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Authors

Migliorini, Adriana
Nostro, Maria Cristina
Sneddon, Julie B

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Human pluripotent stem cell-derived insulin-producing cells: A regenerative medicine perspective

Adriana Migliorini[1], **Maria Cristina Nostro**[1,2,3,#], **Julie B. Sneddon**[4,5,6,7,#]

[1]McEwen Stem Cell Institute, University Health Network, Toronto, M5G 1L7, Ontario, Canada.

[2]Department of Physiology, University of Toronto, Toronto, M5S 1A8, Ontario, Canada.

[3]Toronto General Hospital, Ajmera Transplant Centre, University Health Network, Toronto, M5G 1L7, Ontario, Canada.

[4]Departments of Cell and Tissue Biology, San Francisco, San Francisco, CA 94143, USA

[5]Anatomy, University of California, San Francisco, San Francisco, CA 94143, USA

[6]Diabetes Center, University of California, San Francisco, San Francisco, CA, 94143, USA.

[7]Eli and Edythe Broad Center for Regeneration Medicine and Stem Cell Research, University of California, San Francisco, San Francisco, CA 94143, USA

Summary

Tremendous progress has been made over the last two decades in the field of beta cell replacement therapy as a curative measure for diabetes. Transplantation studies have demonstrated therapeutic efficacy, and cGMP grade cell products are currently being deployed for first in human clinical trials. In this perspective, we discuss current challenges surrounding the generation, delivery, and engraftment of stem cell-derived islet-like cells, along with strategies to induce durable tolerance to grafted cells, with an eye towards a functional cellular-based therapy enabling insulin independence for patients with diabetes.

Generation of stem cell-derived insulin-producing cells

Since their identification, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs), have been extensively studied and used to generate *in vitro* many of the diverse cell types of the body (Takahashi et al., 2007; Thomson et al., 1998). While a variety of protocols have been

[#]Corresponding authors: cnostro@uhnresearch.ca, julie.sneddon@ucsf.edu.

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developed to drive the differentiation of hPSCs towards the primary germ layers (ectoderm, mesoderm and endoderm), the generation of fully mature cell types is still far from optimal. Despite this challenge, hPSCs hold great promise for the generation of specialized cells for cell replacement therapies that we expect to become mainstream lines of treatment in the years to come. Indeed, hESC- and hiPSC-derived cell products are currently being evaluated in a variety of clinical trials targeting macular degeneration (da Cruz et al., 2018), Parkinson disease (Doi et al., 2020), and type 1 diabetes (T1D) (ClinicalTrials.gov identifier: NCT02239354 and NCT03163511), among other diseases.

This year, we celebrate 100 years since the life-saving discovery of insulin, and 15 years since the landmark publication of the first multistep protocol to generate pancreatic endocrine cells *in vitro* from hESCs (D'Amour et al., 2006). This first protocol combined empirical and informed approaches (based on developmental biology) to generate insulin-producing cells by mimicking normal pancreatic development. While it had its limitations (polyhormonal cells, very low yield, poor functionality), this work marked a turning point for regenerative medicine approaches for diabetes. Notably, it led the way to a novel developmental paradigm aimed at directing the conversion of hESCs into pancreatic epithelial progenitors capable of maturing to endocrine cells *in vivo.* Driven by this approach, hESC-derived pancreatic cells were shown to mature *in vivo* into mono-hormonal, glucose-responsive insulin-producing cells (Jiang et al., 2007; Kroon et al., 2008). These studies provided the first evidence that hESC-derived pancreatic cells may serve as an unlimited supply of pancreatic hormone-secreting cells for diabetes cell replacement therapy and fueled many scientists worldwide to further develop fine-tuned step-wise protocols to direct the successive differentiation of hESCs towards mono-hormonal islet cells.

hESC differentiation protocols have been designed to mimic the specification of pancreatic beta cells during embryonic and fetal gestation (Fig. 1). Beta cell development starts in the embryo with the generation of the definitive endoderm that forms the primitive gut tube and is subsequently followed by posterior foregut patterning and specification of pancreatic progenitors, endocrine progenitors, and finally hormone-positive cells (Zorn and Wells, 2009). Based on this knowledge, enormous work has been done over the last two decades to understand the physiological developmental cues that promote the differentiation at each stage, and to translate these findings into step-wise protocols capable of directly controlling the differentiation of hESCs towards islet-like cells (Fig. 1). Indeed, *in vitro* modulation of the Nodal and WNT signalling pathways in hESCs and iPSCs has been employed to efficiently generate definitive endoderm cells marked by the expression of cell surface endoderm markers CXCR4, SOX17, and FOXA2. To restrict definitive endoderm developmental potential to the pancreatic fate, patterning of the primitive gut tube to the posterior foregut has been achieved by modulating FGF10/KGF signaling. Further, activation of Retinoic Acid (RA), combined with inhibition of BMP and SHH signalling, has been used to specify posterior foregut and generate pancreatic endoderm that expresses PDX1, a key transcription factor required for pancreas development (Nostro and Keller, 2012).

Beyond these early stages of lineage commitment, the lack of more detailed information about how the downstream events of pancreatic specification and endocrine commitment are

executed *in vivo* resulted in gaps in knowledge that prevented accurate *in vitro* differentiation of cells to these later developmental stages. To bypass this issue, investigators turned to empirical high-throughput screening (HTS) approaches. Indeed, using HTS techniques, Chen and colleagues reported that activation of the PKC pathway, using the compound Indolactam V either alone or combined with FGF10, was required for many hESC lines to induce the efficient generation of PDX1+ cells (Fig. 1) (Chen et al., 2009). Although there is no report on the role of PKC signaling prior to the formation of the pancreatic bud, it has been speculated that PKC activation may act synergistically with RA to induce PDX1 expression (Chen et al., 2009). Other studies also found that activation of the PKC pathway further enhanced the development of pancreatic cells from hESC-derived posterior foregut cells, while minimizing the formation of hepatic or intestinal lineages (Fig. 1) (Rezania et al., 2012).

The findings from these early studies clearly demonstrated that differentiation occurs asynchronously, resulting in heterogeneous cultures. To address this issue, several groups attempted to purify cells at specific stages of development using cell surface markers, such the anterior definitive endoderm marker CD177 (Mahaddalkar et al., 2020), pancreatic progenitor markers CD142, CD24, and Glycoprotein2 (Ameri et al., 2017; Cogger et al., 2017; Jiang et al., 2011; Kelly et al., 2011), and beta-like cell markers CD49a (Veres et al., 2019) and CD9 (Li et al., 2020). While successful at enriching for desired populations, these purification steps may be challenging to integrate into future large-scale manufacturing processes. Importantly, by sorting for CD142 and enriching for pancreatic endoderm, Kelly et al. demonstrated that mono-hormonal cells arose from a progenitor population co-expressing PDX1 and NKX6-1, consistent with the important role played by NKX6-1 during pancreatic specification and beta cell development (Kelly et al., 2011; Sander et al., 2000).

To gain full control of the developmental paths induced *in vitro*, several groups focused on the optimization of each stage of differentiation to synchronize lineage specification while limiting the development of off-target cells and maximizing the generation of pancreatic progenitors with potential to generate mono-hormonal islet-like cells. In line with this, Rezania et al. proposed a novel protocol to generate pancreatic endoderm enriched for PDX1+/NKX6-1+ cells. By adding Noggin and TPB after PDX1 induction, they reported highly efficient generation of pancreatic progenitor cells at the expense of other non-pancreatic endodermal lineages and minimized teratoma formation after transplantation (Rezania et al., 2013). Importantly, they also validated the capacity of pancreatic progenitors to mature to beta-like cells *in vivo* in a diabetic model. Subsequent studies demonstrated that following PDX1 induction, treatment of cells with EGF, coupled with either nicotinamide or KGF, BMP and WNT inhibition could efficiently generate PDX1+/NKX6-1+ pancreatic progenitor cells from a variety of hESC and hiPSC lines (Fig. 1) (Nostro et al., 2015; Russ et al., 2015; Yung et al., 2019).

In 2014, new reports incorporated beneficial culture conditions (low adhesion; 3D culture in spinner flasks) with a novel combination of small molecules and growth factors to achieve sequential differentiation protocol that generated mono-hormonal, insulin-producing beta-like cells closely resembling primary beta cells (Pagliuca et al., 2014; Rezania et al., 2014). These optimized protocols required approximately 5 weeks to reach the final beta-like cell

stage and involved serial culture steps culminating in the use of factors including TGF-β receptor inhibitors, Notch and BMP signaling inhibitors, and thyroid hormones (Fig. 1). Fine-tuning of these protocols yielded approximately 30-60% beta-like cells and variable frequencies of other endocrine cells, including some poly-hormonal populations. These remarkable findings demonstrated the ability to generate mono-hormonal insulin-expressing cells *in vitro* from hESCs, albeit lacking the dynamic glucose-responsive insulin secretion characteristic of primary beta cells. Nevertheless, compared to previously reported transplantation studies of hESC-/hiPSC-derived pancreatic progenitors, the transplantation of beta-like cells substantially shortened the time to see a therapeutic effect in diabetic models from 4-5 months to 6 weeks (Rezania et al., 2014; Schulz et al., 2012).

Unraveling hPSC pancreatic differentiation using single-cell RNA-Sequencing

Recent advances in bulk and single-cell transcriptional analyses have helped us to understand how close we are to mastering modulation of pancreatic developmental cues *in vitro.* An early study from Petersen et al. performed the first single-cell gene expression profiling of hPSC-derived pancreatic progenitors and their *in vitro* progeny (Petersen et al., 2017). Using a combination of cell sorting and single-cell qPCR, the authors delineated discrete steps along the path towards pancreatic differentiation *in vitro.* Not surprisingly, they identified subpopulations of cells present at each of the pancreatic progenitor, endocrine progenitor, and endocrine cell stages, indicating asynchronous differentiation within the cultures. These data confirmed the hypothesis posited in previous beta cell differentiation studies that several lineage bifurcations can occur during *in vitro* culture, suggesting that multiple paths can be taken to reach the same cell fate. Interestingly, the authors speculated that a population of cells lacking NKX6-1 expression may still commit to the endocrine lineage and generate insulin-expressing cells (Petersen et al., 2017).

Seminal work from Veres et al., reported single-cell transcriptional profiling of the cell types produced during the *in vitro* differentiation of PDX1+ endoderm to beta-like cells (Veres et al., 2019). The authors observed increasing heterogeneity as the differentiation proceeded and identified a previously undetected population of enterochromaffin cells expressing TPH1 and SLC18A1. These cells are not thought to be normally found in the adult or fetal human islet, but do exist within the intestinal epithelium. One possibility for their origin is that some of the PDX1+ endoderm cells could undergo intestinal differentiation at the expense of pancreatic development and in response to endocrine specification, these intestinal cells could upregulate genes involved in enterochromaffin development. These findings highlighted once more the importance of tight control of the differentiation cultures to avoid the generation of off-target cells. Indeed, Andersen et al. performed transcriptomic and epigenomic analyses of pancreatic progenitors derived *in vitro* using modified versions of three different differentiation protocols (Nostro et al., 2015; Rezania et al., 2014; Russ et al., 2015) to assess their similarity to one another and to embryonic development (Wesolowska-Andersen et al., 2020). Gene expression profiles of pancreatic progenitor cells generated with the Rezania 2014 and Nostro 2015 protocols showed significant similarity to pancreatic progenitor cells isolated from human fetal pancreas, suggesting that the timing

and modulation of signalling pathways reported by these protocols indeed recapitulated several aspects of human pancreas development and limited the formation of off-target cells, like CDX2-expressing endoderm. These studies constituted a great resource for the beta cell field, as they provided the transcriptional profiling of progenitor cells and their trajectory to beta-like cells using current differentiation protocols and can support hypothesis-driven experiments to improve differentiation protocols to specific cell types (Wesolowska-Andersen et al., 2020).

From these studies, it was clear that end-stage cultures are not just composed of beta-like cells, but a variety of endocrine cells, uncommited progenitors, and possibly off-target populations, such as the enterochromaffin cells, which could limit further beta cell maturation *in vitro.* To date, the transcriptional profile of hPSC-derived beta-like cells is not equivalent to that of primary beta cells (Augsornworawat et al., 2020; Pagliuca et al., 2014). Transplantation into immunocompromised mice has been shown to improve beta cell maturation, but even after 6 months *in vivo* additional developmental steps need to occur for these cells to acquire a transcriptional signature representative of *bona fide* human beta cells (Augsornworawat et al., 2020).

Improving functionality of hPSC-derived beta-like cells *in vitro*

Various approaches have been taken to enrich for beta-like cells and improve dynamic glucose-responsive insulin-secretion *in vitro,* as this assay has become the gold standard in the field to test for beta-like cell functionality. Beside optimization of medium components designed to activate or repress signalling pathways driving hESC towards pancreatic endocrine commitment, cell culture systems and microenvironment have been observed to affect pancreatic differentiation and maturation. Indeed, recent studies have shown that enrichment for insulin-expressing cells and cytoskeleton manipulation, achieved either by mechanical or chemical stimulation, are beneficial for generating functional hPSC derived beta-like cells *in vitro* (Hogrebe et al., 2020; Nair et al., 2019; Velazco-Cruz et al., 2019). Specifically, using an $INS^{GFP/w}$ hESC reporter line in which *GFP* is expressed under the insulin promoter, Nair and colleagues demonstrated that sorting and re-aggregating for INS:GFP^{+} cells led to the formation of enriched beta cell clusters that showed dynamic glucose responsiveness, but still a lower magnitude of insulin secretion compared to cadaveric islets (Nair et al., 2019). Studies from Velazco-Cruz reported that controlling cellular clusters size combined with removal of ALK5 inhibitor II at the late stages of differentiation can be used to improve dynamic glucose response, without the need to enrich for hESC-derived insulin-expressing cells (Velazco-Cruz et al., 2019). Importantly, these studies showed that prolonged culture conditions and dissociation and re- aggregation of endocrine cells improved beta cell functionality, possibly through cytoskeleton modulation, the depletion of dead cells, and the formation of smaller clusters (Nair et al., 2019; Velazco-Cruz et al., 2019).

With the goal of enriching for insulin-producing cells, single-cell RNA sequencing (scRNA-seq) studies have been applied to identify novel cell surface markers that could be employed for stage-specific sorting strategies to enrich for glucose-responsive beta-like cells. Specifically, scRNA-seq revealed that *CD49a* is expressed at higher levels in hESC-derived

beta-like cells compared to other endocrine cells. Sorting for CD49a was successfully used to enrich for mono-hormonal beta-like cells and resulted in increased glucose-responsivness compared to unsorted populations (Veres et al., 2019). Similarly, scRNA-seq analysis of beta-like cells performed by Xisheng et al., identified a negative correlation between *CD9* and *INSULIN* expression in hESC-derived beta-like cells. In this case, negative selection against CD9 was used to enrich for glucose responsive beta-like cells (Li et al., 2020). Other studies have used a variety of chemical approaches to improve endocrine specification to beta-like cells or to increase beta-like cell functionality after endocrine commitment (Alvarez-Dominguez et al., 2020; Ghazizadeh et al., 2017; Rosado-Olivieri et al., 2019; Sui et al., 2021; Yoshihara et al., 2020). Despite their immaturity, hPSC-derived islet-like cells and pancreatic progenitors have been shown to restore normoglycemia in diabetic models and therefore hold great promise for beta cell replacement therapies in patients with T1D and T2D, as well as patients who have lost beta cell mass due to pancreatitis or cystic fibrosis (Fig. 2).

Current challenges in cell replacement therapy for diabetes

Eligible patient populations

Traditionally, allogeneic islet transplantation has primarily been indicated for the treatment of insulin-dependent patients (T1D and select cases of T2D) with poor metabolic control and hypoglycemia unawareness or with end-stage renal disease (Choudhary et al., 2015). Inclusion criteria typically require a longer than 5 year duration of T1D and age of greater than 18 in order to limit the risks of chronic immunosuppression to pediatric patients, with possible exceptions granted for patients with severe hypoglycemic episodes that cannot be controlled by other means (Hathout et al., 2003; Shapiro et al., 2017). Patients are also often excluded based on high BMI (>30 kg/m^2), weight >90 kg, and/or daily insulin requirement >1.0 U/kg due to the finding that insulin independence has been more difficult to achieve in these patients (Balamurugan et al., 2014; Barton et al., 2012; Shapiro et al., 2017). Among the population of patients who meet these stringent eligibility criteria, only a fraction will undergo islet transplantation due to the shortage of donor islets and the anticipated risks of chronic immunosuppression, which must be weighed against the perceived benefit of islet therapy relative to other treatment modalities. As islet transplantation can lead to a higher degree of metabolic control and improved quality of life, we expect that the advent of functional hPSC-derived material, combined with independence from long-term immunosuppressive therapy, will vastly increase the eligible recipient patient population.

Recent coordination in the transplant community has led to an emerging evidence-based consensus that beta cell replacement therapy could be considered a viable treatment option for patients with beta cell failure (regardless of the etiology of their disease) accompanied with glycemic instability that occurs despite adherence to high-quality medical care (Rickels et al., 2018). Indeed, successful pancreas transplantation of T2D patients suggests that, once unfettered by limitations in supply, beta cell replacement could provide substantial therapeutic benefit for many people with T2D (Kandaswamy et al., 2018; Sneddon et al., 2018). As patients with T2D do not struggle with the challenge of autoimmunity, autologous iPSC-derived beta-like cell transplantation could prove feasible for this population if the cost

of such an approach can be reduced (Fig. 2). Importantly, ongoing concerns remain regarding both how long autologous beta cell grafts would maintain euglycemia in a setting in which insulin dependence has emerged and whether autologous hiPSCs have acquired rejection-inducing mutations in their mitochondrial DNA (Deuse et al., 2019a; Melton, 2021).

Beyond advanced insulinopenic patients with T2D, those with cystic fibrosis-related diabetes or chronic pancreatitis could also be considered candidates, particularly if autologous islet-after-pancreatectomy transplantation fails due to insufficient quality or quantity of islets (Fig. 2) (Rickels et al., 2018). As strategies for immunosuppression continue to improve, or ideally are even obviated altogether, stem cell-derived beta cell replacement therapy should expand to include pediatric patients younger than 18 years old (Hathout et al., 2003; Shapiro et al., 2017). The ability to intervene as early as possible during the process of disease progression will likely translate into meaningful differences in prevention of the chronic morbidity and complications associated with long-term diabetes. As always, prospective randomized clinical trials will be critical to evaluate the safety and efficacy of hPSC-derived beta cell replacement therapy in these additional populations, particularly in comparison to alternative strategies such as artificial pancreas technologies, which themselves have advanced markedly in recent years (Cengiz et al., 2011; Rickels et al., 2018).

Cell manufacturing

To meet the demands of what will become a substantially larger patient pool, manufacturing processes will need to focus on reducing the variability in stem cell-derived islet composition and function. This is being pursued by fine-tuning differentiation protocols, using more reproducible sources of small molecule factors to induce robust differentiation at each step, performing purification or enrichment to select for desired cell types, and genetically encoding so-called “suicide genes” into undesirable cell types (Di Stasi et al., 2011; Liang et al., 2018). As groups continue to refine the generation of stem cell-derived insulin-producing cells according to clinically-relevant current Good Manufacturing Practices (cGMP), some of the same quality metrics used in assessing islets for reversing T1D can be applied with respect to viability, sterility, and pre-transplant assessment (Shapiro et al., 2017). Opportunities exist for improving protocols by reducing the duration and number of steps required and/or increasing the yield of the transplantable product by promoting cell expansion (Melton, 2021; Oakie and Nostro, 2021; Sneddon et al., 2012). To achieve cost-effective manufacturing using industrial processes, synthetic small molecules are likely to replace purified proteins (Kieffer, 2016). Recent reports modeling the cost-effectiveness of hPSC-based therapy for T1D compared to intensive insulin therapy have highlighted the need for scalable manufacturing platforms for stem cell therapies (Bandeiras et al., 2019; Wallner et al., 2016).

It is not yet known what dose of hPSC-derived insulin-producing cells would be needed to achieve insulin independence in human patients. The endocrine compartment comprises only about 1-2 % of the total volume of a normal human pancreas, containing on the order of 3 million islets (Ionescu-Tirgoviste et al., 2015). For islet transplantation, evidence-based

studies have resulted in a recommendation that greater than 5,000 islet equivalents (IEQs) per kg of the recipient body mass be transplanted for effective glycemic control, with yields of greater than 7,000 IEQ/kg from a single donor correlating with higher likelihood of resulting in insulin independence (Shapiro et al., 2017). For a 75 kg adult, even the higher end of dose range of 7,000 IEQ/kg (translating to 525,000 IEQ) would represent only approximately 10-20% of his or her normal islet mass (17.5% of 3 million), suggesting that a fraction of endogenous beta cell mass may be sufficient to significantly improve glycemic control upon transplantation (Kieffer, 2016). This IEQ dose would correspond to roughly 1-5 x 10^8 hPSC-derived beta-like cells, depending on the frequency of beta cells within a hPSC-derived islet-like cluster (Melton, 2021). Scalable suspension-based cultures have been reported for a number of years, enabling the generation of stem cell-derived beta cells on this scale (Pagliuca et al., 2014; Schulz et al., 2012).

Cell delivery and engraftment

Beta cell survival after transplant remains a major barrier to cell replacement therapy for diabetes. Significant loss of islet mass is observed during the peri-transplant period, with recent work demonstrating greater than 60% loss within the first few days after transplant (Davalli et al., 1996; Faleo et al., 2017). As with islets, low engraftment has also plagued hPSC-derived pancreatic progenitors (Henry et al., 2018) and insulin-producing cells (Faleo et al., 2017) . Beta cells have been reported to be especially susceptible to hypoxia (Bensellam et al., 2016; Cantley et al., 2010). To address the susceptibility of hPSC-derived beta-like cells to ischemic injury, work from Faleo et al. showed that supplementation with amino acids, along with pre-exposure of differentiating cells to physiological levels of oxygen, led to improved graft viability and preservation upon transplant *in vivo* (Faleo et al., 2017).

Islets are highly vascularized tissue, with every beta cell believed to be adjacent to a capillary; this proximity to vasculature is a crucial property that permits rapid sensing of fluctuations in blood glucose levels and delivery of insulin to maintain euglycemia (Bonner-Weir and Orci, 1982; Cottle et al., 2021; Henderson and Moss, 1985; Stan et al., 2012). The process of islet isolation results in disconnection of islets from their vasculature as well as damage to the endothelial cells that do remain, leading to prolonged ischemic injury and cell death. In contrast to islets, engraftment of another highly vascularized endocrine tissue, the parathyroid gland, is highly successful (Lo and Lam, 1998). Co-transplantation of islets with parathyroid gland was recently shown to vastly improve the engraftment of both human islets and hPSC-derived insulin-producing cells(Kelly et al., 2019). Based on these impressive preclinical data, clinical trials have recently entered phase 1 to test the efficacy in human patients (IND18596, ClinicalTrials.gov identifier: NCT03977662). Similarly, co-transplantation of islets with human amniotic epithelial cells or umbilical cord perivascular cells has been reported to improve vascularization (Forbes et al., 2020; Lebreton et al., 2019). As stromal cells, such as vascular cells, support islet viability and functionality beyond their vascular supply role (Nikolova et al., 2006), elucidating the molecular mechanisms underlying the improvement in beta cell engraftment will generate valuable insights into how such signals may be recapitulated with defined cocktails of factors (Fig. 2, **left panel**).

In addition to solving the issue of low beta cell survival upon transplantation, the field has also been focused on cell delivery strategies that would ensure protection of transplanted cells from immune-mediated graft rejection (Fig. 2). In the case of islet or hPSC-derived beta cell replacement for T1D, such strategies must ultimately address the challenges of both allo-rejection and autoimmune attack. Even if autologous iPSC-derived cells were transplanted into patients with T1D, autoimmune attack would still need to be overcome (Melton, 2021).

Encapsulation is one such approach that could potentially address both challenges, without requiring the chronic use of systemic immunosuppression that puts patients at risk of infection, organ damage, and cancerous growth. Encapsulation devices provide a physical barrier between the host and the transplanted beta cells, while ideally still allowing physiological glucose-stimulated insulin secretion and yet preventing infiltration of host immune cells. Thus, encapsulation devices entail an inherent engineering challenge: contain pore sizes that permit exchange of oxygen, glucose, and insulin, but prevent the transit of cells and molecules responsible for mediating immune rejection. Additional parameters to optimize include proximity to vasculature, viability of encapsulated cells, biocompatibility, and the containment of potentially tumor-forming cells (Desai and Shea, 2017).

Over several decades, multiple types of encapsulation devices have been developed, generally divided into the categories of macro- and micro-encapsulation (Fig. 2). Macroencapsulation devices are of a scale that accommodates many islets-worth of cells, and can be implanted intravascularly or extravascularly (Schweicher et al., 2014). Macrocapsules offer the advantages of tunability of porosity and pore size, along with retrievability of the graft should any issues arise. Challenges of macroencapsulation devices include more limited vascularization of the cells within, which can result in decreased cell survival, as well as delays in oxygen and nutrient diffusion and cell function as a result of device thickness. Out of concern for hypoxia within the center of the device, membranes must be thin, while maintaining mechanical integrity (Schweicher et al., 2014). Another limitation of macroencapsulation is the potential for host-mediated fibrosis that can surround the microcapsule, effectively walling off the contained cells from exchange of glucose, insulin, oxygen, and nutrients and leading to cell death (Schweicher et al., 2014).

Examples of macroencapsulation devices that have been developed include the Theracyte device (Scharp and Marchetti, 2014), which is immunoisolating and comprises a two-membrane pouch (Kumagai-Braesch et al., 2013; Sörenby et al., 2006). Long-term *in vivo* studies using a nanoporous polymer thin film cell encapsulation device have demonstrated immunoprotection of hPSC-derived islet-like cells, along with favorable biocompatibility indicated by evidence of neovascularization and minimal foreign body response in mouse models (Chang et al., 2017). ViaCyte's Encaptra device is composed of a single immunoisolating membrane that protects cells within from direct physical contact with host immune cells. Encaptra is designed to be transplanted subcutaneously and it is currently in clinical trials with hPSC-derived progenitor cells (ClinicalTrials.gov identifier: NCT02239354).

As vascularization of implanted devices typically takes on the order of two to three weeks, several approaches are being taken to prevent or address the resulting hypoxia during the peri-transplant period (Schweicher et al., 2014). These include attempts to accelerate vascularization with growth factors such as VEGF, (Trivedi et al., 2000) the incorporation of oxygen carriers, oxygen-generating biomaterials or access port to deliver oxygen, such as the βAir device developed by Beta-O_2, (Pedraza et al., 2012; Schweicher et al., 2014) (Barkai et al., 2013; Neufeld et al., 2013) and the prevascularization of the macrocapsule before addition of cells, such as Defymed's MailPan (Magisson et al., 2020; Pileggi et al., 2006). A thin-film cell microencapsulation device fabricated from polycaprolactone has demonstrated favorable vascularization, limited fibrosis, and immunoprotection, leading to improved viability of encapsulated hPSC-derived islet-like cells (Chang et al., 2017; Nyitray et al., 2015).

In contrast to macroencapsules, microcapsules only contain on the order of one to three islets and are typically approximately 300-1500 μm in diameter (Schweicher et al., 2014; Wilson and Chaikof, 2008). Microcapsules offer the advantages of high surface area to volume ratio, which in principle should confer improved diffusion characteristics compared to extravascular macrocapsules. They can be implanted via minimally invasive procedures (potentially even through injections). One of the most significant disadvantages of microcapsules is the difficulty of retrievability after implantation, raising safety concerns (Schweicher et al., 2014).

The most popular material for microcapsules is alginate, a natural polysaccharide typically extracted from seaweeds that shows favorable biocompatibility and can be used to encapsulate cells under mild, cell-friendly conditions (Rabanel et al., 2009). Some groups have incorporated factors into alginate or synthetic hydrogels, paving the way for new approaches for immunomodulation of encapsulated cells after transplant of allogeneic cells (reviewed in (Stabler et al., 2019)). When applied to hPSC-derived islet-like cells, one study demonstrated that incorporation of CXCL12 into alginate prolonged both the survival and function of hPSC-derived beta-like cells in mice to 150 days after transplant without the use of immunosuppression (Alagpulinsa et al., 2019). Long-term rescue of immunocompetent streptozotocin-treated diabetic mice was reported using hPSC-derived beta-like cells that were encapsulated with immunomodulatory alginate derivates and then implanted into the intraperitoneal space. Euglycemia was achieved without immunosuppression for 174 days until graft removal (Vegas et al., 2016). Several groups have developed techniques to deposit very thin (1-50 μm), so-called conformal coatings on the surface of islets (Wilson and Chaikof, 2008). This conformal coating approach reduces delays in diffusion and reduces total graft volume, which can be beneficial when anatomical real estate is an issue. A recent study demonstrated that conformally coated hPSC-derived beta cells showed similar function to unencapsulated counterparts *in vitro,* and *in vivo* rescued diabetes in NODSCID mice for more than two months (Stock et al., 2020).

In the absence of immunoisolating encapsulation, cells could be transplanted with immunosuppression and infused in the portal vein, as with human islets. Semma Therapeutics/Vertex recently received approval to launch a clinical trial to test the safety, tolerability, and efficacy of their hPSC-derived beta cell product (VX-880) infused into the

hepatic portal vein of patients with T1D (ClinicalTrials.gov identifier: NCT04786262). Alternatively, cells could be transplanted in the subcutaneous space, omentum, or muscle using scaffolds or open devices, such as PEC-Direct from Viacyte (Clinical Trials.gov identifier: NCT03163511) (Fig. 2). Such approaches have been advanced as a method to prevent cell clumping, control cluster size, and provide mechanical support as well as three-dimensional context to implanted cells. Scaffolds have been generated from diverse materials and can be either permanent (e.g., made of silicone) or degradable and resorbable (e.g., made of hydrogel or plasma clot) (Tomei et al., 2015). For instance, matrices made of fibrin have been used to promote islet function and to enhance angiogenesis in the site of transplantation (Beattie et al., 2002). Scaffolds may be used alone or within a macroencapsulation device, and they offer the potential advantages of better mimicking tissue context and possibly of retrievability if made of permanent materials (Fig. 2, **middle panel**).

Site of transplantation

Multiple transplantation sites are being pursued as options for stem cell-derived insulin-producing cells. In scenarios involving encapsulation of transplanted cells, the feasibility of various anatomical sites for transplantation depends on the type of device. For instance, microcapsules are often transplanted into the peritoneal cavity. Intravascular macrocapsules are connected as a shunt to systemic blood circulation, whereas extravascular microcapsules are typically implanted subcutaneously or intraperitoneally (Schweicher et al., 2014).

Although portal infusion is used for human islet transplantation, this approach limits the amount of tissue that can be delivered secondary to the development of portal hypertension (Sneddon et al., 2018). An additional concern is that cells delivered to the portal vein cannot be retrieved, should overgrowth or transformation occur. Thus, alternative sites for islet transplantation are being explored, including subcutaneous, intramuscular, and omental/intraperitoneal sites, and these sites are similarly candidates for hPSC-derived insulin-producing cells (Bertuzzi et al., 2018; Sneddon et al., 2018) (Fig. 2, **right panel**). Although subcutaneous implantation offers the advantages of retrievability and simplicity of delivery, it has also posed challenges due to poor spontaneous vascularization. Induction of local neovascularization before transplantation has been reported to enhance subsequent maturation and function of hPSC-derived pancreatic progenitors (Pepper et al., 2017). Several groups have reported successful transplantation of pancreatic islets into recipient rodents, non-human primates, and human patients when delivered to the highly vascularized and surgically accessible omentum (Baidal et al., 2018; Berman et al., 2016; Schmidt, 2017).

Tolerance

A number of promising strategies have emerged in recent years to meet the goal of achieving tolerance to transplanted hPSC-derived beta-like cells. Genetic engineering approaches are currently being developed by multiple groups to enable hPSCs and their differentiated progeny to evade detection from alloimmune effectors following transplantation. For instance, HLA mismatch between donor and recipient is a major obstacle leading to graft rejection in the absence of immunosuppression. As one potential solution, gene editing

techniques have been used to decrease the expression of HLA proteins on the surface of hPSCs and their derivatives, a strategy that could lead to the establishment of hypoimmunogenic, universally compatible hPSC lines as sources of cells for transplantation (Deuse et al., 2019b; Gornalusse et al., 2017; Han et al., 2019; Kong and Zhou, 2021; Lanza et al., 2019; Xu et al., 2019). Genome editing approaches are also being used to control the expression of various immunomodulatory factors by the implanted hPSC-derived cells, including PD-L1, CD47, CTLA4Ig, IL10, IL4, among others (Deuse et al., 2019b; El Khatib et al., 2015; Gaddy et al., 2012; Han et al., 2019).

A complementary approach is the induction of immunological tolerance using cellular immunotherapy. Using a short-term treatment with monoclonal antibodies targeting T cell co-stimulatory pathways, Szot and colleagues demonstrated that immune tolerance to allogeneic hPSC-derived pancreatic endoderm could be achieved in a humanized immune model. Using this strategy, the transplanted endodermal cells survived long enough to differentiate into islet-like structures that maintained normoglycemia in diabetic mice, while untreated animals promptly rejected their grafts and became hyperglycemic. While a short duration of co-stimulatory blockade led to long-term tolerance in this model, further studies will be required to delineate the effectiveness and durability of this strategy, given the limitations of humanized mice in modeling mature human immune systems (Szot et al., 2015). Alternatively, hematopoietic mixed chimerism (HMC) could be employed to induce immune tolerance to allogeneic hPSC-derived islet-like cells, possibly with augmentation by adoptive transfer of regulatory T (Treg) cell populations. Using HMC, it has been demonstrated that long-term allogeneic solid organ graft tolerance can be achieved by transplantation of hematopoietic cells from the same donor (Zuber and Sykes, 2017). As conditioning regimens to induce donor cell engraftment typically require the use of risky chemotherapeutic or radiation protocols, recent work pioneering a less toxic, antibody-mediated conditioning regimen shows great promise for induced mixed chimerism to be applied to a broader patient population (Chhabra et al., 2016). The addition of Treg cells has also been shown to further enhance HSC engraftment (reviewed by (Pilat et al., 2017)). Further innovations in developing safer conditioning approaches for hematopoietic stem cell transplant, together with adoptive transfer of Tregs, will move this field even closer to achieving durable tolerance to allogeneic hPSC-derived insulin-producing cells, reducing or even eliminating the need for chronic immunosuppression or encapsulation.

Future perspectives:

The convergence of multi-disciplinary fields including endocrinology, stem cell biology, immunology, bioengineering, and transplantation has now led to an explosion of possibilities for the use of hPSC-derived islet-like cells for cell replacement therapy for diabetes. Further work is needed in several areas to make this dream a reality. First, the application of single-cell RNA-sequencing to developing human pancreatic tissue should provide a critical benchmark for current *in vitro* protocols for generating hPSC-derived islet-like cells, both by advancing our understanding of the intermediate progenitor populations that should be recapitulated *in vitro* and by evaluating the transcriptional fidelity of *in vitro*- to *in vivo*-derived endocrine populations. Second, the ideal composition of the final islet replacement product is still unknown. As human islets contain not only beta cells, it will be important to

test whether incorporation of hPSC-derived alpha cells (Peterson et al., 2020; Rezania et al., 2011), other hPSC-derived hormone-expressing cells, and/or non-epithelial niche cells such as vascular, mesenchymal or neuronal cells, leads to improved engraftment and function *in vivo.* After all, the pioneering work in islet transplantation entails the transfer of whole islets (not purified populations of beta cells), and paracrine interactions may be critical for islet engraftment, survival, and function, as well as optimal recapitulation of the natural kinetics of insulin release. Third, if human hPSC-derived islets are to be engineered *in vitro*, a better understanding of the human islet tissue structure will be needed. Innovation in high-resolution 3D imaging will be important for generating a human islet blueprint that could then be used as a guide for tissue engineering strategies to design hPSC-derived human islet tissue. Fourth, ongoing advances in genome editing technologies and synthetic biology will enable the exploration of alternative strategies for generating hypoimmunogenic cell populations with enhanced functionality. Fifth, advances in bioengineering approaches will be crucial for providing improved strategies for cell manufacturing and cell delivery. And lastly, insights and new approaches in the field of transplant immunology will be critical to develop combination therapies that will support long-lasting grafts.

In conclusion, 100 years after the discovery of insulin, the field now finds itself at a watershed in the history of the treatment of patients with diabetes. Following a burst of innovation in pharmacotherapy and devices that enable automated insulin delivery, patients with diabetes now have many options to help control blood glucose levels and minimize the risk of symptomatic hypoglycemia and irreparable organ damage. As these technologies require constant vigilance and monitoring from patients and their families, however, a reliable, fully automated therapy that restores physiologic glucose control remains an elusive dream outside of cadaveric organ transplantation. In a remarkable effort leveraging breakthroughs across multiple fields, a scalable and universal beta cell replacement therapy now seems within grasp. As the global scientific community intensifies its rally to solve the final outstanding challenges, we look forward to witnessing even greater breakthroughs in generating surrogate islets *in vitro*, and most importantly, improving outcomes for patients with diabetes.

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Stem cell-derived insulin-producing cells hold great promise for advancing diabetes treatment. To fully benefit from such technology, however, improving cell survival post-transplant is crucial. Migliorini et al. discuss progress in stem cell differentiation protocols and the impact of new technologies to prevent immune-rejection and improve engraftment to enhance therapeutic efficacy.

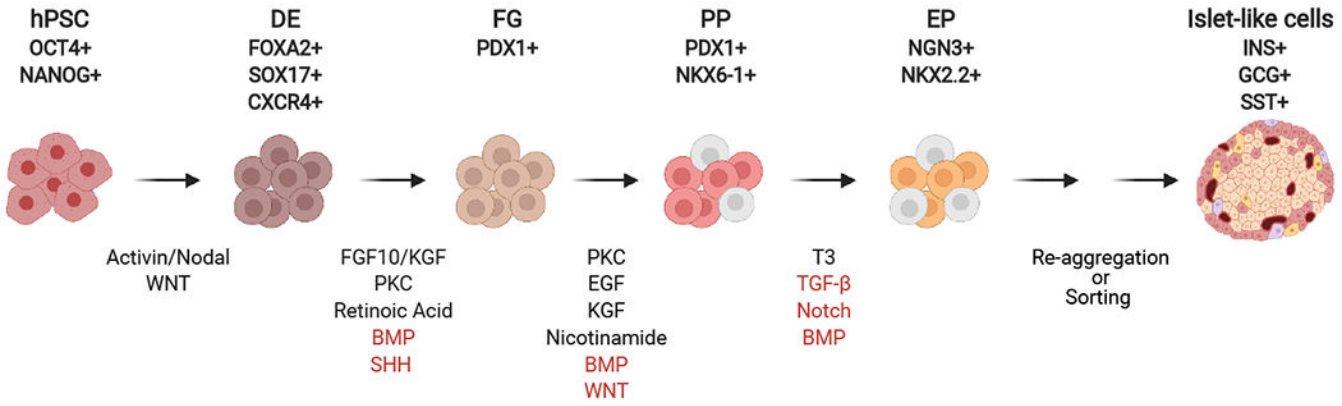

Figure 1: Schematic showing differentiation of human pluripotent stem cells to pancreatic lineages.

Directed differentiation of human pluripotent stem cells (hPSCs) to pancreatic cells is achieved through a series of sequential steps that recapitulate *in vivo* development. Such protocols lead to the formation of definitive endoderm (DE, characterized by expression of the transcription factors FOXA2 and SOX17 and the cell surface marker CXCR4), patterning of the posterior foregut (PF) to generate PDX1-expressing endoderm and specification of the PDX1+ endoderm to pancreatic progenitors (PP) expressing PDX1 and NKX6-1. Endocrine commitment, leading to the formation of endocrine progenitors (EP), is characterized by the up-regulation of transcription factors NGN3 and NKX2-2, followed by the expression of lineage-specific gene, including the hormones insulin (INS) expressed by beta-like cells, glucagon (GCG) expressed by alpha-like cells, and somatostatin (SST) expressed by delta-like cells. Each developmental step is controlled by activation (black) and/or inhibition (red) of specific signalling pathways. Activin/Nodal and Canonical Wnt signaling are required for definitive endoderm formation. Retinoic Acid and FGF10 signaling are important for PDX1 induction. PKC activation or EGF and Nicotinamide treatment are required for the formation of PP cells. Endocrine lineage commitment is achieved in the presence of BMP/TGF-β/NOTCH inhibition.

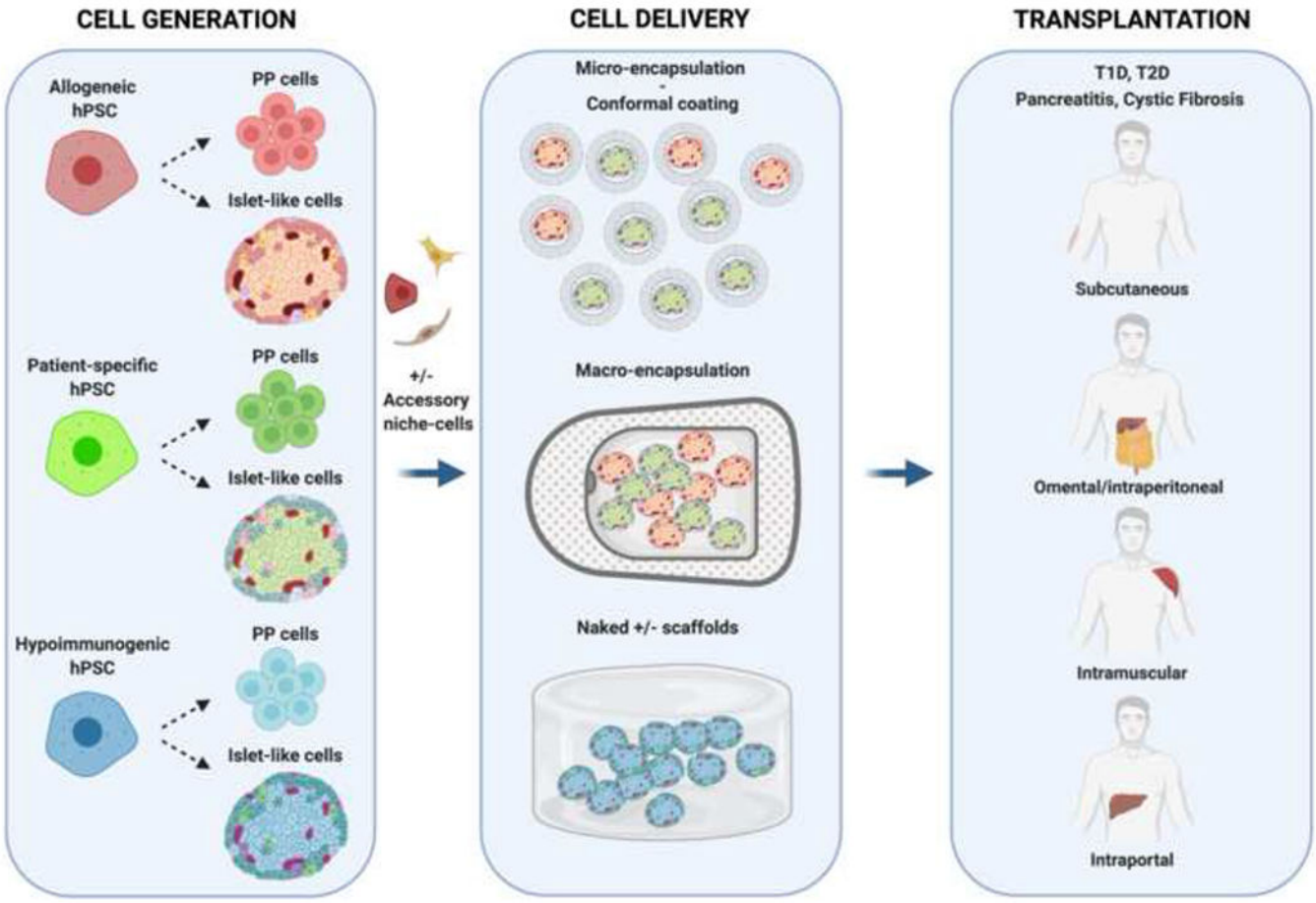

Figure 2: Strategies for beta cell replacement using human pluripotent stem cells (hPSCs).

Cell Generation: hPSCs can be differentiated *in vitro* to generate pancreatic progenitors (PPs) or islet-like cells for functional restoration of euglycemia. Potential sources of hPSCs include allogeneic hESC or iPSC lines, autologous iPSCs derived from the patient's own cells, and hESC or hiPSC lines that have been genetically engineered to be hypoimmunogenic. ***Cell Delivery:*** To protect the graft from allorejection and/or autoimmunity, allogeneic and autologous cells can be delivered inside micro- or macroencapsulating devices, possibly supplemented with accessory niche cells to improve engraftment and functionality. Hypoimmunogenic pancreatic cells could be transplanted in the absence of immunoprotective devices but may still require scaffolds and/or accessory cells to facilitate engraftment. ***Transplantation:*** Potential anatomical sites for transplantation include the subcutaneous, omental/intra-peritoneal, intramuscular, or intraportal locations. Target patient populations include patients living with type 1 diabetes (T1D), type 2 diabetes (T2D), pancreatitis, or cystic fibrosis. This figure was created using BioRender.com