin in real time, in a glucose concentration-dependent manner. Once glucose enters the β-cell via specialized glucose transporters, the carbohydrate is promptly metabolized via glycolysis to pyruvate, which is shuttled into the mitochondria for oxidative phosphorylation (OxPhos). The ATP generated in the process changes the phosphate potential (ATP to ADP ratio) of the cell leading to closure of ATP-sensitive K^+ (K_{ATP}) channels, which in turn causes membrane depolarization and the influx of calcium ions (Ca^{2+}) from both the extracellular environment and intracellular stores. The increase in Ca^{2+} concentration induces translocation of insulin-packed secretory granules to the plasma membrane, vesicle–membrane fusion and release of the hormone. This rapid K_{ATP}-dependent insulin secretion is further sustained and augmented by 'amplifying' pathways, which are K_{ATP}-independent but β-cell metabolism-dependent, thereby leading to the observed biphasic glucose-stimulated insulin secretion (GSIS)[15–19].

The metabolic configuration of β-cells is attuned to couple insulin secretion to glucose metabolism. Accurate glucose sensing is facilitated in β-cells by the low-affinity (high K_M) glucose sensor — glucokinase (GCK) — instead of other hexokinases (for example, HK1 or HK2) present in other tissues, as well as the low-affinity (high K_M) transporter GLUT1 (GLUT2 in rodents). Similarly, proteins that interfere with the stimulus–secretion coupling are suppressed in mature β-cells. Such proteins include lactate dehydrogenase (LDHA), which shunts glucose to lactate instead of pyruvate, or the monocarboxylate transporter 1 (encoded by *SLC16A1*), which mediates pyruvate and lactate efflux from the cell[20,21]. Notably, these 'disallowed genes' (*HK2*, *HK1*, *LDHA* and *SLC16A1*) are expressed at higher levels in immature β-cells and/or neonatal islets that hyper-secrete insulin at low glucose concentrations[20,21]. Thus, a switch in gene expression occurs during maturation of β-cells, where disallowed genes are repressed and those essential for insulin transcription (for example, *NEUROD1, NKX6–1, PDX1, PAX6, MAFB, MAFA* and *GLIS3*), processing and packaging (*SLC30A8, CHGA, CHGB, PCSK1/3* and *PCSK2*) and secretion (*ABCC8, KCNJ11, KCNK3, GCK* and *GLUT1*) are upregulated[22]. In particular, the transcription factors NKX6.1, NEUROD1, MAFA and PAX6 facilitate maturation and maintain β-cell identity[23–26]. Chromogranin-A, chromagranin-B (encoded by *CHGA* and *CHGB*, respectively), PCSK1/3, *PCSK2* and ZnT8 (encoded by *SLC30A8*) are localized in the dense core insulin granules. Furthermore, chromogranin-A and chromogranin-B are involved in the generation of insulin granules, ZnT8 is a transporter that specifically imports zinc, which is essential for crystallization of insulin, and PCSK1/3 and PCSK2 are prohormone convertases that process proinsulin to insulin. K_{ATP} channels, the upsteam mediators of secretion, consist of four sulfonylurea receptor 1 (encoded by *ABCC8*) subunits and four KCNJ11 subunits.

Of note, immature β-cells (present in neonatal islets) display a greater sensitivity to calcium at low basal glucose concentrations, which causes increased basal

insulin secretion and poor GSIS. Calcium sensitivity itself is plastic and decreases during β-cell maturation in mice, via rising levels of synaptogamin 4 (REF.[27]). Additional intracellular mechanisms and processes essential for full β-cell functionality are listed in BOX 1.

β-cell maturation

Neonatal β-cells (from infants <1 year of age) are immature in that they secrete insulin at low glucose concentrations, thus having a reduced glucose threshold for GSIS. Unlike neonatal β-cells, juvenile β-cells (from children between 1 and 9 years of age) behave like cells from adults in dynamic GSIS assays, except that they release lower quantities of insulin[28]. Like adult β-cells, juvenile β-cells also respond to stimulation with various secretagogues[29–31], thereby indicating that human β-cells are functionally mature by 1 year after birth.

Despite their similarities in function, deep sequencing reveals differences between the transcriptomes of juvenile and adult β-cells. For example, although the transcription factor MAFB is expressed at comparable levels in both populations, the other closely related member of the family — MAFA — is highly expressed only in adult cells[28]. In addition, proliferative markers are reduced and transcription factors such as SIX2 and SIX3 that mediate insulin secretion are enriched in adult β-cells[28]. Whether transcription factors that are enriched in adult β-cells have other roles besides conferring increased secretory capacity is unclear. Further studies are needed to elucidate additional changes in β-cell functionality and/or physiology that might occur upon assuming the adult state, including the observation made in a 2020 study that adult β-cells contain lipid droplets, whereas juvenile islets do not[32].

Interestingly, despite being fully differentiated, adult β-cells are heterogeneous with regard to their insulin secretory capacities, mitochondrial function, calcium signalling and proliferative properties. Maturity is thus not solely defined by the expression of key markers such as PDX1, NKX6.1 and MAFA, or the presence of high insulin expression. For example, a diverse subtype of adult β-cells known as 'hub' cells coordinate and synchronize islet-wide calcium and insulin secretory responses. These cells display hyperpolarized mitochondria and high expression of GCK but contain reduced levels of insulin, PDX1 and NKX6.1 (REF.[33]). Furthermore, two subpopulations of adult β-cells are distinguished by the novel Wnt/PCP effector Flattop (FLTP) and have distinct functional properties. FLTP$^-$ β-cells are more proliferative and respond to physiological demands such as pregnancy and convert to FLTP$^+$ β-cells, which are more glucose-responsive[34]. This plasticity is becoming apparent among various β-cell subtypes and is essential in maintaining islet functionality during metabolic stress and diseased states, as detailed elsewhere[35]. What controls the plasticity and interconversion of subtypes is less clear and requires further investigation.

Box 1 | **Properties of a functional β-cell**

- Dynamic biphasic insulin secretion upon stimulation with nutrients[147]
- Responsiveness to signals from the gut and nervous system[148,149]
- Rapid shutdown of insulin secretion upon removal of stimulus[147]
- Expression of the full component of genes regulating insulin synthesis, packaging into granules, glucose sensing, stimulus–secretion coupling and insulin exocytosis[150]
- Repression of disallowed genes that could cause aberrant insulin secretion[20]
- Induction of mitochondrial oxidative phosphorylation[52,151]
- Active mitochondrial redox shuttles for the production of mitochondrial coupling factors[152]
- Formation of SNARE protein complexes involved in docking and exocytosis of insulin granules, and genes regulating proper calcium sensitivity[27,153]
- Emerging evidence of co-secretion of amylin and urocortin 3 with insulin to regulate the activity of other endocrine cells in the islet[13,153]

Paths to a functional β-cell

Several routes are envisioned to generate functional β-cells from progenitor cells. One option is to replicate the signalling events that control β-cell formation during human pancreas development. Another is to exploit the plasticity of closely related endoderm-derived cell types such as pancreatic non-β-cells, and cells residing in the liver, stomach and intestine, coaxing them to adopt a β-cell phenotype. Lastly, the concept of engineering a synthetic 'cell' that possesses the functional properties of a bona fide β-cell is gaining traction, given accumulating advances in biomolecular engineering.

Directed differentiation from hPSCs. Human PSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Human ESCs (hESCs) were first derived from the inner cell mass of blastocysts more than two decades ago and are capable of extensive self-renewal and differentiation to cell types of all three germ layers[36]. By contrast, iPSCs are obtained by reprogramming somatic cells such as peripheral blood mononuclear cells or dermal fibroblasts to the pluripotent state using defined factors[37,38]. Human PSCs ushered in the era of regenerative medicine, promising an unlimited source of all types of therapeutic cells, including pancreatic β-cells. Since their discovery, several groups have invested considerable effort and resources in trying to generate functional β-cells from these cells. In early studies, hESCs were cultured as embryoid bodies that allow spontaneous differentiation to occur. Importantly, scattered insulin-positive cells were observed in such cultures, thereby proving the possibility of β-cell formation from hESCs[39,40]. However, spontaneous differentiation methods also meant that the direction of differentiation was largely uncontrolled.

Later efforts focused on translating the knowledge gleaned from mouse embryonic development and signalling pathways to direct the specification of hESCs towards the pancreatic lineage in a stepwise manner. Directed differentiation entails the exposure of hESCs to physiologically relevant cues, which prompts fate transition through definitive endoderm[41] and pancreatic endoderm[42] to hormone-expressing cells[43,44]. Most of the resulting cells generated early on were polyhormonal (for example, simultaneously expressing both insulin and glucagon), thus resembling immature endocrine cells rather than mature islet cells[45]. Additional signs of immaturity included the absence of essential β-cell markers such as the NKX6.1 transcription factor. Further

optimization of directed specification protocols led to the emergence of monohormonal insulin-producing cells, co-expressing NKX6.1 (REFS[46–48]) through modification of the composition and the timing of addition of the soluble signalling factors, as well as utilization of small molecules with less variable activity than regular growth factors and cytokines. Transplantation of these insulin-positive cells in streptozotocin-treated rodents reversed diabetes within 40 days of engraftment[46]. This finding was a considerable improvement over previous reports wherein prolonged engraftment of pancreatic progenitor cells for more than four months was necessary to rescue diabetes[42,49]. However, the β-cells obtained from these protocols were only marginally functional in vitro, exhibiting partial GSIS, and did not resemble fully mature human β-cells[46–48].

Single-cell RNA sequencing (scRNA-seq) has provided a more comprehensive view of the wide variety of populations obtained in hPSC differentiations[50,51]. Surprisingly, in addition to β-cells, two major fractions of cells present were α-like cells expressing markers such as *GCG*, *ARX* and *IRX2*, but also *INS* and enterochromaffin-like cells normally found in the intestine expressing markers including *CHGA, TPH1, LMX1A* and *SLC18A1*. The enterochromaffin cells might represent a previously unknown lineage of the pancreas or their appearance in β-cell cultures indicates the need for stricter control over hPSC commitment to the pancreas versus the intestine to obtain organ-specific endocrine cells. A population of non-endocrine (*SOX9*$^+$) cells was also detected[50]. These *SOX9*$^+$ cells could constitute either uncommitted pancreatic progenitors or cells fated to the pancreatic ductal tree. The heterogeneity in the composition of cells obtained at the end of the stem cell differentiation process, spanning various lineages not present in native human islets, suggests that enrichment strategies to form islet-like clusters as well as additional steps for improving the efficiency of differentiation must be taken.

Exciting advances in the field in 2019 have led to the generation of hESC-derived β-cells that display dynamic insulin secretion properties that largely mirror those of native human islets[52,53] (FIG. 1). Islets consist of pseudo-epithelial hormone-producing cells, whose optimal function necessitates close contact with other islet cells. The native architecture of islets is largely recapitulated in 3D rather than 2D differentiation cultures. Moreover, sorting and re-aggregation of stem cell-derived immature endocrine cells at the final stage of differentiation more closely mimic conditions that exist during embryonic islet formation[52]. In addition to dynamic function, β-cells derived under these conditions exhibit active calcium signalling, functional K_{ATP} channels and mitochondrial OxPhos upon glucose stimulation[52]. Specifically, endocrine cell clustering induces metabolic maturation by activating mitochondrial respiration, a central component of stimulus–secretion coupling in mature β-cells. Another key feature of the latest differentiation approaches is the inclusion of steps to reduce cell cluster size during the process[52,53]. Elimination of TGFβ inhibitors, usually added to differentiating cultures, following re-aggregation[53] was also found to increase maturation. Of note, there are conflicting reports indicating that TGFβ signalling either supports[54,55] or impairs GSIS[56].

A serious concern with stem cell-derived therapeutic products is the presence of undifferentiated or partially differentiated cells that might interfere with the activity of the desired cell types or even be tumorigenic. Optimizing the generation of desired cell populations, whilst minimizing that of unwanted cell types during in vitro differentiation, is critical for clinical translation. Clinical trials investigating the use of transplantation of pancreatic endoderm have been initiated in patients with T1DM; however, in vivo maturation of cells from such early stages of differentiation in animal models is highly variable and depends on the site of transplantation, delivery device and the circulatory microenvironment[57–59]. Preliminary results from the first clinical trial with pancreatic endodermal cells in immunoprotective macroencapsulation devices highlighted the difficulties for this approach with minimal tissue engraftment and differentiation into insulin-producing cells[60]. In a subsequent trial, modification of the device allowing direct vascularization of the engrafted cells resulted in better differentiation; however, the level of insulin production and the number of insulin-positive cells remained low[61]. Grafts in mice from further differentiated hormone-expressing populations can also form cysts, which are structures consisting of cells with duct properties that might continue to grow over time, as long as progenitor cells are present in the final mix[52,62]. These observations argue for the need to generate fully mature endocrine cells for cell therapies.

Given the inability to differentiate cells at 100% efficiency, sorting strategies to yield cell clusters that contained only differentiated cell types resembling native islets were implemented in studies published in 2019. For example, using a GFP reporter under the control of the insulin promoter or antibodies against the cell surface marker CD49a (otherwise known as ITGA1), stem cell-derived β-cells can be enriched by more than 80–90%[50,52]. Although the strategies were designed to purify β-cells, they in fact resulted in enrichment of immature pan-endocrine cells that are capable of giving rise to other hormone-producing islet cell types as well. Increasing evidence points to intricate regulation of glucose via the interplay of α-cells, β-cells and δ-cells[63]. Arguably, generating the full spectrum of islet endocrine cells, rather than just β-cells, is likely to improve glucose control upon transplantation into patients with diabetes mellitus. Directed differentiation of hESCs towards α-cells, albeit immature, has been reported[64], and similar efforts are underway to generate δ-cells (BOX 2). The ultimate goal of assembling the various islet cell types into a functional unit with defined size, architecture and composition that resembles the endogenous human islet, would benefit from the identification and use of cell surface antibodies specific to each endocrine cell type.

In conclusion, the aforementioned advances in stem cell-derived β-cell differentiation protocols underscore the need to finely tune fate decisions, in order to obtain highly pure populations of endocrine cells. The latest

Macroencapsulation devices
Sealed devices constructed out of a selectively permeable membrane that are filled with cells either free floating or in a matrix, wherein the cells can still exert their therapeutic effect.

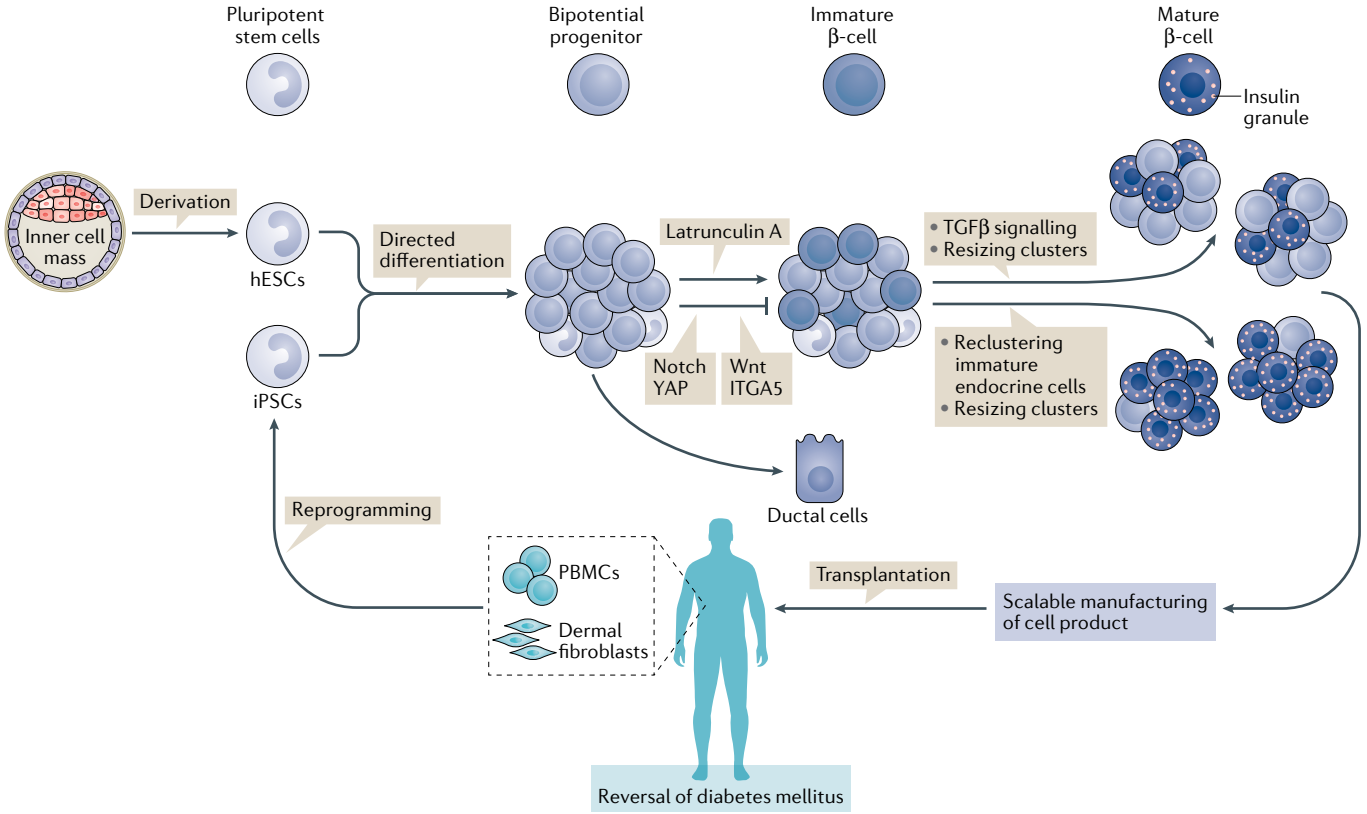

Fig. 1 | **Advances in the generation of mature β-cells from hPSCs and their application for diabetes mellitus cell therapy.** Human pluripotent stem cells (hPSCs) include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived from the inner cell mass of blastocyst stage embryos and induced pluripotent stem cells (iPSCs) are obtained by reprogramming somatic cells of patients such as peripheral blood mononuclear cells (PBMCs) or dermal fibroblasts. hPSCs can be converted to mature β-cells by directed differentiation through modulation of signalling pathways active during human pancreas formation. Efforts from 2015 onwards have focused on further promoting endocrine commitment from bipotential progenitors by inhibition of actin polymerization[126], YAP[125,145], Wnt[146], ITGA5[125] or Notch, and by adding steps that closely mimic islet formation in vivo to the final stage of differentiation. Isolation of immature endocrine cells and re-aggregation into smaller islet-like assemblies promotes functional and metabolic maturation[52]. New surface markers have been discovered that allow enrichment of endocrine cells[50]. Resizing of clusters and removal of TGFβ inhibitor after re-aggregation causes β-cells to acquire dynamic insulin secretion properties[53]. Ultimately, mature β-cell products need to be manufactured at the clinical scale and transplanted in immunoprotective devices and/or with immunosuppression to reverse diabetes mellitus in patients. hESCs, human ESCs.

protocols permit the generation of other islet hormone-producing cells in addition to β-cells, and reconstructing the functional equivalents of human islets from stem cells for cell therapy seems within reach.

Transdifferentiation from closely related cell types. The pancreas arises from the posterior foregut region of the developing embryo, an area that also gives rise to the posterior stomach, liver and proximal gut[65]. Given the close ontogenetic relationship among these tissues, the potential for interconversion between cell types from these regions is not surprising (FIG. 2). Plasticity is especially pronounced between pancreatic α-cells and β-cells[66–70]; for example, inhibition of ARX or overexpression of PAX4, MAFA and PDX1 in mice induces α-cell to β-cell conversion, suggesting a possible alternative approach to replacing β-cells in diabetes mellitus. A 2019 study demonstrated such plasticity in human α-cells by lineage tracing and reprogramming with MAFA and PDX1. The converted human insulin-producing cells retained α-cell features as evidenced by transcriptome and proteome analysis, including the expression of ARX but secreted insulin and reversed diabetes for 6 months in mice[71].

Of note, α-cell to β-cell transdifferentiation was also reported to occur naturally upon β-cell loss in mice from puberty to adulthood, although almost complete ablation of β-cells is required to elicit this response[72]. By contrast, prior to puberty, β-cell loss upon injury is compensated for by conversion of somatostatin-producing δ-cells[73]. β-Cells also originate from α-cells located at distinct niches within the periphery of rodent islets[74]. Furthermore, treatment of mice with GLP1-expressing adenovirus, or an α-cell line with GLP1 agonists led to proliferation of α-cells and their conversion to new β-cells[75]. These studies were, however, conducted in rodents and hence the applicability of the findings to human cells needs to be ascertained.

Long-term administration of γ-aminobutyric acid (GABA) mediated the neogenesis of β-cells from human islet α-cells transplanted in mice, paving the

Box 2 | **Advances in the generation of non-β-cell islet cell types from hPSCs**

- Human embryonic stems cells (hESCs) have been differentiated into α-cells via a 4-week, six-stage protocol[64]. About 65% of cells were monohormonal for glucagon at the end of the differentiation process. Cells also expressed ARX, a key α-cell transcription factor.
- Low glucose levels, arginine, potassium chloride or carbachol triggered glucagon secretion. Conversely, treatment with a somatostatin analogue or high glucose levels suppressed glucagon release.
- Glucagon secretion was induced in vivo upon arginine challenge and transplanted cells maintained the α-cell phenotype in grafts.
- The production of GLP1 and GLP2 derived from proglucagon indicates the immature state of hESC-derived α-cells.
- Efforts to develop protocols to generate δ-cells, ε-cells and pancreatic polypeptide cells are underway.

hPSCs, human pluripotent stem cells

way towards clinical trials[76]. A supporting report showed that artemisinins, an anti-malarial class of drugs, disrupt α-cell identity in immortalized rodent cell lines by inhibition of ARX and by increasing GABA signalling[77]. Unfortunately, the success of this approach is not yet clear, as other studies confirmed the inhibition of ARX after artemether (a derivative of artemisinin) treatment but did not find any α-cell to β-cell conversion in primary mouse islets[78,79]. The contradictory results could be due to GABA-stimulated neogenesis of endocrine cells from ducts rather than direct α-cell to β-cell transdifferentiation. Moreover, artemisinins were used on immortalized cell lines rather than primary islet cells in the initial study[77]. These discrepancies warrant efforts towards the discovery of novel regulators for efficient and reliable human α-cell to β-cell transdifferentiation.

Similarly, the exocrine cells of the pancreas retain a degree of plasticity. Several groups have proposed the existence of progenitors among the ductal epithelial tree that serve as a source of islet tissue expressing markers, such as CD133, CD49f^{hi}, DCLK1 and ALDH^{hi} in humans[80–83]. Details on various factors that induce duct to β-cell transdifferentiation is reviewed elsewhere[84]. In vivo reprogramming of acinar cells to β-cells was also found following injection of an adenovirus delivering a cocktail of the key β-cells genes *Pdx1*, *MafA* and *Ngn3* (referred to here as PMN-cocktail) into mice[85]. In addition, experiments with human acinar cells showed promising results upon addition of *PAX4* to the PMN-cocktail and suppression of *ARX*; however, the conversion rate remained low[86,87].

The transdifferentiation of liver cells to β-cells following ectopic overexpression of *PDX1* has been studied extensively in mice[88,89] and in cultured human hepatocytes[90]. Human hepatocytes can assume a partial β-cell phenotype and ameliorate hyperglycaemia in a mouse model of streptozotocin-induced diabetes mellitus[90]. Certain subpopulations of liver cells seemingly have a predisposition for transdifferentiation to β-cells and active Wnt signalling is obligatory for retaining this plasticity[91]. In line with the close lineage association between hepatocytes and pancreatic cells, induced expression of TGFβ-induced factor homeobox 2 (TGIF2) activated the pancreatic progenitor programme in mouse adult hepatocytes. TGIF2 induced the expression of markers such as PDX1 and SOX9, and the cells further differentiated into glucagon-producing and insulin-producing cells when co-cultured with mouse embryonic pancreas explants[92]. Also other tissues of the extrahepatic biliary tree are susceptible to transdifferentiation into β-like cells ex vivo after treatment with the PMN-cocktail and *PAX6* overexpression, for example, human gallbladder and cystic duct[93].

The intestinal and antral stomach niches are rich in endocrine cells that possess a high degree of similarity to pancreatic β-cells. Ablating the Forkhead box protein O1 (FOXO1) transcription factor specifically in enteroendocrine cells gave rise to functional β-like cells that could revert streptozotocin-induced diabetes mellitus in mice[94]. The gastrointestinal tract is considered an immune-privileged site, which raises the possibility that reprogrammed insulin-producing cells in the gut of patients with T1DM could potentially evade immune rejection[95]. Gut-resident immune cells might also induce systemic tolerance to insulin. Similarly, either in vivo reprogramming of stomach cells with the PMN-cocktail or transplanting in vitro reprogrammed bioengineered stomach-organoids suppressed hyperglycaemia in streptozotocin-induced diabetic mice[96]. Interestingly, in contrast with reprogrammed intestinal cells, the reprogrammed stomach cells expressed NKX6.1 and were monohormonal[97].

Despite these promising findings, transdifferentiating non-β-cells into insulin-producing cells raises several unresolved questions. It is unclear how similar reprogrammed cells are to endogenous β-cells. For example, whether these cells express all the key β-cell factors remains to be elucidated. Furthermore, important functional aspects of reprogrammed cells remain to be determined; such as whether the cells terminate insulin secretion under low glucose conditions, or if the cells respond to various physiological stimuli such as an increased metabolic demand. In addition, further research is required to understand whether reprogrammed cells are locked into their new differentiation state or whether can they relapse to their prior fate. Moreover, the safe adoption of viral reprogramming techniques to the human setting has to be explored further. Small molecules for reprogramming are more attractive than virus-based methodologies, but they are difficult to develop for the regulation of several transcription factors. In addition, a limitation to clinical translation of the transdifferentiation approaches is the low conversion rate to insulin-secreting cells.

Synthetic β-cells. Beyond the approaches described thus far, an emerging concept centres on forming a β-cell-like cell de novo using just the key elements required for proper functionality. A 'synthetic cell' can be defined as a cell or a bioengineered particle with rudimentary components that are sufficient to perform one or several important functions of a specialized cell. β-Cell-mimetic designer cells were developed by engineering human embryonic kidney 293 (HEK293) cells with a glucose-sensing system based simply on glycolysis; glucose sensitivity was conferred by linking an ectopically

TGFβ-induced factor homeobox 2
A transcription factor whose expression separates the pancreatic from the liver lineage early in embryonic development.

expressed Ca^{2+} channel, Cav1.3, to the glucose-sensing system. In turn, Ca^{2+} entry was coupled to an excitation–transcription system that controls transgenic expression of insulin or GLP1. Remarkably, insulin-expressing and GLP1-expressing glucose-inducible designer cells ameliorated hyperglycaemia and improved insulin secretion in rodent models of T1DM and T2DM, respectively[98]. Other examples of designer cells include an engineered system with only glucose homeostasis-modulating properties without glucose-inducibility, such as HEK293 cells with light-inducible expression of GLP1. These cells improved glucose tolerance following a glucose challenge in T2DM mice[99,100]. In summary, although synthetic β-cells are a promising approach, as yet, they have been unable to fully restore normoglycaemia in mouse models. Also, the long-term effects of transplanting these proliferative somatic cells (for example, HEK293) remain to be evaluated.

Simpler than designer cells are acellular bioengineered constructs. These are non-living biomimetic assemblies, for example, vesicles carrying a drug payload or cell membrane-cloaked nanoparticles. Particles delivering insulin dynamically in response to glucose concentrations could theoretically act as β-cell surrogates for diabetes mellitus therapy. Eliciting a specific activity in response to external stimuli is a highly complex and difficult to replicate feature of natural cells. Nonetheless, a multilayered vesicle-in-vesicle superstructure was reported that resembles insulin granules enclosed in a cell[101]. The inner vesicles were packed with insulin and the outer vesicle was lined with glucose-sensing moieties (GLUT2). Oxidation of glucose into gluconic acid following its entry into the outer vesicle was mediated by glucose oxidase. The glucose concentration-dependent drop in internal pH mediated the fusion of the inner vesicle to the outer membrane, thereby releasing the insulin 'cargo' in a glucose-stimulated manner[101]. It is worth noting that this assembly replicates only the most basic function of a β-cell, whereas insulin synthesis, amplifying signals and fine control of the relative insulin content

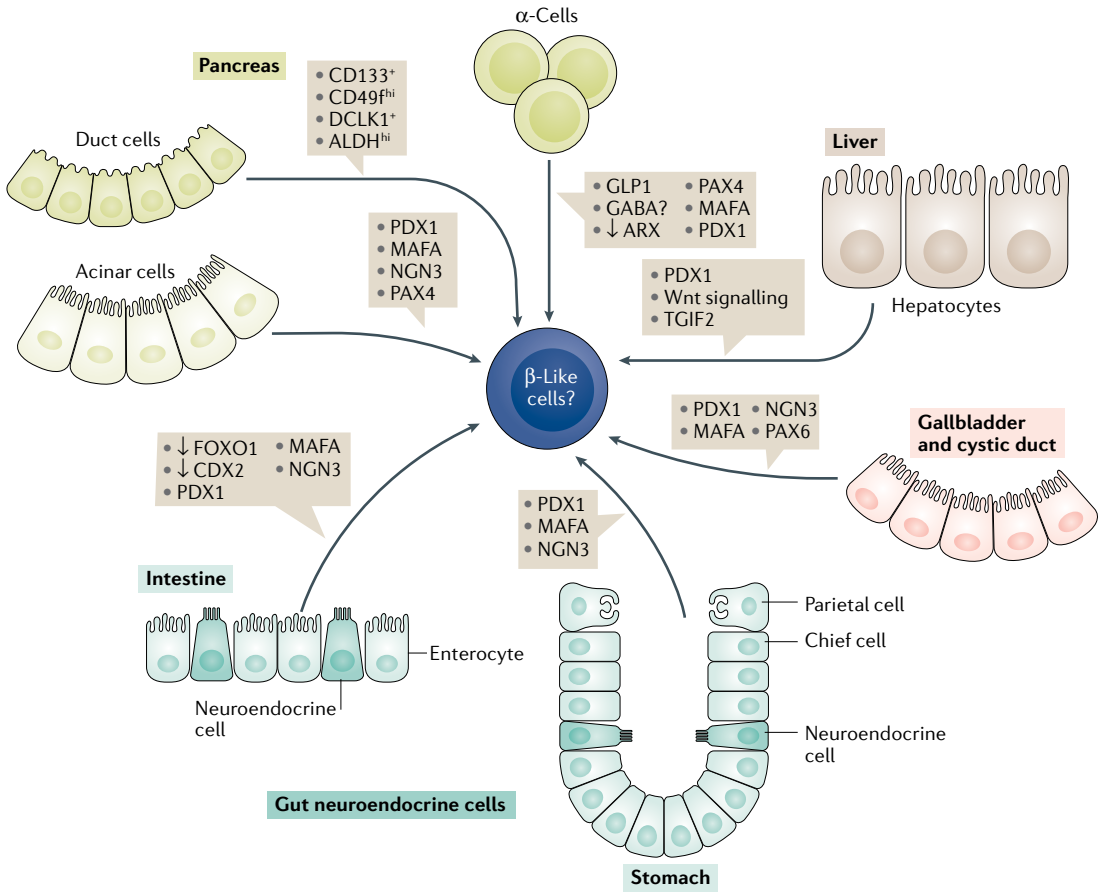

Fig. 2 | **Transdifferentiation of closely related endoderm-derived somatic cells to β-like cells.** β-Like cells can be derived from other pancreatic cell types such as: acinar cells, by adenoviral reprogramming with PDX1, MAFA, NGN3 (referred to as the PMN-cocktail) and PAX4 (REFS[85–87]); from α-cells by overexpression of PAX4, MAFA, PDX1 and inhibition of ARX[70,75,76]; or from progenitor cells expressing CD133, $CD49f^{hi}$, DCLK1 and $ALDH^{hi}$ lining the ductal tree[84]. Hepatic and associated extrahepatic tissues share similar developmental programmes with the adjacent pancreas, and hence activation of few key pancreatic markers such as PDX1 (REFS[88,89]) or TGIF2 (REF.[92]) or Wnt signalling[91] is sufficient to convert liver or gallbladder tissue to β-like cells[93]. Another source of β-like cell generation are the gut neuroendocrine cells that highly resemble pancreatic endocrine cells. Treatment of gastric endocrine or enteroendocrine cells with the PMN cocktail[96,97] in addition to FOXO1 (REF.[94]) and CDX2 inhibition induces transdifferentiation into cells that possess β-cell characteristics.

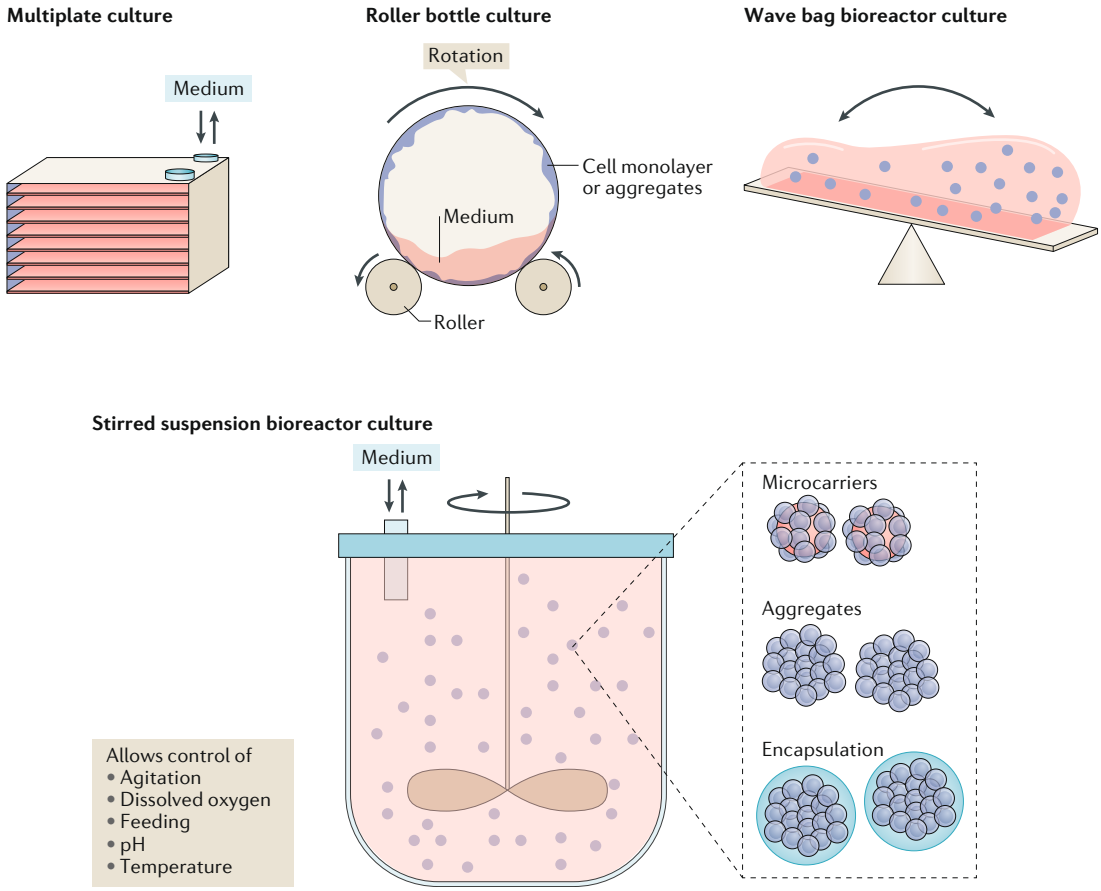

Fig. 3 | **Stem cell bioprocessing for pancreatic islet cell manufacturing.** Multiplate systems, roller bottles, bag bioreactors featuring a rocking motion and stirred suspension bioreactors are candidate cultivation modalities for the generation of clinically relevant quantities of islet cells from hPSCs. Stirred suspension bioreactors afford flexibility as cells can be grown and differentiated as aggregates, on microcarriers or following encapsulation. Moreover, these systems allow monitoring and control of the culture environment.

released are lacking. Yet, such approaches present exciting steps in the direction of building β-cell surrogates that are impervious to autoimmune or alloimmune rejection. Adverse effects such as fibrosis and foreign body-induced response might also be avoided by the selection of appropriate biomaterials.

Bioprocessing and associated challenges

The marked progress in the derivation of functional β-cells from hPSCs has intensified efforts to develop bioprocesses for cellular therapies for diabetes mellitus. However, several challenges must be addressed for the scalable manufacturing of these products at a reasonable cost and of high quality. Present biomanufacturing systems are designed and optimized for making biologics, which differs substantially from the production of cell therapeutics. Whereas in traditional biopharmaceutical processes cells are the means to generate recombinant proteins or vaccines, in stem cell bioprocesses the cells are the actual products. Moreover, the lengthy development of cell lines for high-titre production of biologics might not be applicable to stem cell bioprocessing, which is constrained by time-frames spanning a few weeks; for example, from harvesting cells from patients, to reprogramming and differentiation to the desired progeny.

Various cultivation modalities. Most studies on pancreas specification of hPSCs have been carried out in traditional dish or T-flask cultures. Larger 2D systems that comprise multiple parallel plates (for example, cell factories) have also been used for cell expansion prior to differentiation[102]. Although 2D culture modalities have been employed in clinical protocols[103,104], they are characterized by poor mass transfer of soluble factors that becomes more pronounced as the surface area for growth increases. This issue can be partly alleviated by differentiating hPSCs as aggregates in low-adhesion multi-well plates under orbital stirring[52,102]. Still, these culture vessels are not well suited for current good manufacturing practice production and are limited by a low surface area-to-volume ratio when compared with other cultivation systems, such as stirred suspension bioreactors (SSBs). Roller bottles, which are utilized in the production of recombinant proteins and vaccines, have been accepted as suitable for the clinical manufacturing of pancreatic endoderm cells from hPSC aggregates[105] (FIG. 3). As the scale of roller bottle-based production increases, however, procedures such as medium changes and product harvest become labour intensive with a greater risk of contamination. Another cultivation platform, the wave-agitation bioreactor, is increasingly

Stirred suspension bioreactors
Vessels for cultivation of cells that feature an impeller for mixing, probes for monitoring the culture environment, ports for sampling and exchange of medium, and assemblies for aeration and maintenance of temperature.

employed in the production of cellular therapeutics[106,107] (FIG. 3). In the wave-agitation bioreactor, cells can be cultured in single-use bags with probes for monitoring the culture environment and in volumes suitable for single-patient batch production. To our knowledge, there are no reports to date on generating islet cells from hPSCs using this platform.

SSBs are an appealing modality for the scalable expansion and differentiation of hPSCs to pancreatic islet cells. Self-renewing hPSCs have been successfully cultivated in SSBs as aggregates[108,109], on microcarriers (beads with typical size 100–150 μm made of different materials such as polystyrene, glass, alginate or dextran)[110,111] or after encapsulation (for example, in alginate)[112], permitting various specification regimes to be accommodated in the same vessel. The use of SSBs presents a considerably lower barrier for translating relevant laboratory-scale differentiation protocols to current good manufacturing practice production of hPSC products in a commercial setting, as this bioreactor type is already the workhorse in biopharmaceutical production facilities.

Several reports have focused on optimizing critical parameters for the propagation of self-renewing hPSCs in SSBs, including the seeding concentration, cell passaging, aggregate size distribution and stirring rate[113,114]. As a result, hPSC concentrations of 10^6–10^7 cells/ml of SSB culture have been reported by several groups. Given that insulin independence in humans is noted with the transplantation of 7,000 islet equivalents (IEQs) per kilogram[115], and considering that each IEQ contains ~1,100 β-cells[116], ~5×10^8 β-cells/70 kg body weight are needed for reconstituting normal glucose homeostasis in patients with T1DM. The actual numbers could be three-fold to four-fold more than this estimate after considering quality of cells and loss following engraftment. This estimate suggests that culture volumes of several hundred millilitres to a few litres might be adequate for treating a single patient, after adjusting for the efficiencies of differentiation and downstream processes.

Box 3 | **Quality-by-design attributes for developing bioprocesses for pancreatic cell therapy products**

Quality profile of hPSC-derived β-cells (or islets)

- Potency: glucose sensing, GSIS with first-phase and second-phase hormone secretion
- Identity: insulin-positive, PDX1$^+$, GLUT1$^+$, MAFA$^+$, CHGA$^+$ (pan-endocrine marker), UCN3$^+$
- Safety: exclusion of pluripotent and partially differentiated cells, polyhormonal cells
- Cell quantity: approximately 5×10^8 β-cells/70 kg body weight, or >7,000 IEQ/kg body weight. Numbers may be higher subject to quality of cells/islets
- Cell transplant: allogeneic (encapsulated cells)

Major process variables and parameters involved in quality-by-design and control of envisaged bioprocesses for manufacturing islet cells from hPSCs

- Temperature
- pH
- Dissolved O_2
- Feeding regimen
  - Substrate or substrates
  - Differentiation stimuli
- Cell seeding density and/or microcarrier seeding density
- Aggregate size distribution
- Agitation rate

GSIS, glucose-stimulated insulin secretion; hPSCs, human pluripotent stem cells.

Differentiation in SSBs. In contrast to extensive reports on the expansion of hPSCs in SSBs, fewer studies exist showing coaxing of hPSCs in these systems towards various therapeutically useful cell types (for example, cardiomyocytes[109] or neural cells[117]), particularly to pancreatic cell progeny. Functional human β-cells were generated upon differentiation of ~3×10^8 cells over 4 weeks in 500-ml spinner flasks[47], which are convenient as laboratory-scale surrogates of SSBs. Others have also reported the conversion of hPSCs to pancreatic islet cell progeny in spinner flasks[118]. However, neither continuous monitoring nor active control of bioreactor culture conditions were carried out in these studies, and both are important for reducing batch-to-batch variability. A 2017 study found the growth and differentiation of iPSCs as aggregates towards pancreatic progenitor cells in spinner flasks fitted with sensors for temperature, pH and dissolved oxygen[119]. The pH was maintained (7.2) through adjustments of the CO_2 level inside the incubator and medium exchanges. Dissolved oxygen was regulated by modulating the composition of the gas feed (air, O_2 or N_2). Cell clumps seeded in spinner flasks were maintained at 60% dissolved oxygen during expansion and differentiation to definitive endoderm (6 days). Subsequent differentiation towards pancreatic progenitors (days 6–17) was carried out under 40% dissolved oxygen resulting in 1.6×10^8 cells/100 ml of culture with 22% of the cells co-expressing PDX1 and NKX6.1 (REF.[119]).

In contrast to fully instrumented bioreactors, however, spinner flasks are not ideal for testing various feeding strategies[120] and continuously surveying and regulating culture parameters. This point is particularly important when considering that differentiation is carried out for 20–30 days. Adaptation of existing differentiation regimens to SSB cultivation will require optimization of bioprocess parameters. For hPSC-derived islet cell manufacturing, the implementation of bioreactor scale-up heuristics[121] combined with the rational linking of bioprocess conditions to critical cell product attributes such as identity (for example, expression of β-cell markers), potency (for example, level of GSIS) and purity (for example, minimizing undifferentiated or partially differentiated cells) will be necessary. This process is akin to the quality-by-design framework implemented in the design of processes for the manufacturing of biopharmaceutical products[122,123] (BOX 3). The intensified interest by academia and industry in diabetes mellitus cell therapies increases the likelihood of additional breakthroughs in the mass production of β-cells in the not so distant future.

Bioprocess environment and hPSCs. The expansion of hPSCs in SSBs has largely drawn on protocols used for the cultivation of animal cells (for example, Chinese hamster ovary cells) used for recombinant protein production. Yet, detailed knowledge of the effects of the bioreactor environment on hPSC physiology is

essential, as the cells are the products instead of their secreted molecules. For instance, large gaps still exist in our knowledge of how mechanical cues (for example, agitation-induced shear) can affect cell commitment along a particular lineage trajectory[124]. In 2018, a mechanotransduction cascade was delineated that involves integrin/focal adhesion kinase (FAK) signalling activation of Yes-associated protein 1 (YAP1) as a regulator of bipotent pancreatic progenitors derived from hESCs[125]. Reduction in FAK signalling in the presence of laminin-enriched or collagen-enriched extracellular matrix resulted in endocrine specification. By contrast, FAK activation of YAP1 suppressed endocrine gene expression and enhanced HES1 expression, yielding ductal cells[125] (FIG. 1). Confirming these findings, the depolarized state of the actin cytoskeleton induced by small molecules such as latrunculin A also favoured endocrine differentiation from pancreatic progenitors[126]. Conceivably, the bioreactor environment can be tuned to facilitate commitment towards pancreatic islet cells, for example with the use of xeno-free laminin peptide-coated or collagen peptide-coated microcarriers[127]. Xeno-free factors and culture environment are necessary to avoid the transmission of zoonotic diseases to human patients receiving the cells.

Although most cells situated inside clusters can be shielded from external shear in a bioreactor, the exchange of oxygen, nutrients and metabolic products is hindered between the cells within oversized clusters and the bulk of the culture medium, signifying the importance of aggregate size control. Although attenuated in SSBs compared with static cultures, mass transfer limitations for aggregates >200 μm[128] still remain a problem, during both the stage of expansion of undifferentiated hPSCs, where proliferation rates are high, and the differentiation to pancreatic endoderm. In fact, increased oxygen tension (pO_2) activates β-cell differentiation in cultured pancreatic explants[129], and mouse and human PSCs[130]. These findings warrant the closer examination of how the pO_2 level in a bioreactor influences the commitment of hPSCs to pancreatic islet cells, thereby informing strategies for dissolved oxygen control. Besides proliferation, differentiation imparts other changes to cell physiology, many of which are uncharted to date. Undifferentiated hPSCs rely mainly on glycolysis for utilization of glucose, whereas differentiating cells shift to OxPhos[131]. Even whilst maintained as undifferentiated, cultivation of hPSCs in SSBs induces a switch from glycolysis to OxPhos[120]. In addition, hPSCs also produce more lactate than differentiated cells[132]. Accommodating the dynamic metabolic profile and associated hPSC fate decisions in SSBs will require a shift from the techniques employed in traditional cell culture.

Downstream issues. Downstream processing of hPSC-derived pancreatic cells presents challenges and opportunities for new technologies, since state-of-the-art methods were developed for the separation of molecules (for example, monoclonal antibodies) rather than cells. In fact, the effects of applying current bioprocess procedures for cell separation and retention (for example, acoustic settlers, tangential or alternating tangential flow filtration) to hPSC-derived cells are unknown. Surface markers for sorting β-cells[133] or endocrine cell progenitors following hESC differentiation[50] have been reported. However, existing methods for sorting cells or islets[134] such as fluorescence or magnetic activated cell sorting, lack the throughput necessary for rapid processing of even single-patient cell batches. Cell sorting should also be coupled to online evaluation of functional attributes, for example glucose sensing and biphasic insulin secretion. Incorporation of such online monitoring in the bioproduction will require novel analytical tools given that existing laboratory methods (for example, enzyme-linked immunosorbent assay) for assessing insulin secretion are characterized by long processing times.

Immune modulation

The most obvious application of functional β-cells is in cell-replacement therapy for patients with T1DM or late-stage T2DM. Allograft rejection — and in the case of T1DM, autoimmune rejection — remains a major barrier to clinical translation of therapies derived from stem cell differentiation or transdifferentiation approaches. Despite the remarkable restoration of normoglycaemia upon cadaveric islet transplantation, patients require lifelong immunosuppression with drugs carrying unwanted side effects[135]. Although graft rejection could be avoided with the use of autologous tissues and patient-specific iPSCs in patients with T2DM, the wide-scale application of this approach would be labour intensive and cost prohibitive. To address these concerns, biobanks of a limited number of iPSC lines with the human leukocyte antigen (HLA) types matching the majority of potential recipients in specific ethnic populations are being considered[136–138]. However, challenges still exist in differentiating iPSCs as efficiently as hESCs, and the differentiation propensity of iPSC clones derived from even one individual is highly variable[139].

In addition to allorejection, autoimmunity in T1DM is a serious challenge. Taking advantage of a combination of immunosuppressive drugs, bioengineering advances and gene-editing tools will be necessary to overcome the barriers posed in T1DM. Bioengineering advances include macroencapsulation in devices made of polymers such as polytetrafluoroethylene or polycaprolactone, and/or microencapsulation of β-cells in materials including alginate, polyacrylate, collagen or agarose for immunoprotection[140]. These devices prevent immune attack, but poor vascularization of the grafts as well as delayed insulin release kinetics present major limitations. T cell therapies to induce immune tolerance by activating regulatory T cells (T_{reg}) have shown preliminary success in preclinical studies. For example, graft-specific T_{reg} can be isolated, cultured and expanded ex vivo in therapeutically relevant numbers; however, hurdles still exist that prevent high yields being obtained whilst maintaining purity[141]. The principles used in T cell cancer therapy using chimeric antigen receptors could also be applied to T_{reg} cell therapy to improve tolerogenic outcomes[142]. Another prospect is engineering β-cells with CRISPR–Cas9 gene editing tools to confer immunoavoidance by dismantling the MHC components that

Agitation-induced shear
Shear in the liquid phase of bioreactor cultures arising from spatial gradients of velocity due to stirring.

Chimeric antigen receptors
(CARs). Novel receptors designed to bind to specific proteins on cells (for example, cancer cells). T cells are engineered with CARs to provide new targeting ability.

typically present autoantigens and alloantigens to the immune system. Deletion of the highly polymorphic MHC class 1 genes (*HLA-A*, *HLA-B*, *HLA-C*) can render the transplanted cells hypoimmunogenic and maintaining HLA E/G and/or overexpressing CD47/PDL1 can prevent macrophage and natural killer cell-mediated killing[143,144]. This approach is being pursued as an off-the-shelf or universal cell therapy. However, reducing immunogenicity of the graft also increases the probability of neoplastic growths and pathogenic infections, serious issues confronting the field.

Conclusions

Given the impressive progress in converting hPSCs to β-cells, the path from bench to bedside seems more feasible through the use of hPSC-derived β-cells than transdifferentiation approaches. Improvements in bioprocesses for efficiently manufacturing stable, functionally mature and large quantities of desired cells will accelerate ongoing efforts to develop cell therapy for diabetes mellitus.

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G.G.N. and E.S.T. researched data for the article. All authors made substantial contributions to the discussion of the content, wrote the article and carried out review/editing of the manuscript before submission.

Competing Interests
M.H. is affiliated with Semma Therapeutics (Consultant and SAB member) and Encellin Inc. (SAB member, stock holder). M.H. also holds stocks from Viacyte Inc.. The other authors declare no competing interests.

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