Review

Developments in stem cell-derived islet replacement therapy for treating type 1 diabetes

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SUMMARY

The generation of islet-like endocrine clusters from human pluripotent stem cells (hPSCs) has the potential to provide an unlimited source of insulin-producing β cells for the treatment of diabetes. In order for this cell therapy to become widely adopted, highly functional and well-characterized stem cell-derived islets (SC-islets) need to be manufactured at scale. Furthermore, successful SC-islet replacement strategies should prevent significant cell loss immediately following transplantation and avoid long-term immune rejection. This review highlights the most recent advances in the generation and characterization of highly functional SC-islets as well as strategies to ensure graft viability and safety after transplantation.

A CELL REPLACEMENT THERAPY FOR TREATING TYPE 1 DIABETES

Diabetes as an ideal candidate for cell replacement therapy

Insulin is an essential regulator of energy metabolism throughout the body, directing the usage of carbohydrates, fats, and proteins.[1–4] In particular, the binding of insulin to cell surface receptors facilitates the entry of glucose from the blood stream into many cell types within the body, most notably in muscle and fat, so that they can use it as energy. Insulin signals for excess glucose to be stored for future use as glycogen in the liver and muscle as well as converted to fat in adipocytes. In parallel, insulin signaling slows the breakdown of fats and proteins, as they are not needed much when glucose is plentiful. The amount of insulin circulating in the blood changes dynamically in response to the constantly changing energy needs of the body and the availability of each fuel source. Specialized endocrine cells called β cells, located in the islets of Langerhans within the pancreas, are responsible for this tightly regulated production and release of insulin. After a meal, for example, blood glucose levels rise as carbohydrates are broken down into glucose and absorbed into the blood stream. β cells can rapidly sense this increasing blood glucose level and secrete the appropriate amount of insulin in response, allowing cells throughout the body to utilize this glucose for energy production. Blood glucose levels fall as glucose enters cells, causing the β cells to slow their release of insulin. This reduction in insulin secretion in conjunction with an increase of the counter-regulatory hormone glucagon by α cells prevents blood glucose levels from dropping below a set threshold (~70 mg/dL in humans), as a minimum concentration is required for tissues of the central nervous system to function properly. By rapidly changing the amount of insulin and glucagon in circulation, β and α cells maintain blood glucose levels in a narrow optimal range.

In type 1 diabetes (T1D), β cells are selectively destroyed by an autoimmune process, resulting in the inability to produce and secrete insulin.[5,6] Without insulin, glucose homeostasis and energy metabolism balance in the body are completely disrupted. As many cell types can no longer import glucose for energy, they must switch to metabolizing free fatty acids that are liberated from the breakdown of triglycerides in adipocytes as their main energy source. Importantly, as the liver processes free fatty acids, it generates ketones that can also be used as an energy source by other tissues. While this process is normal during periods of fasting and low-carbohydrate dieting, the complete absence of insulin signaling in T1D results in uninhibited fat breakdown and uncontrolled ketone production. The rapid buildup of ketones changes blood pH, resulting in the life-threatening condition called ketoacidosis.[7,8] Thus, patients with T1D must inject exogenous insulin in order to survive and restore the necessary signaling pathways that properly regulate their energy metabolism.

While injecting insulin allows T1D patients to stay alive, replicating the precise insulin secretion dynamics of β cells can be difficult. Not only do patients need to consider what they are eating when calculating an insulin dose, but because insulin requirements are intimately linked to energy metabolism, other factors such as the intensity and duration of physical activity as well as stress levels can influence how much insulin the body requires at a given time. Incorrectly estimating the amount of insulin needed at a particular time can create both short and long-term issues. For example, not giving enough insulin will cause the blood glucose level of a patient to be higher than it should be. In the extreme, high glucose levels can change blood osmolarity and result in life-threatening dehydration.[8] Moderately high glucose levels are not a serious issue in the short term, though they may make the patient feel suboptimal. Over the lifetime of the patient, however, this chronic elevation of blood glucose concentration can damage tissues throughout

the body. Therefore, it is critically important to try to keep blood glucose levels as close to normal as possible to avoid long-term complications, such as cardiovascular, kidney, and eye diseases.[9,10] On the other hand, giving too much insulin is dangerous in the short term because it can cause blood glucose levels to dip below the normal lower limit. The central nervous system requires a constant supply of glucose to function, and a certain concentration in the blood is required to facilitate sufficient transport of glucose across the blood-brain barrier.[11] Thus, as blood glucose levels drop below this threshold, a person can start to act abnormally as their brain essentially starves. If blood glucose levels continue to fall, the person can lose consciousness and ultimately die.[12] Consequently, patients taking insulin must try to navigate between these two extremes and estimate the amount of insulin their body needs based on their current metabolic state.

The treatment of T1D has made great strides in recent years. In particular, new tools such as insulin pumps and continuous glucose monitors have undoubtedly made living with diabetes easier and have helped many patients achieve better control over their blood glucose levels.[13] Despite these advances, however, many patients still fail to achieve their target goals. Moreover, even if they do meet their blood glucose targets, the therapy can remain burdensome, as it still requires constant monitoring and adjustment. Because of the difficulty in replicating the precise insulin secretion dynamics of β cells with exogenous insulin injections, a potentially better treatment alternative consists of replacing the lost β cells with new ones, allowing these transplanted cells to monitor blood glucose levels and secrete the appropriate amount of insulin in response. Such a transplant would provide a "functional cure" for T1D patients, as they would no longer have to manage their blood glucose levels with insulin injections. Type 2 diabetics who rely on insulin injections may also benefit from such a transplant. Intriguingly, T1D is a potentially ideal candidate for cell replacement therapy. Because individual β cells can sense extracellular glucose changes and secrete insulin, there is less of a need for a complex working structure of multiple integrated cell types, as would be required in tissue engineering whole organs such as a heart or kidney. Rather, as long as the β cells secrete insulin properly in response to glucose stimulation and are transplanted in a location that facilitates adequate exchange of these molecules with the blood stream, such a cell replacement therapy could provide a functional cure for T1D.

Improvements in whole pancreas transplantation since the 1960s demonstrated that donor pancreatic tissue could restore glucose homeostasis in diabetic patients.[14] Transplantation of pancreatic islets alone to restore β cell mass, however, had limited success until the development of the Edmonton Protocol in 2000, which demonstrated that infusion of islets into the liver via the portal vein was sufficient to restore glucose homeostasis.[15,16] Their initial trial led to insulin independence of all seven patients 1 year after transplantation, vastly improving previous islet transplantation outcomes due to optimization of their immunosuppression regimen as well as benefiting from improvements in islet isolation techniques. Although all but one of these patients required supplemental insulin 10 years post-transplant, all recipients retained some level of graft function that provided substantial benefits to glucose control and elimination of severe hypoglycemic events.[17] Subsequent transplantation studies continued to show improved patient outcomes, with islet transplantation approaching the success of whole pancreas transplantation without the corresponding surgical risk.[18–23] Importantly, these remarkable studies demonstrated that the concept of replacing β cell mass by transplanting pancreatic islets was relatively safe and effective in treating T1D.

Recent clinical trials with SC-derived pancreatic tissue

While transplantation of human islets from deceased donors has demonstrated the feasibility of a cell replacement therapy for treating diabetes, there are a number of issues that limit its more widespread use. In particular, graft success increases when a high number of islets are transplanted, often resulting in the need for multiple donor pancreases.[15,20,24] Not only are the number of pancreases available for islet isolation limited, but transplanting cells from multiple genetic backgrounds per patient could exacerbate immune rejection and graft failure.[25,26] Furthermore, the stress of purification from the pancreas can damage islets before transplantation.[27] This sourcing problem could be circumvented by differentiating stem cells (SCs) into β cells in vitro, generating an unlimited supply of insulin-producing cells for transplantation.[28] These stem cell-derived islets (SC-islets) could be generated from a single-cell source using a standardized process, and the resulting cell product could be well characterized to allow for more predictable transplant outcomes. Furthermore, a SC-derived product could also be genetically engineered to have advantageous features not found in primary islets, such as resistance to stressors like hypoxia or being able to evade the host immune system.

Recent advances in SC technologies have led to the first human clinical trials using SC-derived pancreatic products. In particular, ViaCyte developed a SC-derived pancreatic endoderm cell population known as PEC-01, which they demonstrated to mature over several months in vivo into insulin-producing endocrine cells in rodent models.[29–31] In conjunction, they developed several iterations of a retrievable macroencapsulation device to contain the transplanted cells. An initial 2014 human clinical trial (clinicaltrials.gov: NCT02239354) used the Encaptra device, which was designed to fully immunoprotect the cells using a cell-impermeable membrane.[32] While the transplanted PEC-Encap product was well tolerated with few adverse effects, the trial was halted due to insufficient functional product engraftment.[33] While some endocrine cells were observed, fibrosis around the capsule resulted in graft loss, and no insulin secretion from the device was detected.[33,34] To overcome this issue, the newer PEC-Direct device contained openings in the membrane to allow for vascularization to enhance nutrient exchange and promote cell survival. Because host cells were able to penetrate the device, however, immunosuppression was required after transplantation. An ongoing clinical trial was started in 2017 at seven sites to test this device (clinicaltrials.gov: NCT03163511), and the first round of results have recently been published.[33,35] They demonstrated glucose-responsive C-peptide production 6–9 months post-transplant as the grafted cells matured from pancreatic progenitors into pancreatic endocrine cells. Upon graft removal, a majority of these graft-derived cells immunostained for the general endocrine marker chromogranin A (CHGA). Interestingly, while there were regions of C-peptide-positive β-like cells, a majority of these endocrine cells stained for

the α cell marker glucagon. Ductal cells and rare acinar cells were also observed. As intended, the porous design promoted the growth of host-derived blood vessels inside the device, and this phenomenon was more prominent in regions containing the graft-derived cells. In other regions of the device, however, host fibroblasts were the predominant cell type, resulting in the deposition of fibrotic tissue rather than host-derived vasculature. While the observed circulating C-peptide levels in these studies were too low to induce a measurable clinical benefit attributable to the transplanted cells, these clinical trials have demonstrated that facilitating host-derived vascularization into the graft promoted long-term endocrine cell survival and function in humans. In parallel, they highlight the importance of limiting the fibrotic response of the body to a transplanted device in order to ensure these outcomes. Importantly, these studies did not detect any serious safety concerns related to the transplanted cells, such as tumor formation.

In the ViaCyte clinical trial, the explanted grafts after months *in vivo* were observed to be highly heterogeneous between patients since the cells were transplanted as pancreatic progenitors and allowed to finish differentiating *in vivo*.[33,35] Ideally, the β cells could be terminally differentiated *in vitro* and capable of glucose-responsive insulin secretion before transplantation, which would allow for a more consistent cell population that would facilitate a relatively rapid restoration of blood glucose control. In 2014–2015, several groups published protocols for generating stem cell-derived β (SC-β) cells that secreted insulin in response to glucose stimulation.[36–38] Because this differentiation process also generates other endocrine cell types, such as glucagon-producing α cells, these clusters of differentiated cells are now often referred to as SC-islets. These cells have been shown to improve glycemic control in diabetic mice, highlighting their potential utility *in vivo*.[36,37,39–44] Recently, these SC-islets were also used to help treat diabetic non-human primates.[45,46] While the primates did not achieve complete insulin independence, the transplanted SC-islets significantly reduced their insulin requirements and greatly improved their glycemic control, further illustrating the potential utility of SC-islets as a therapy. SC-islets have also now been transplanted into human T1D patients in a clinical trial by Vertex Pharmaceuticals, which began in 2021 (clinicaltrials.gov: NCT04786262). The first two patients were given half the anticipated target dose to assess the safety profile of their SC-derived product, VX-880. Furthermore, these cells were transplanted without an immune-protective device to avoid problems with nutrient exchange and fibrosis, and thus, the patients required immunosuppressive drugs to ensure graft survival. While the initial findings have not yet been peer reviewed, positive preliminary results reported in a press release have indicated that transplantation of these SC-islets improved glycemic control in these two T1D patients (https://www.businesswire.com/news/home/20220606005424/en/). The transplanted SC-islets took longer to improve glycemic control than in rodent models, however, and the reason for this delay has yet to be explored.

Remaining challenges for developing a widely used cell therapy

These promising initial clinical trial results highlight the great promise SC-islets hold for treating T1D. However, there are several remaining challenges to be addressed before this cell therapy can become a routine procedure (Figure 1). First, SC-β cells generated with current directed differentiation protocols are still not quite as functional as primary human β cells, and their transcriptional and chromatin landscape remains immature. Furthermore, the current differentiation methodologies still produce off-target cell types, notably cells that resemble a type of intestinal endocrine called the enterochromaffin cell (EC).[47] The presence of these off-targets as well as the observed lower insulin secretion per cell compared with primary islets indicates that there is still room to improve these differentiation protocols. If the insulin secretion per cell can be increased further, fewer cells will be needed to cure a patient. Reducing the required cell number is significant in terms of both the graft volume that needs to be transplanted as well as cell production costs. To this end, significant progress has been made at improving the function of SC-islets when compared with the original protocols,[39,40,44,48,49] and recent advances in single-cell sequencing technologies have allowed for unprecedented characterization of these cells to further elucidate ways to refine SC-β cell differentiation strategies.[43,47,50,51]

Second, there would ideally be a strategy to circumvent the need to take immunosuppressive drugs after transplantation. For the typical T1D patient that is able to achieve their blood glucose targets with insulin injections as indicated by their hemoglobin A1c levels, the potentially severe adverse side effects of life-long immunosuppression may not outweigh the benefits of further improved glycemic control. Thus, several strategies are currently being pursued to protect the transplanted SC-islets from immune attack. One attractive method is to encapsulate the cells in a device with finely controlled pore sizes that facilitates the diffusion of small molecules such as glucose and insulin but blocks immune cells from infiltrating the device.[52] Biomaterials may also be fabricated with instructive chemistry to induce local immune tolerance around the transplanted cells.[53] Alternatively, the SC-islets themselves can be genetically engineered to both remove antigens that would signal them for destruction by the host immune system as well as add surface signaling molecules that induce immune tolerance.[54,55]

Lastly, there are several practical factors that need to be addressed for this cell-based therapy to become widely used in the clinic. Specifically, it is crucial to reduce the stress response within islets immediately following transplantation to reduce initial cell death, which can result in significant graft loss.[56] Facilitating rapid vascularization is key in alleviating these stresses to promote cell survival and long-term health of the transplanted SC-islets. Genetically engineering the cells to be resistant to these non-ideal conditions could also aid in their survival during the period before vascularization can occur. Furthermore, developing large-scale manufacturing methods for generating SC-islets as well as methods for their cryopreservation and distribution will be crucial for the widespread adoption of this procedure. In the remainder of this review, we will be discussing the most recent advances in addressing these issues associated with (1) generating highly functional SC-islets, (2) solving the immune rejection problem, and (3) overcoming practical challenges associated with transplanting these cells.
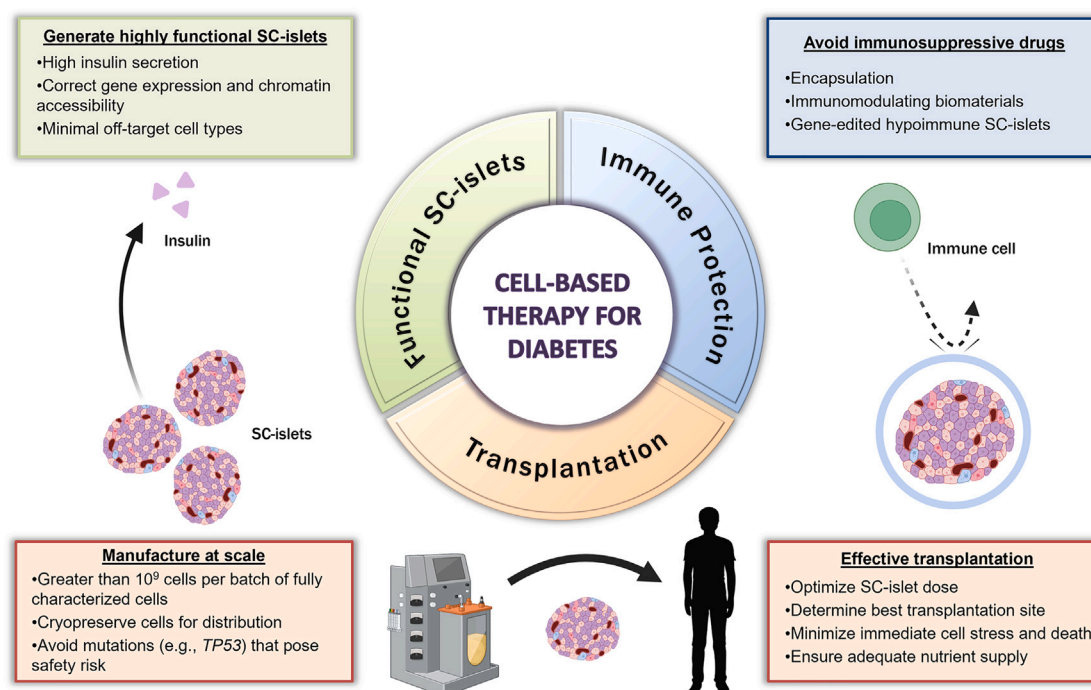
Figure 1. Key pillars of a successful SC-islet therapy for treating T1D
The remaining challenges limiting the application of SC-islets as a treatment option for diabetes cover a broad range of considerations. The first major component of a successful SC-islet therapy is the ability to produce a highly functional, uniform cell-based product for transplantation. Further optimization of differentiation protocols should aim to limit the generation of off-target cell populations and improve the gene expression and chromatin accessibility profiles to match those of human adult islets. These advancements would ideally improve the amount of insulin secreted by SC-β cells and thus reduce the number of cells required per patient. Furthermore, improved transplant survival and a reduced, or eliminated, need for immunosuppressive drugs can be achieved by applying bioengineering strategies. Specifically, using immunomodulating biomaterials or genetically engineered hypoimmune SC-islets could maximize the efficacy of transplanted grafts while minimizing the immune response commonly associated with the transplantation of allogeneic materials. Finally, regulatory standards must be established to maximize the safety and efficacy of SC-islet transplantation. This includes improving methods for large-scale manufacturing of SC-islets with sufficient quality control characterizations, such as avoiding mutations that pose safety risks, and developing standardized methods for storage and distribution. Additionally, as these challenges are addressed and SC-islet products are improved, guidelines to optimize dosing, transplantation site, and graft survival must be established.

GENERATING HIGHLY FUNCTIONAL SC-ISLETS

Strategies for improved SC-islet generation

The ongoing clinical trial led by Vertex Pharmaceuticals has indicated that existing protocols generate SC-islets that are capable of improving glycemic control in human T1D patients. However, further enhancing SC-islets to better mimic the functionality of primary adult islets would reduce the number of cells required for transplantation and make it easier to manufacture sufficient cell numbers. For example, conceptually, if insulin secretion per cell is doubled, then potentially only half as many cells are needed to cure a patient. Reducing graft volume facilitates an easier transplantation procedure, reduces nutrient exchange requirements at the transplantation site, and opens alternative transplantation site possibilities. Furthermore, fewer cells would need to be manufactured, which is significant in terms of both production costs and logistics for a cell-based therapy.

SC-islets are generated through a multistep differentiation process that attempts to mimic stages of embryonic development with the timed application of growth factors and small molecules, driving SCs through several intermediate cell types on their way to becoming pancreatic endocrine cells (Figure 2).[57] This differentiation methodology takes approximately a month

or more to complete, and functional maturation can continue for months once the cells are transplanted *in vivo*.[43,51] The developmental pathways targeted by this process can be very sensitive to changes in timing and signaling intensity, and thus, small signaling changes at specific times can lead to large changes in cell fate selection. Consequently, many attempts to improve SC-islet generation consist of iterations with different combinations and timings of soluble factors. The latest generation of protocols are better at generating islet-like clusters consisting of mostly endocrine cells, though not all of these are β cells. A substantial fraction can be α cells along with some δ cells. Interestingly, the role or importance of these other endocrine cell types to β cell function within the context of SC-islets or if there is an optimum cell ratio to target during differentiation is unknown. Current protocols also produce a significant percentage of SC-derived ECs (SC-ECs), which resemble a type of intestinal endocrine.[47] With less optimized protocols or cell lines, there is also the potential to retain progenitor cells in the final islet clusters as well as produce other endodermal cell types, such as those of hepatic origin.[39,58] Consequently, increasing insulin secretion per cell in SC-islets can be achieved through improvements in either SC-β cell maturation or by decreasing the percentage of these off-target cell populations.
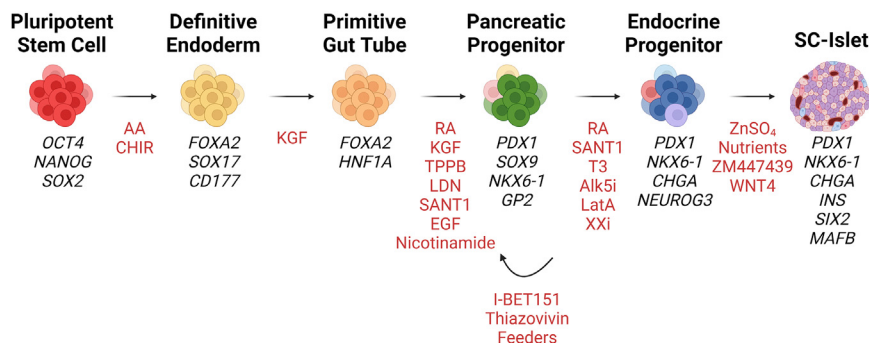
Figure 2. Growth factors and small molecules used during the multistage differentiation of hPSCs to SC-islets

The generation of SC-islets from hPSCs is a multistage process that involves the temporal application of small molecules and growth factors (highlighted in red). At each stage, specific developmental pathways are targeted to mimic human pancreatic development. While multiple differentiation protocols have been reported, each follows the same general differentiation trajectory by first specifying hPSCs into definitive endoderm using Activin A (AA) and WNT pathway activation, most often with CHIR99021. Next, definitive endoderm cells are guided into a primitive gut tube state using keratinocyte growth factor (KGF), also known as fibroblast growth factor 7 (FGF7). Notably, some protocols include additional compounds that target WNT (e.g., IWP2[49]) and TGF-β signaling pathways to improve primitive gut tube specification. Generating pancreatic progenitor cells requires additional activation of protein kinase C with TPPB and retinoic acid (RA) signaling pathways while simultaneously inhibiting bone morphogenetic protein (BMP) signaling with LDN193189 and sonic hedgehog (SHH) signaling with SANT1. Epidermal growth factor (EGF) and nicotinamide have also been shown to improve the generation of PDX1^{+}/NKX6-1[+] pancreatic progenitors.[59] Interestingly, it has been reported that the use of a selective bromodomain and extraterminal domain (BET) inhibitor can maintain pancreatic progenitors cells in a proliferative and expandable state.[60] This can potentially accelerate SC-islet manufacturing by providing an intermediate point from which to generate SC-islets. During the later stages of SC-islet differentiation, reported protocols begin to diversify both in nomenclature of cell populations and compounds applied. For example, latrunculin A (LatA) has been used to specify endocrine cells in planar culture.[39] Additional nomenclature differences arise with some protocols describing the initial SC-islet product as "immature" and include an additional stage to generate "mature" SC-islets using an aurora kinase inhibitor (ZM447439)[43] or WNT analogs.[61]

Since the original SC-islet protocol developments in 2014–2015,[36–38] there have been a number of reports that have significantly improved SC-islet function and characterized their maturation. The first significant advance established dynamic function in SC-islets.[42,44] Biphasic insulin secretion is a well-known characteristic of human islet function,[62,63] but SC-islets generated from early protocols notably lacked this feature. An enriched serum-free media combined with cluster resizing through a single-cell dispersion and aggregation technique generated SC-islet clusters that achieved biphasic insulin secretion kinetics resembling that of human islets.[42] Notably, the exclusion of Alk5 inhibitor II in this media greatly improved insulin secretion. While this inhibitor is necessary during the previous endocrine induction step, the benefits of its removal in the subsequent maturation stage strongly indicated that TGF-β signaling was important for SC-β cell functional maturation. Similarly, enriched β-clusters (eBCs) generated by sorting INS[GFP+] β-like cells and aggregating them into clusters demonstrated improved function.[44] These eBCs were composed exclusively of SC-derived endocrine cells, with approximately 90% of the cells being C-peptide[+]/glucacon[−] and the remainder expressing multiple hormones. Mitochondrial respiratory function, mitochondrial energization, and mitochondrial membrane potential during glucose stimulation assays suggested metabolic maturation of eBCs as a driver of improved insulin secretion. Further adding to these improvements, single-cell dispersion and reaggregation of SC-islet clusters promoted enrichment of C-peptide[+] endocrine cell types[42,47] along with sorting based on the surface marker CD49a.[47]

The 3D architecture of human islets is known to be important for glucose responsiveness and insulin secretion.[64] Consequently, early differentiation protocols for SC-islets were developed using 3D cell clusters, either from the outset of differentiation in suspension culture[36,38] or prior to endocrine specification on an air-liquid interface.[37] Interestingly, PDX1^{+}/NKX6-1[+] pancreatic progenitors could be efficiently generated from human pluripotent SCs (hPSCs) in traditional planar culture, but these protocols required

3D cell aggregation to efficiently generate endocrine cells from these progenitors.[37,59] Recently, the state of the actin cytoskeleton and downstream yes-associated protein (YAP) signaling was found to be vital to the pancreatic progenitor program.[39,65–67] In particular, a polymerized cytoskeletal state resulting from culture on tissue culture polystyrene inhibited NEUROG3-mediated endocrine induction in PDX1^{+}/NKX6-1[+] pancreatic progenitors.[39] To circumvent the need for a 3D format during endocrine specification, a planar methodology was developed whereby the actin cytoskeleton was depolymerized with the compound latrunculin A (LatA) during the first 24 h of endocrine specification.[39] This short treatment was sufficient to initiate robust endocrine specification of PDX1^{+}/NKX6-1[+] pancreatic progenitors in planar culture. These differentiated endocrine cells could subsequently be dispersed from the culture surface and aggregated into islet-like clusters for use in downstream assays. SC-islets generated with this approach demonstrated improved *in vitro* dynamic insulin secretion and faster reversal of severe diabetes in mice compared with cells generated with a suspension differentiation protocol. Importantly, this planar methodology was also more amenable to differentiating SC-islets from a wide range of SC lines with various genetic backgrounds.[40,57]

Several other unique approaches have been used to improve the function of SC-islets. For example, adhering to a strict feed/fast cycle activated circadian rhythm programs in maturing SC-islets.[48] This feeding schedule triggered rhythmic transcription of genes involved in energy metabolism as well as genes related to the synthesis, transport, and release of insulin, resulting in improved SC-islet function. In several studies, modulation of WNT signaling at various time points in the differentiation protocol has been shown to improve endocrine cell fate selection and maturation. In one report, different subpopulations of hPSC-derived definitive endoderm displayed inverse activation of canonical and noncanonical WNT signaling.[49] Specifically, definitive endoderm cells expressing CD177 were distinguished by increased noncanonical WNT signaling and their tendency to differentiate into a pancreatic lineage, whereas

CD275-expressing cells upregulated canonical WNT signaling and specified a liver fate. Treatment with IWP2, a small-molecule inhibitor of WNT ligand secretion, after endoderm specification promoted a pancreatic lineage. Furthermore, sorting the definitive endoderm population to enrich for CD177^{+} cells led to the generation of SC-islets with improved maturation and function. An alternative differentiation approach attempted to recreate multicellular interactions during pancreatic organogenesis by combining human adipose-derived SCs and human umbilical vein endothelial cells with human induced pluripotent stem cell (hiPSC)-derived endocrine progenitors in a polysaccharide-based gel.[61] Interestingly, these multicellular spheroids demonstrated that noncanonical WNT signaling drove maturation of the β cells. Addition of exogenous WNT4 helped these islet-like organoids achieve glucose-stimulated insulin secretion, implicating the need to promote noncanonical WNT signaling during SC-islet maturation. Conversely, another study demonstrated that inhibiting canonical WNT signaling during endocrine differentiation improved outcomes, further highlighting that the type and timing of WNT signaling during differentiation can drastically influence the specification and maturation of SC-islets.[68]

Utilizing a differentiation protocol that incorporated several of the improvements mentioned here, one study presented a comprehensive analysis of SC-islets compared with primary human islets.[43] These SC-islets developed robust dynamic insulin secretion overtime *in vitro*, corresponding to changes in islet architecture but without changes in β cell mass. Furthermore, SC-β cells demonstrated transcriptional maturation over the 6-week culture period and had the correct ion channel profile and exocytosis machinery to support proper insulin secretion. Aspects of glucose metabolism, however, differed in SC-islets when compared with primary human islets. In particular, glucose processing was abnormal and not correctly coupled to mitochondrial respiration, which is normally key to canonically triggering insulin secretion in β cells. Similarly, another report demonstrated that defects of glucose metabolism in SC-β cells prevented proper insulin release rather than originating from any deficiencies in the exocytosis machinery.[69] Treatment with cell-permeable metabolites to bypass this bottleneck in glycolysis resulted in greatly improved insulin secretion in SC-islets. These studies highlight that while current generation SC-islets can secrete significant amounts of insulin in response to glucose stimulation *in vitro*, fundamental differences still exist between SC-islets and primary human islets, resulting in the observed lower functional performance of SC-islets. Consequently, it is crucial to better characterize the cells generated with these protocols in order to inform the next generation of differentiation strategies.

Using single-cell sequencing to obtain new insights into SC-islet biology
In addition to profiling the functional characteristics of SC-β cells to assess their maturation state, insights into deficiencies in their gene expression and chromatin accessibility profile can reveal additional signaling pathway interactions that can be targeted to further improve SC-β cell lineage specification and maturation. The rise of single-cell sequencing technologies has steered much of the recent SC-islet research toward thoroughly characterizing their transcriptional and chromatin landscape.

Single-cell RNA sequencing (scRNA-seq) has become one of the most widely used single-cell sequencing approaches, enabling high-throughput, multi-dimensional characterization of cellular diversity by transcriptionally profiling each individual cell.[70] In parallel with the boom in scRNA-seq technology, the assay for transposase-accessible chromatin using sequencing (ATAC-seq) has facilitated robust and sensitive epigenomic profiling of open chromatin sites that are accessible for transcription.[71] On the individual cell level, this ATAC-seq technology can use either single cells (scATAC-seq)[72] or single nuclei (snATAC-seq).[73] These technological advances have enabled new insights into the differentiation of SCs into SC-islets, which is a transcriptionally dynamic process that often produces heterogeneous cellular populations. Furthermore, multimodal analysis through combined scRNA-seq and snATAC-seq using integrative computational methods has provided an even deeper understanding of SC-islet composition and maturation.

Recently, two separate studies utilized scRNA-seq to map transcriptional changes during SC-islet differentiation.[47,50] Despite the use of different pluripotent SC lines and protocols, both roadmaps observed well-known markers of pancreatic progenitors (e.g., *PDX1* and *NKX6-1*) during the middle stages of differentiation as well as islet-specific endocrine identity markers in their final cell populations (e.g., *INS* for β cells, *GCG* for α cells, and *SST* for δ cells). These results validated well-established knowledge of pancreatic organogenesis that inspired early protocols for SC-islet differentiation. Interestingly, both scRNA-seq roadmaps detected significant cell diversification following the pancreatic progenitor stages. After sequencing 40,444 cells during the second half of SC-islet differentiation, the first study reported a mostly homogeneous population of *PDX1*^{+} progenitors at end of stage 3.[47] In subsequent stages, high expression of *PDX1* in combination with expression of *NKX6-1* and *PTF1A* was predictive of endocrine induction potential. Notably, the non-endocrine cell populations of ductal-like, acinar-like, and mesenchymal-like cells were derived from populations that retained expression of cell-cycle-associated genes. The second report sequenced 87,769 cells across 12 points during the differentiation of a SC line known to result in highly heterogeneous SC-islets.[50] Using a semi-supervised method to construct a lineage tree of cell fate, it was demonstrated that divergent cell populations that arose during early stages did not significantly contribute to the off-target populations observed later. In contrast, the final non-endocrine off-target populations, including duct-like and pancreatic stellate-like cells, branched during pancreatic progenitor stages. 1,150 endocrine-specific switch genes were identified, and it was inferred that 656 pairs of transcription factor and target gene interactions potentially influenced endocrine specification. Of note, NOTCH signaling via *HES1* was implicated as a driver of a non-endocrine fate.

One of the most interesting findings from these scRNA-seq studies was the identification of SC-EC cells, which are absent from primary human islets and resemble an endocrine cell type normally found in the intestines.[47] These cells are characterized by the expression of enterochromaffin gene markers, such as *TPH1* and *LMX1A*, as well as the secretion of serotonin rather than insulin. SC-β cells and off-target SC-EC cells appear to emerge from a common *NEUROG3*^{+} progenitor population.[47] These cells have been subsequently identified in SC-islets

generated from various differentiation protocols and SC lines by multiple groups.[43,50,51] Despite distinguishable differences in gene expression between SC-EC and SC-β cells, the two populations appear to have a relatively similar overall transcriptional profile. Because SC-EC cells comprise a significant percentage of the final cell population in SC-islets but do not secrete insulin, differentiation efficiency and SC-islet insulin secretion on a per cell basis could be greatly improved by diverting these SC-EC cells toward a SC-β cell lineage during either the cell specification or maturation stages.

To gain further insights into the nature of these cells and other lineage fate decisions during SC-islet differentiation, several groups have recently implemented snATAC-seq analysis to ascertain the chromatin accessibility profiles of differentiating cells in combination with their transcriptional data obtained from scRNA-seq. Of note, these findings are currently published as pre-prints and still under peer-review due to the novelty of both snATAC-seq itself as well as the computational methods used to analyze the integrated scRNA-seq and snATAC-seq datasets. In one approach, a canonical correlation analysis algorithm was used to integrate chromatin accessibility and gene expression data from different cells *in silico* to generate pseudo-cells with matched epigenomic and transcriptomic information within differentiating SC-islets.[74] In contrast, another study reported the simultaneous multiomic sequencing of RNA expression and chromatin accessibility from the same cell.[75] Using canonical gene markers of islet cell types, both multiomic studies identified distinct populations representing SC-β, SC-α, SC-δ, and SC-EC cells. Notably, both studies also provided greater resolution of cellular heterogeneity by describing specific subpopulations of expected cell types. Specifically, one report identified two pancreatic progenitor populations based on differences in *NKX6-1* expression and defined four distinct endocrine progenitor populations identified by the expression of *NEUROG3*, *ARX*, *LMX1A*, and *RFX3*.[74] Similarly, the second study identified two SC-EC populations based on the integrated analysis of both mRNA and chromatin accessibility data.[75]

Both studies performed trajectory analysis using Monocle3[76] to characterize lineage selection during SC-islet differentiation. Previous analysis of scRNA-seq data alone had suggested that SC-β and SC-EC cells are distinct cell types that share a common progenitor lineage during *in vitro* differentiation of SC-islets,[47,50] but examining the combined gene expression and chromatin accessibility data for each cell revealed a gradient of cell states between SC-EC and SC-β cells.[75] Specifically, there appeared to be SC-β cell subpopulations that expressed *TPH1*, a known marker of SC-EC cells, and that had increased accessibility of binding sites for *LMX1A*, a transcription factor associated with the SC-EC cell fate. They also reported a potentially novel role for the chromatin remodeling transcription factor *CTCF* in regulating cell fate selection toward the enteroendocrine lineage. Additionally, the other study identified *CDX2* as the earliest transcription factor expressed during SC-EC lineage specification.[74] Interestingly, they also reported a novel *CDX2*+ β cell-precursor-like population in the human fetal pancreas that resembled SC-EC cells, leading them to suggest that SC-EC cells are in fact of pancreatic rather than intestinal origin. Collectively, these studies highlight the utility of single-cell multiomic analysis to gain greater resolution of cell identity during SC-islet

differentiation. Importantly, both studies indicated that epigenetic regulators are important drivers of cell identity, particularly in the cell fate choice between SC-EC and SC-β cells.

Recent applications of single-cell sequencing technologies have not only improved our understanding of SC-islet composition but have also elucidated the transcriptional and epigenetic differences between SC-derived and human adult islets. SC-islets generated *in vitro* remain transcriptionally and functionally immature compared with adult human islets.[44,77,78] The multiomic studies described here further demonstrated that SC-β cells were less similar to their primary counterparts than other SC-derived endocrine cell types.[74,75] Moreover, the chromatin accessibilities of primary adult islet cell types were much more restricted than those in SC-islet cell types, where the *INS* gene remained open in SC-α and SC-δ cells in addition to SC-β cells.[75] Transplantation of SC-islets into mice improves their insulin secretion and glucose responsiveness, suggesting that SC-islet immaturity can be resolved.[36,37,41–44] scRNA-seq analysis of transplanted SC-islets revealed that improved function corresponded with transcriptional maturation.[43,51] Specifically, known markers of β cell maturation, such as *INS*, *MAFA*, *IAPP*, *MNX1*, and *G6PC2*, became more highly expressed in a greater proportion of SC-islet cells after transplantation. These transcriptional changes appeared to occur temporally, as some genes (e.g., *G6PC2*) increased in expression shortly after transplantation, while others (e.g., *MAFA*) took several months to upregulate. Interestingly, SC-EC cells also persisted after transplantation and exhibited increased expression of key gene markers of their identity, such as TPH1.[51,75] Despite transcriptional improvements, SC-islets nonetheless had significantly reduced activity of metabolic pathways compared with primary islets.[43] Furthermore, a multiomic analysis identified over 600 genes, 350 promoter regions, and 250 transcription-factor-binding motifs that increased after 6 months *in vivo*, while only a small fraction of these were also increased during *in vitro* maturation.[75] These findings suggested that extended time *in vivo* tended to open chromatin regions associated with cell-specific identity and maturation, while *in vitro* culture methods remained relatively ineffective at promoting the same maturation effect. Overall, comparison of scRNA-seq and snATAC-seq data from transplanted SC-islets and primary islets has indicated that SC-islets become more similar to human islets overtime *in vivo* in terms of both their transcriptional and chromatin accessibility profiles, though some important differences still remain. These strategies for highly detailed characterization of SC-islet cell types will be invaluable for elucidating the mechanisms driving islet development and the factors that are currently limiting further SC-islet maturation.

OVERCOMING IMMUNE REJECTION

Encapsulation and immune-modulating biomaterials

As with the transplantation of any allogenic material, transplanted SC-islets are targeted by the host immune system as foreign, resulting in graft rejection. An additional complication is that T1D itself is an autoimmune disease that specifically targets β cells. Thus, even if autologous SC-islets were generated from patients using an iPSC intermediate, it is possible that they would still be targeted by immune cells. This issue is

currently addressed with the use of immunosuppressive drugs that are used for whole organ transplants, and the prescribed regimen has improved in recent years.[79] While these drugs can successfully retain graft viability, they also can have serious side effects, including an increased risk of infection and cancer as well as less severe but uncomfortable symptoms.[80,81] For many patients, these adverse side effects may not outweigh the benefits of increased glycemic control, and thus, there has been much research focused on alternative methods for protecting the transplanted cells from the host immune system.

One of the most researched approaches is to encapsulate the cells in a biomaterial with finely tuned pore sizes that allow for the diffusion of small molecules such as glucose and insulin but shield the transplanted SC-islets from contact with host immune cells. This field has been progressing for several decades and excellently reviewed in-depth elsewhere,[52,53,82,83] and so the focus here will be on several recent examples to highlight the current status of these technologies. In the macroencapsulation strategy, many islets are put into a single device.[84] These encapsulation devices are most often constructed of either a polymer film such as polytetrafluoroethylene (PTFE) or an alginate hydrogel owing to their excellent biocompatibility and ease of fabrication. Not only does the device membrane provide protection against contact with host immune cells, but it also prevents the graft cells from spreading to other parts of the body should any unwanted cell growth or tumor formation occur. Furthermore, the device can be easily retrieved if any other safety or efficacy concerns arise after transplantation. One popular option for islet macroencapsulation is the commercially available TheraCyte device, which is composed of a 0.4-μM inner PTFE membrane to block entry of host immune cells and an outer 5-μM PTFE membrane that promotes vascularization around the graft. While these devices have had success in preventing overt immune rejection of pancreatic tissue,[85–88] these devices can be limited by a lack of proper nutrient exchange, particularly until vascularization has formed.[89] As highlighted by the ViaCyte clinical trials with similar devices[33,34] as well as other studies,[90,91] these macroencapsulation devices can also promote fibrosis around the implant, further preventing nutrient diffusion.[84,92]

New systems are being developed to combat these issues associated with nutrient delivery. For example, a PTFE macroencapsulation device that actively pumped fluid through a hollow fiber running through its center demonstrated enhanced nutrient exchange that promoted cell survival and allowed for a greater cell density to be successfully loaded into the device.[93] Insulin secretion and blood glucose control was also enhanced when transplanted into diabetic rats. Interestingly, they also observed a decreased fibrotic response with their active fluid flow setup. While advancements need to be made to replace the external pump used to perfuse the device once implanted, this study highlights that relying on active fluid flow rather than passive diffusion for nutrient transport can greatly enhance the viability and performance of islets within a macroencapsulation device.[93,94] Because oxygen is one of the most important of these nutrients to the metabolically active β cells, several oxygen delivery systems and oxygen generating materials have been incorporated into encapsulation device designs.[91,95,96] In a recent iteration, CaO_2 was integrated into a polydimethylsiloxane (PDMS) slab that was encased in an agarose hydrogel con-

taining islets.[97] The CaO_2 reacted with the water in the hydrogel to generate oxygen that became available to the surrounding cells. In another example, Li_2O_2 particles were suspended in a perfluorocarbon oil and encased in silicone tubing.[98] This core was surrounded by an islet-containing alginate hydrogel. The Li_2O_2 reacted with the CO_2 waste generated from cellular respiration to generate O_2, resulting in a self-sustaining source of oxygen within the hydrogel that was only limited by the amount of Li_2O_2 in the device. In both these examples, the oxygen generated by the device significantly enhanced the viability and function of the islets after transplantation.

One method to passively alter nutrient transport is to control the geometry of the device. For instance, a network of interconnected hydrophobic channels inside an islet-filled alginate hydrogel greatly increased the diffusion of oxygen throughout the device from the surrounding tissue.[99] This design greatly enhanced islet survival upon transplantation into diabetic mice and allowed the device to be scaled up to 6.6 mm thick. Alternatively, increasing the surface-to-volume ratio and decreasing the distance of cells from the outer surface of the device can facilitate better mass transport to the encapsulated cells. For example, a durable polymer thread was coated in a thin alginate hydrogel layer containing islets, generating a long thread-like device with a high surface-to-volume ratio.[100] Rat islets encapsulated within the device were able to survive and reverse diabetes in immune-competent diabetic mice. To further improve the mechanical strength of their device concept in a follow-up study, they developed an alternative design that placed the alginate hydrogel inside of an immunoprotective tube composed of a strong nanofibrous mesh that was electrospun from a medical-grade thermoplastic silicone-polycarbonate-urethane.[101]

This concept of controlling device geometry to enhance nutrient transport can be further scaled down to the level of encapsulating individual islets in a strategy known as microencapsulation. One popular approach uses alginate microcapsules, and various iterations have been utilized.[83] Interestingly, while decreasing the size of encapsulation devices down to the scale of individual islet microcapsules increases nutrient diffusion shortly after transplantation when compared with macroencapsulation devices, smaller capsule sizes have been shown to also induce a robust foreign body response and subsequent fibrosis.[102] To this end, there has been some excellent recent work altering the surface chemistry of these microcapsules to minimize the fibrotic response. For example, a combinatorial biomaterial screen identified three different triazole-containing covalent modifications to alginate that significantly reduced the foreign body response in both rodents and non-human primates.[103] One of these formulations, triazole-thiomorpholine dioxide alginate, was then used to encapsulate SC-β cells within 1.5-mm spheres.[41] These encapsulated cells restored glycemic control in diabetic, immune-competent mice for at least 174 days without any signs of fibrosis. This formulation was subsequently shown to prevent fibrosis and facilitate cell survival of allogeneic islets in non-human primates.[104] Another group added a zwitterioinic modification to alginate microcapsules to significantly reduce fibrosis because of its ability to resist protein adsorption and cell attachment.[105] They then combined this zwitterionic concept with the previously discovered triazole

modification to further improve the ability of the alginate microcapsules to resist fibrosis and as well as enhance their mechanical stability.[106]

These studies utilizing modified alginate chemistries point to an interesting shift in thinking whereby a biomaterial is not only thought of as a physical barrier to keep out immune cells but rather as a source of instructive signals to induce local immune tolerance, either by releasing molecules as a drug delivery system or by having a biofunctional immunomodulatory surface.[53] By only targeting the area directly surrounding the implant, these strategies can increase the efficacy of the desired immune modulation while simultaneously avoiding systemic side effects. For example, alginate microcapsules fabricated to release the immunomodulatory chemokine CXCL12 improved encapsulated SC-islet survival and reduced the fibrotic response in mice.[107] Similarly, alginate microcapsules designed to release exosomes derived from umbilical cord mesenchymal SCs were also shown to induce local immune tolerance by altering signaling in multiple immune cell types.[108] The incorporation of the CSF1R inhibitor GW2580 in its crystallized form within alginate microcapsules allowed for its controlled release overtime, resulting in long-term reduction of fibrosis and significant improvements in islet transplantation outcomes in mice, even within the subcutaneous space.[109] In an alternative approach, immune-modulating proteins such as programmed death-ligand 1 (PD-L1) or Fas ligand (FasL) can be immobilized onto the surface of biomaterials to dampen local T cell adaptive immune responses. Both of these proteins play important roles in immune tolerance, as the binding of PD-L1 to PD-1 on effector T cells reduces their proliferation and cytokine production, while the binding of FasL to the Fas receptor induces T cell apoptosis. To this end, PD-L1[110] or FasL[111,112] proteins were immobilized onto the surface of poly(ethylene glycol) microgels, which were then mixed together with allogeneic islets and co-transplanted into either mice[110,111] or non-human primates.[112] In conjunction with a transient rapamycin treatment, these functionalized microgels greatly improved islet survival without the need for long-term immunosuppression or encapsulation.

Engineering hypoimmune SC-islets

This concept of inducing local immunity can be pursued even further by engineering the transplanted cells themselves to hide from the immune system. Avoiding the use of biomaterials altogether eliminates the inherent diffusion barriers of encapsulation systems as well as circumvents the biomaterial-induced fibrotic response. For example, isolated mouse islets were engineered to transiently display FasL on their surface through a biotinylation technique.[113,114] Allografts were able to survive indefinitely if also treated for the first 15 days with rapamycin, whereas those without FasL were rejected within 30 days. They demonstrated that FasL induced local immune tolerance by causing apoptosis in alloreactive T cells and as well as promoting the development of regulatory T cells (Tregs) for long-term maintenance. The development of local rather than systemic immune tolerance was further highlighted by the transplantation of a second set of unmodified, donor-matched cells that survived when grafted to the original transplantation site but were rejected when transplanted to the other kidney of the same mouse. In another example, mesenchymal stromal cells

were engineered using a lentiviral vector to overexpress PD-L1 and the cytotoxic T lymphocyte antigen 4 immunoglobulin (CTLA4-Ig) fusion protein. These gene-edited cells were co-transplanted with allogenic mouse islets to reverse diabetes significantly longer than without the genetic modifications.[115] These accessory mesenchymal stromal cells reduced infiltration of CD4$^+$ and CD8$^+$ effector T cells and increased the number of Tregs to induce local immune tolerance. This PD-L1 strategy was pushed even further by directly overexpressing PD-L1 in islet-like organoids made from hiPSCs using a lentiviral system.[61] These engineered cells were found to evade the immune system in both immune-competent mice and in a humanized mouse model.

The immune response to allogeneic material leading to graft rejection is complex, as multiple cell types and pathways are involved.[116] The main cause of rejection in allogeneic transplantation is T cell recognition of human leukocyte antigens (HLAs). The HLA class I molecules A, B, and C are present on all nucleated cells and present antigens to CD8$^+$ cytotoxic T cells, while HLA class II proteins activate CD4$^+$ helper T cells. Thus, one successful strategy to eliminate the T cell-mediated adaptive immune response against allogeneic material is to remove HLAs from transplanted cells so that they cannot activate host T cells. Deleting HLAs from cells, however, causes them to be targeted by cells of the innate immune system, such as natural killer (NK) cells and macrophages. Therefore, various strategies have been pursued to simultaneously eliminate both adaptive and innate immune responses. For example, in contrast to prior studies, it was recently reported that PD-L1 alone was not sufficient to protect against xeno- and allorejection in transplanted SC-islets.[54] Instead, they reported that HLA depletion facilitated improved cell survival. To further enhance the survival of these transplanted cells, the SC-islets were also engineered to secrete several factors to induce local immune tolerance, including IL-2 mutein to facilitate Treg expansion, as well as IL-10 and TGF-β to help the immunosuppressive function of Tregs. These cells reversed diabetes and survived for at least 8 weeks in an autoimmune diabetes mouse model. Overexpression of CD47 has also been successfully implemented to mitigate innate immune responses after HLA deletion.[117] Using the CRISPR-Cas9 system in human iPSCs, the β2-microglbulin (B2M) and CIITA genes were deleted to remove HLA class I and class II molecules, respectively, while a lentiviral vector was used to induce overexpression of CD47. These gene-edited iPSCs could differentiate into endothelial cells and cardiomyocytes that demonstrated long-term survival in humanized mouse models without any immunosuppression.

In another strategy, the CRISPR-Cas9 system was used to selectively remove HLA-A, B, and C in hPSCs, and HLA class II molecules were eliminated by targeting the CIITA gene.[118] In parallel, CRISPR-Cas9 was also used to overexpress PD-L1 to further suppress T cells, HLA-G to modulate NK cells, and CD47 to inhibit macrophages. These cells could be differentiated into endothelial cells or vascular smooth muscle cells and demonstrated improved survival in co-cultures with T cells, NK cells, and macrophages. Similarly, CRISPR-Cas9 was used to delete all HLA-A, B, and C genes in hPSCs with the exception of HLA-A2, which helped retain HLA-E expression.[55] Importantly, HLA-E expression in hPSCs has been

demonstrated to inhibit killing by NK cells.[119] In addition, HLA class II molecules were also eliminated by removing the *CIITA* gene. These hPSCs could differentiate into SC-islets that demonstrated protection from T cell-mediated rejection and reduced NK immune responses in allogeneic humanized mice.[55] Lastly, in a unique approach, scRNA-seq and a CRISPR screen of SC-islets transplanted into a humanized mouse model revealed that SC-islets upregulated genes in the interferon (IFN) pathway during graft rejection.[120] Knockout of *CXCL10*, which is induced by IFN signaling and appeared to be essential for early IFN-triggered immune responses in transplanted SC-islets, improved survival during allogeneic transplantation in humanized mice.

FURTHER CHALLENGES ASSOCIATED WITH THE CLINICAL TRANSPLANTATION OF SC-ISLETS

Reducing stress responses within islets after transplantation

Early investigation of primary human islet transplants[56] and animal studies[121,122] demonstrated that 50% or more of the islet graft was lost during the first few days after transplantation. Because of this major cell loss during the immediate aftermath of transplantation, a high islet mass from multiple organ donors and several infusions was used in the clinic.[123] Furthermore, primary islets often demonstrated progressive functional decline in the years following transplantation, necessitating patients to resume exogenous insulin injections overtime. Advances in islet isolation methodologies, immunotherapies, and transplantation techniques have improved islet transplantation outcomes in recent years, improving 5-year insulin independence rates to 50%–80%.[23] In particular, the amount of islet mass that is lost immediately following transplantation has been reduced closer to 25%, and thus fewer islets have been needed to achieve insulin independence.[79,124] Discovering new ways to further address the acute stressors experienced by islets in the immediate aftermath of transplantation could further reduce the degree of β cell loss and dysfunction that impedes long-term restoration of normoglycemia.[125]

Due to the high metabolic demands of insulin production and secretion, β cells are particularly susceptible to stress. Perturbations in normal metabolism and environmental conditions can induce endoplasmic reticulum (ER) stress and initiate the unfolded protein response (UPR).[126] While this pathway aims to restore protein homeostasis under mild stress conditions, it will induce apoptosis if the trigger is more severe. Inflammatory cytokines, hypoxia, and hyperglycemia can all induce an ER stress response in β cells that leads to cell dysfunction and apoptosis.[126–128] This stress response is thought to be involved in the progression of both T1D and type 2 diabetes (T2D).[129] Hypoxic conditions and oxidative stress can also induce other stress pathways that result in β cell functional failure and apoptosis.[130–133] Stress from a high glucose environment can cause downregulation of key islet genes and decreased function in human islets[134] and animal models,[122] implicating that adequate glucose control is important for β cell health in the immediate aftermath of transplantation. Furthermore, while SC-islets appear to be generally more stress-resistant than isolated primary islets,[135] they nevertheless respond to inflammatory cytokines with similar stress response pathways,[136] highlighting the need to mitigate exposure to inflammation during transplantation.

Native islets are highly vascularized within the pancreas to receive the proper nutrients and high oxygen supply required for the production and secretion of large amounts of insulin. Consequently, primary islet transplantations have leveraged the portal vein for islet infusion to ensure an adequate initial blood supply. One consequence of this direct blood contact is an immune response called the instant blood-mediated inflammatory reaction (IBMIR).[137–139] Within 15 min of contact with host blood, islets are encapsulated within a layer of platelets, reducing nutrient diffusion. Furthermore, leukocytes infiltrate the islets within an hour, leading to cell death. It is thought that a major cause of the initial islet death observed in the immediate aftermath of portal vein islet infusion is due to IBMIR. This initial cell death of transplanted cells can also cause release of molecules that initiate other immune responses, further exacerbating the issue by causing other transplanted cells to exhibit stress responses.[140,141]

A successful SC-islet transplantation strategy will need to adequately address these stressors, particularly those associated with hypoxia, nutrient deprivation, and inflammation immediately following the transplantation procedure. Similar to primary islet infusion, transplantation of SC-islets into mice has demonstrated that a significant portion of the cells can die shorty after transplantation due to the synergistic effects of nutrient deprivation and hypoxia.[142] Consequently, many attempts to alleviate this issue have focused on modulating the graft environment by optimizing the transplantation site and promoting vascularization. In humans, several alternative transplantation sites to the liver have been proposed,[143] including the subcutaneous space,[144] intramuscular space,[145] and the omentum.[146,147] These sites require a less invasive procedure for transplantation and provide easier access to the graft so that it can be monitored and possibly removed if problems arise. These alternative sites may also potentially circumvent the intense IBMIR response that is observed with portal vein infusion.[148] To date, however, these alternative sites have yet to lead to improved transplant outcomes when compared with portal vein infusion in humans.[148] For SC-islet transplantations, site and dose have been shown to influence cure efficacy in mice, with placement underneath the highly vascularized kidney capsule producing much better results than subcutaneous infusions.[142,149] Similarly, mice transplant outcomes for SC-islets within a microencapsulation device were more favorable in the intraperitoneal cavity than in the subcutaneous space due to a much lower fibrotic response.[101] In non-human primates, transplantation of SC-islets between the rectus abdominis muscle in the abdomen and its surrounding rectus sheath demonstrated better cell survival than in intramuscular or subcutaneous locations.[46] Furthermore, this location facilitated robust vascularization by week 12 and promoted an environment that allowed for SC-islet maturation.

Once an optimal transplantation site is chosen to minimize initial cell loss, other strategies can be pursued to further increase vascularization and nutrient transport. For example, implanting a device into the subcutaneous space 1 month prior

to cell transplantation pre-vascularized the graft site and avoided the several week lag time that it takes for vascularization to occur.[144,150] Incorporating vascular endothelial growth factor (VEGF)-releasing capsules into a similar pre-vascularization strategy[151] or transplanting islets within VEGF-releasing hydrogels[152] were shown to further improve vessel formation. Alternatively, islets embedded within sub-millimeter collagen cylinders coated with endothelial cells[153] or islets combined with microvascular fragments[154] were demonstrated to become rapidly vascularized and connected to the host vasculature when transplanted into rodents. Transplanting islets with either human umbilical cord perivascular cells[155] or amniotic epithelial cells[156] also improved islet engraftment and vascularization. To specifically improve oxygen levels at the transplantation site, oxygen generating materials have been inserted alongside the islet graft,[95,97,98] while another strategy directly pumps oxygen into an encapsulation device.[91,96]

In contrast to modulating the transplantation environment, the SC-islets themselves can be altered to make them more resistant to different stressors. For example, to improve SC-islet resistance to low oxygen conditions following transplantation, SCs were differentiated under hypoxic conditions.[142] Transplanting these preconditioned SC-islets in combination with supplemental amino acids to mitigate nutrient deprivation drastically improved SC-islet survival within the first several weeks, highlighting how cell-targeted interventions can improve graft outcomes. One unique approach is to genetically engineer SC-islets to be resistant to different stressors, which has been largely unexplored in the context of SC-islets until recently. For example, SC-islets differentiated from iPSCs generated from a patient with Wolfram syndrome, which is characterized by ER stress, exhibited poor function and increased stress responses.[40] Correcting this stress-causing pathogenic variant with CRISPR-Cas9, however, allowed this iPSC line to generate highly functional SC-islets that were capable of rapidly reversing severe diabetes in mice, illustrating that gene-editing strategies can be used to mitigate stress responses. Furthermore, a recent study manipulated normal stress responses in SC-islets to make them more resistant to transplantation conditions.[135] Specifically, SC-islets exhibited dysfunction, apoptosis, and increased expression of genes related to stress and immune interaction when exposed to a variety of stressors, including a cytokine mix to mimic inflammatory stress, thapsigargin to induce ER stress, and high glucose to replicate metabolic stress. Lentiviral shRNA knockdown of *XBP1*, *CDKN1A*, *NLRC5*, and *B2M* decreased the level of apoptosis in SC-islets when treated with these stressors as well as downregulated the expression of genes associated with stress responses and immune interaction. Furthermore, these gene knockdowns decreased T cell activation and T cell-induced apoptosis when SC-islets were co-cultured with peripheral blood mononuclear cells.

Long-term safety of the cell product

Differentiation of hPSCs to SC-islets poses potential challenges for ensuring their safety after transplantation. While optimized protocols used on research cell lines can often avoid these problems in animal models, special care is needed when protocols and cell lines are converted for clinical use in humans. In particular, not all SC lines differentiate with the same efficiency using the current generation of SC-islet protocols.[57] In-depth characterization of the composition and genomic heterogeneity of the final cell population is especially important, particularly to identify subpopulations that may proliferate uncontrollably. Given the pluripotent nature of these SCs and the complexity of the differentiation process, there is a risk that cells may differentiate into unintended or dangerous off-target cell types. In the context of SC-islet differentiation, SC-EC cells are one of the most common off-targets. Fortunately, while they may be detrimental to SC-β cell potency,[47] no safety issues have been reported to date. Hepatic, mesenchyme, and pancreatic exocrine are also possible off-targets reported from SC-islet differentiations.[47,51,75] Of greatest concern is the potential for highly proliferative uncommitted progenitors or residual hPSCs to be present in the final SC-islet product that have the potential to form tumors.[157–159] Interestingly, it was demonstrated that ensuring the pancreatic progenitor population expressed the glycoprotein GP2 prevented tumor formation upon transplantation, suggesting that dangerous cell populations in the final cell product can potentially be identified earlier in the differentiation.[160] Additional refinements to the differentiation methodology can help eliminate specification of these off-target cell types in clinical-grade cell lines. Furthermore, thorough characterization of the final SC-islet cell product with flow cytometry and single-cell sequencing technologies can identify differentiation batches that generate potentially harmful off-target cell types, such as uncommitted or highly proliferative cells, to ensure that these problematic cell populations are not transplanted into patients.

Accumulation of unsafe genetic variation, particularly oncogenic mutations, in the final cellular drug product can pose a significant safety risk for SC-islet transplantation. For example, a clinical trial in Japan for macular degeneration was paused due to genetic variation.[161] Variants can be acquired through standard culture of the cells and reprogramming.[162] Loss of function of the p53 protein is common in the majority of cancers,[163] and six specific variants in *TP53* have been identified in several hPSC lines.[164] Furthermore, numerous karyotypic abnormalities have been observed in hPSCs.[165] Substantial genomic variation has also been observed specifically in hiPSCs,[166] particularly for *BCOR* mutations that are involved in many cancers.[167] To further complicate this issue, reprogramming and genetic engineering itself have also been implicated in increasing genomic variation.[168–170] Therefore, it is crucial to test the genomic status of cells at critical stages of product development, such as after reprogramming, genetic editing, creation of the master cell bank, and creation of the final cell product.

An additional safety precaution for an SC-islet therapy is the introduction of a safety switch to destroy the graft in the event of adverse outcomes. These often take the form of an inducible gene to encoded for a protein that kills the cell, such as inducible caspase-9[171] or proliferation-induced constructs.[172] This enables precise control over the timing and duration of the therapeutic effect to improve both safety and efficacy. These safety switches will most likely be particularly important when developing hypoimmune SC-islet strategies, as the cells are being purposefully designed to hide from the immune system and cannot be easily retrieved.

Scale-up and distribution

Development of a large-scale, cost-efficient manufacturing methodology for differentiating SC-islets will be necessary to make this therapy available to a substantial number of patients.[173] While 2–5 × 10^6 SC-islet cells are sufficient to reverse hyperglycemia in mice,[149] the required dose for humans is currently unknown and is dependent on SC-islet quality. Assuming a similar dose as with human islets from deceased donors,[174,175] on the order of 10^9 cells will be required per patient. Even a relatively modest number of patients of about 1,000 will require on the order of one trillion cells to be produced. Current differentiation approaches will encounter challenges achieving these yields. SC-islet protocols that are performed completely in suspension culture[36] benefit from the natural three-dimensional scaling of these systems. Large-scale (>1,000 L) bioreactors for mammalian cell culture have been used in the biotechnology industry for decades.[176] Notably, these bioreactors allow for real-time monitoring of critical process parameters, such as dissolved oxygen, that will be critical for quality control during large-scale production. To date, however, such large scales have not been extended to hPSCs, and the largest SC-islet differentiation that has been reported in literature was performed in 500-mL magnetic spinner flasks.[36] A fundamental challenge to scaling hPSC differentiation in suspension culture is the fluid convection necessary to keep cells from settling, which is made more difficult due to hPSCs requiring either microcarriers or culture in aggregates.[177] Critically, PSCs are sensitive to mechanical cues,[178,179] and changes in shear stress due to increases in reactor volume as well as impeller speed and size will require careful optimization. Changes in these mechanical forces could alter crucial cell fate decisions during specific points in the differentiation protocol when the cells are highly sensitive to external signaling perturbations.

Alternatively, several versions of SC-islet protocols use a hybrid approach that employs traditional planar culture for some or all of the differentiation process and only aggregate the cells into clusters in later stages. For example, one popular methodology uses planar culture for making PDX1+/NKX6-1+ pancreatic progenitors and then clusters cells on an air-liquid interface for endocrine induction and β cell maturation.[37] While this protocol is able to robustly generate functional SC-islets in a lab setting, this air-liquid interface culture system would be difficult to adapt to large-scale manufacturing. In contrast, another approach is able to fully differentiate SC-β cells in planar culture.[39,57] The endocrine cells are then single-cell dispersed from the culture surface and aggregated into islet-like clusters in suspension culture. This hybrid methodology may encounter fewer optimization issues than a solely suspension-based approach during scale-up. Specifically, this SC-islet differentiation approach has been shown to scale proportionally to culture surface area,[57] and it could potentially be scaled up further using cell stacks or hyperflask setups. Importantly, parameters such as shear stress that could influence cell signaling during early differentiation stages do not change in planar culture as surface area is increased. Once the cells are differentiated into endocrine cells, they are less susceptible to mechanically induced signaling changes, facilitating an easier transition to suspension culture for the final aggregation step. This methodology has been shown to produce more cells per unit media volume than a fully suspension approach,[57] which is an important cost consideration since many of the differentiation factors are expensive. Furthermore, this approach has also been shown to be more amenable for differentiating a wide range of SC lines,[57] which may be important when this protocol is adapted to clinical-grade cell lines. Though this hybrid methodology seems to have a number of benefits, it is yet to be determined in practice whether a hybrid or fully suspension protocol will be the most effective strategy for generating trillions of SC-islet cells.

All well-established protocols require approximately 1 month of culture to achieve functional SC-islets, with additional time needed for SC-β cell maturation. Several studies, however, have demonstrated the ability to propagate the pancreatic progenitor population that is generated during the middle portion of the differentiation protocol.[180,181] In particular, it was recently reported that defined culture media containing either the TGF-β pathway inhibitor SB431542[182] or I-BET151,[60] an inhibitor of the acetyl-lysine bromodomain-containing proteins, promoted the expansion of PDX1+/NKX6-1+ human pancreatic progenitors. Stable and robust expansion of hPSC-derived pancreatic progenitors that can be efficiently differentiated into functional SC-islets would reduce manufacturing time and costs. Furthermore, this population could be well characterized and facilitate the generation of a master cell bank of high-quality pancreatic progenitors. Importantly, such a system could reduce variation in SC-islet differentiation efficiency between production runs when compared with starting with SCs for every differentiation.

Once the SC-islets have been manufactured, a system will need to be developed for their efficient distribution. An attractive idea is to cryopreserve the SC-islets so that they can be used as an off-the-shelf product. To date, however, freezing whole islets has been met with challenges.[183] Better viability and post-thaw function has been achieved by dispersing islets, freezing them as single cells, and then reaggregating them into clusters upon thaw.[184,185] A similar dispersion freezing method has been successfully used in the context of SC-islet transplantation into non-human primates.[45,46] An optimized vitrification process was also recently developed to successfully freeze whole SC-islets,[186] but scaling this process for the cell numbers needed for therapeutic use may be difficult. Future work will need to focus on scaling up these cryopreservation techniques while mitigating cell loss after thawing. Furthermore, other questions to be addressed relate to how the cells will actually be delivered to the clinic. For example, SC-islets frozen as single cells will need to be aggregated into clusters for several days before transplantation. This process could take place at the site of production, and the SC-islets could be shipped live overnight to the site of transplantation. Furthermore, it is possible that a recovery culture period may be necessary on-site before transplantation in order to maximize SC-islet survival and efficacy.

CONCLUDING REMARKS

The field has come a long way since the discovery of robust generation of definitive endoderm from hPSCs in 2005.[187] Through the excellent contributions of many research groups, protocols were developed to drive these definitive endoderm cells through several intermediate stages, culminating in the
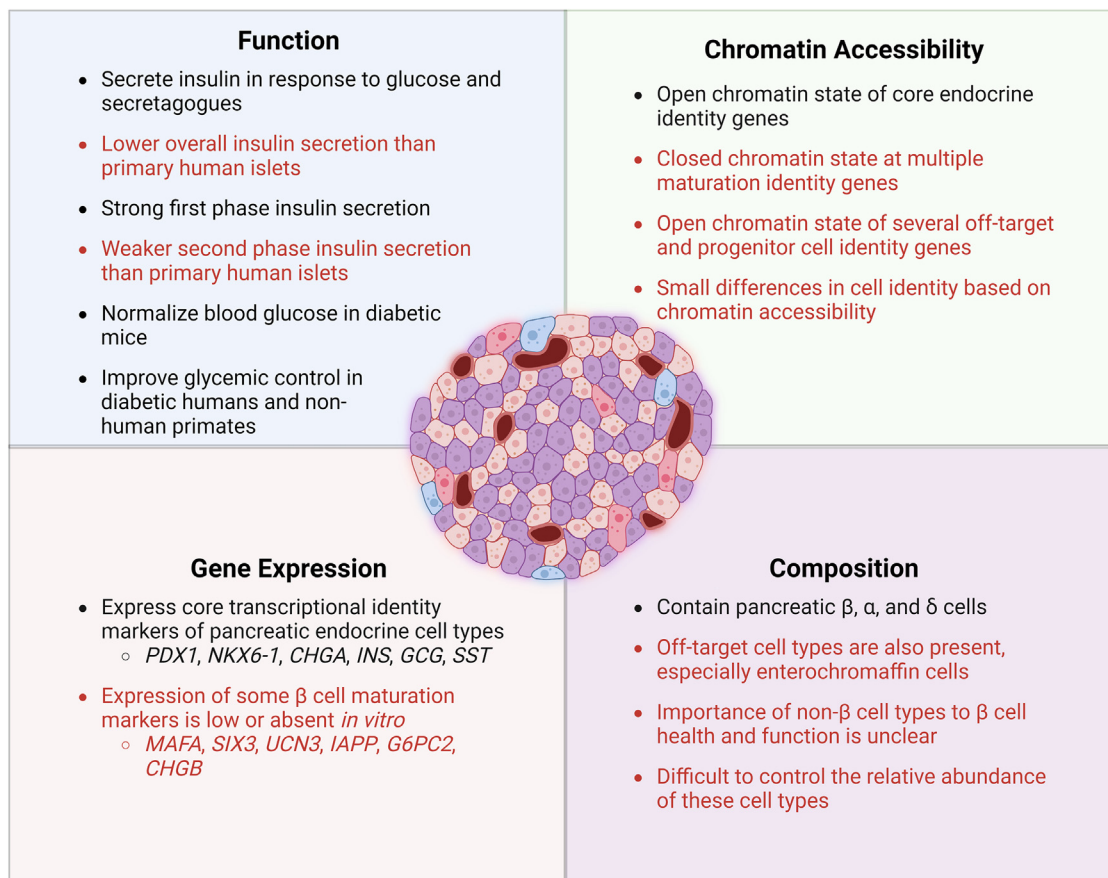
Function
- Secrete insulin in response to glucose and secretagogues
- Lower overall insulin secretion than primary human islets
- Strong first phase insulin secretion
- Weaker second phase insulin secretion than primary human islets
- Normalize blood glucose in diabetic mice
- Improve glycemic control in diabetic humans and non-human primates

Chromatin Accessibility
- Open chromatin state of core endocrine identity genes
- Closed chromatin state at multiple maturation identity genes
- Open chromatin state of several off-target and progenitor cell identity genes
- Small differences in cell identity based on chromatin accessibility

Gene Expression
- Express core transcriptional identity markers of pancreatic endocrine cell types
  - *PDX1*, *NKX6-1*, *CHGA*, *INS*, *GCG*, *SST*
- Expression of some β cell maturation markers is low or absent *in vitro*
  - *MAFA*, *SIX3*, *UCN3*, *IAPP*, *G6PC2*, *CHGB*

Composition
- Contain pancreatic β, α, and δ cells
- Off-target cell types are also present, especially enterochromaffin cells
- Importance of non-β cell types to β cell health and function is unclear
- Difficult to control the relative abundance of these cell types

Figure 3. Critical quality attributes of SC-islets
Despite making up a very small percentage of the pancreas, primary islets function to maintain blood glucose levels within a tightly regulated range. SC-islets have been able to replicate some of the key attributes of native islet function but fall short in some areas. Critical quality attributes that have been achieved in SC-islets are indicated in black, while those that remain unresolved are indicated in red.

generation of glucose-responsive β cells in 2014. Further refinement of these differentiation strategies induced further functional maturation of SC-islets, facilitating the acquisition of biphasic insulin secretion kinetics similar to that observed in primary human islets. These cells are able to rapidly cure severely diabetic mice and improve glycemic control in non-human primates, and continued progress has allowed the field to narrow in on the critical quality attributes of these cells (Figure 3).

As discussed throughout this review, the latest developments in SC-islet generation and immune protection have the potential to increase the efficacy and safety of the final cell product. For example, single-cell sequencing technologies have allowed for unprecedented characterization of SC-islets, providing insight into the identity and maturation state of the cells produced with these differentiation protocols. These data on the transcriptional and chromatin landscape of SC-islet cell types can guide the next generation of protocols for refining SC-islet production in an effort to increase insulin secretion per cell and eliminate off-target cell types. Progress has also been made with immunoprotective strategies, including tailored biomaterials that induced local immune tolerance as well as genetically engineered cells that evade the immune system. These

approaches offer the potential for avoiding immunosuppressive drugs altogether, broadening the applicability of this cell therapy. Finally, further genetic engineering approaches to improve SC-islet resistance to the stressors they experience immediately after transplantation will likely be key to improving graft viability and reducing the number of cells needed for insulin independence.

AUTHOR CONTRIBUTIONS

N.J.H., M.I., and J.R.M. wrote, edited, and reviewed the manuscript.

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