Emerging diabetes therapies: Bringing back the \(\beta\)-cells

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ABSTRACT

Background: Stem cell therapies are finally coming of age as a viable alternative to pancreatic islet transplantation for the treatment of insulin-dependent diabetes. Several clinical trials using human embryonic stem cell (hESC)-derived \(\beta\)-like cells are currently underway, with encouraging preliminary results. Remaining challenges notwithstanding, these strategies are widely expected to reduce our reliance on isolated islets for transplantation procedures, making cell therapies available to millions of diabetic patients. At the same time, advances in our understanding of pancreatic cell plasticity and the molecular mechanisms behind \(\beta\)-cell replication and regeneration have spawned a multitude of translational efforts aimed at inducing \(\beta\)-cell replenishment in situ through pharmacological means, thus circumventing the need for transplantation.

Scope of Review: We discuss here the current state of the art in hESC transplantation, as well as the parallel quest to discover agents capable of either preserving the residual mass of \(\beta\)-cells or inducing their proliferation, transdifferentiation or differentiation from progenitor cells.

Major Conclusions: Stem cell-based replacement therapies in the mold of islet transplantation are already around the corner, but a permanent cure for type 1 diabetes will likely require the endogenous regeneration of \(\beta\)-cells aided by interventions to restore the immune balance. The promise of current research avenues and a strong pipeline of clinical trials designed to tackle these challenges bode well for the realization of this goal.

Keywords
Islet regeneration; Beta cell proliferation; Pancreatic progenitor cells; Human embryonic stem cell transplantation

1. INTRODUCTION

Endocrine cells within the pancreatic islets of Langerhans are responsible for the maintenance of glucose homeostasis in all vertebrates. The targeted autoimmune destruction of insulin-secreting \(\beta\)-cells results in type 1 diabetes (T1D). Insulin administration remains a life-saving therapy for T1D patients. However, exogenous insulin fails to exert the tight regulation of native islets, and daily blood glucose excursions are inevitable. In the long run, the inability of the patient to maintain adequate glycemic control typically leads to vascular disease and a host of derived complications, including blindness and kidney failure [1].

Islet transplantation (IT) from cadaveric donors has proven successful at restoring normoglycemia in a subset of T1D patients, especially since the development of steroid-free immunosuppression regimes [2] and, more recently, targeted immunotherapies that ensure high insulin independence rates many years post-transplantation. However, the widespread application of IT is limited by the need for chronic immune suppression and a worldwide dearth of donor organs. While more than 1,500 recipients have received IT since the turn of the century, this figure pales in comparison with the demand for an effective cell therapy that could be applied to the millions of patients afflicted by this devastating disease.

Initial expectations for IT to become a curative strategy have cooled down with time, and few would expect for this intervention to remain a significant front in our war against the disease throughout the next decade. However, the trailblazing impact of IT in shaping the next generation of cell therapies for T1D cannot be overemphasized. In fact, one of the very first successful attempts at reversing diabetes with human embryonic stem cells (hESCs) has taken the shape of intraportal transplantation of islet-like clusters (ClinicalTrials.gov identifier: NCT04786262).

In this review, we will first discuss the current impetus to phase out islet transplantation in favor of stem cell-based cell products, an effort

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20 years in the making that is finally bearing fruit in the form of multiple ongoing clinical trials. However, the restlessness of the field is evidenced in the emergence of many alternative approaches focused on harnessing the natural potential of the pancreas to regenerate its own β-cells, be it by proliferation, transdifferentiation or by the stimulation of resident progenitor cells. There is a growing perception that we are simultaneously reaching a critical mass of knowledge and the technical maturity to effectively combat the disease. The natural culmination of decades-long research endeavors is stoked and exponentially accelerated by ever more powerful technological platforms. One hundred years after the discovery of insulin, we are arguably entering a new golden era in diabetes research.

2. β-CELL REPLACEMENT

The last two decades have witnessed an unrelenting quest to create an unlimited supply of insulin-producing cells that could be transplanted in lieu of islets. Several cell sources that were actively explored throughout the early 2000s (e.g., mesenchymal, hematopoietic, fetal, xenogeneic, immortalized β-cell lines) are no longer a primary focus for the development of β-cell replacement therapies. Rather, the direct differentiation of human pluripotent stem cells (hPSCs) into pancreatic cell types has taken center stage. hPSCs come in two flavors: Embryonic stem cells (ESCs), which are derived from the inner cell mass of fertilized blastocysts not used in in vitro fertilization treatments; and induced pluripotent stem cells (iPSCs) generated by the reprogramming of somatic cells, a procurement method that circumvents the ethical concerns that hindered the use of ESCs. These two hPSC types share two main characteristics, namely, the capacity to divide rapidly in an undifferentiated fashion, and the ability to differentiate into virtually every cell type present in the body. While the promise of iPSCs remains high in the context of personalized medicine, most translational advances described thus far have focused on the use of hESCs. Seminal work by Viaucye, Inc. between 2006 and 2011 [3–6] pioneered a direct differentiation approach to guide hPSCs through subsequent developmental steps into pancreatic cell types. This was achieved by exposing hPSCs to recombinant proteins and/or small molecules to modulate key signaling pathways in a stepwise manner during the differentiation process. A phenomenal body of work employing predominantly murine models provided important insights into the signaling pathways that orchestrate pancreas organogenesis [7–9]. The early differentiation steps are highly efficient, and subsequent pancreatic endodermal (PE) cells, characterized by the co-expression of PDX1 and NKX6.1, can be generated with high purity. However, inducing endocrine differentiation in PE cells in vitro is inefficient and requires incubation with several inducing factors for a week or longer. Initially, while hormone-expressing cells could be produced using this strategy, those that expressed insulin did not do so in a glucose-responsive manner [4]. In fact, many cells expressed multiple hormones, a reflection of their functional immaturity. In contrast, when PE cells were transplanted into immune deficient mice, grafts gave rise to fully functional, glucose-responsive stem cell-derived β-like cells (sBCs) that can reverse chemically-induced diabetes [5,6,10,11]. The acquisition of functionality was attributed to the complex microenvironmental signaling milieu in the engraftment site, which could not be realistically replicated in vitro. These results using PE cells provided the basis for first-in-human clinical trials using stem cell-derived human pancreatic cells.

Fueled by the demonstration that stem cell-derived PE cells have the potential to differentiate into sBCs in vivo, several groups focused on generating functional, glucose-responsive s- and β-like cells in vitro [12,13]. In 2014, two of them reported the successful generation of functional sBCs within 4 weeks by optimizing the later steps of direct differentiation [14,15]. Interestingly, both teams employed similar factors to achieve this feat. While the Kieffer group described the use of an air-liquid interface culture at the end of the differentiation process, the Melton group established a suspension culture-based format employed throughout differentiation, amenable to commercial scale-up.

Conditions for the efficient generation of pancreatic progenitors were also increasingly refined [16]. In fact, focusing on further improving the generation of PE cells, we reported the generation of glucose-responsive sBCs in only 3 weeks in 2015 [17]. Over the last few years, many studies have further enhanced the in vitro generation of functional sBCs by synchronizing cultures [18], changing signaling pathway modulation [19,20], altering available energy sources in culture media [21], facilitating coalescence of sBCs into islet-like structures [22], and recreating 3D culture systems that evoked pancreatic development [23]. Taken together, published data indicate that sBCs exhibit distinct degrees of functionality (often referred as maturity). Indeed, in-depth characterization has revealed the presence of distinct subpopulations even in sBCs with dynamic insulin secretion, a hallmark of maturity [24]. Additional refinements of late-stage culture conditions might allow the capture of a more mature sBC phenotype, as current medium formulations fail even at preserving function in isolated cadaveric islets. Another often underappreciated aspect of the problem is the inability of current protocols to generate highly pure populations of sBCs. One example was provided by Veres et al., who observed the formation of enterochromaffin-like cells during the reprogramming of human stem cells towards a β-like cell phenotype, affecting overall differentiation efficiency [25]. How other contaminating cell types present in sBC cultures might influence performance is largely unknown. Batch-to-batch differences are also commonly observed, which sometimes stands in the way of consistent reproducibility. Despite these remaining challenges, the direct differentiation of hPSCs into sBCs provides an abundant source of human β-cells with distinct advantages compared to cadaveric islets for basic research, chiefly the elimination of the biological/processing variability observed when working with cadaveric islet preparations.

State-of-the-art genome engineering approaches allow researchers to readily modify hPSCs and cells derived therefrom, effectively enabling the generation of novel human disease models. This is especially significant given the historic difficulty to manipulate human β-cells, albeit recent progress in manipulating cadaveric islets as pseudo islets might provide a complementary model [26]. hPSCs also afford us the opportunity to generate other functional cell types to co-culture with sBCs, such as endothelial cells, thereby approximating the native tissue composition more closely. Indeed, recreating an appropriate niche for sBCs can improve their function and engraftment [27]. Further development of multi cell type hESC/iPSC platforms will enable disease modeling in an isogenic manner, a critical, yet largely unmet need in the wider T1D research space. Isogenic human approaches will be especially important to study interactions between immune and β-cells derived from the same donors.
3. CLINICAL TRANSLATION OF HPSC-DERIVED PANCREATIC CELLS

There are several ongoing clinical trials evaluating the safety and efficacy of either PE cells or sBCs (Figure 1). First-in-human trials were initiated by ViaCyte in 2014. The cellular product (VC-01TM/PEC-EncapTM) was largely composed of PE cells derived from the hESC line CyT49 macroencapsulated in an immunoisolation device, which was transplanted subcutaneously in fully immunocompetent patients (ClinicalTrials.gov identifier: NCT02239354). The device in question, an evolution of the Encaptra/C210 drug delivery system, permits the exchange of nutrients and gas but prevents direct interaction with host immune cells, thus providing immune protection. The study, terminated in December 2017, showed that the treatment was safe and well tolerated, with no teratoma formation. The analysis of explanted sentinel devices demonstrated the in vivo generation of insulin-producing cells after several months. However, the lack of direct interaction with the host vasculature, as well as a considerable foreign-body immune response to the device, reduced the exchange of oxygen and nutrients, negatively impacting the survival of transplanted cells. A phase II clinical trial with an optimized closed device is currently underway (ClinicalTrials.gov identifier: NCT04678557). Another embodiment of the device, containing pore openings at the surface that allow for the penetration of blood vessels, was also being tested with the same cells in Phase II clinical trials (ClinicalTrials.gov identifier: NCT03163511) [28,29]. While this approach provides better oxygenation and overall conditions for grafts, the need for systemic immune suppression is still problematic. Finally, ViaCyte and CRISPR Therapeutics just started a clinical trial with cells genetically engineered to evade detection by the immune system, which would also make use of the same porous version of the device (ClinicalTrials.gov identifier: NCT05210530).

Vertex announced in 2021 a clinical phase I/II trial (ClinicalTrials.gov identifier: NCT04786262) in which functional sBCs (product name: VX-880), rather than PE cell clusters, would be transplanted for the first time via portal vein infusion into the liver, in a manner similar to that used with human islet grafts (Vertex Pharmaceuticals Incorporated [2021, March 10]. Vertex Announces FDA Fast Track Designation and Initiation of a Phase 1/2 Clinical Trial for VX-880, a Novel Investigational Cell Therapy for the Treatment of Type 1 Diabetes [Press release]. Retrieved from: https://www.businesswire.com/news/home/20210310005218/en). Since sBC clusters are naked, immune suppression is required. The results from the first patient were released in October 2021, although this report has not peer-reviewed yet. Again, the treatment was well tolerated, and significant levels of circulating human C-peptide were detected 90 days post transplantation, reaching 560 pM/L at peak stimulation by a mixed-meal tolerance test. This was associated with significant decreases in HbA1C levels and a 90% reduction in exogenous insulin administration, a remarkable result considering that the recipient, a longstanding male T1D patient with no measurable circulating C-peptide prior to the treatment, received only half the sBC dose (Vertex Pharmaceuticals Incorporated [2021, October 18]. Vertex Announces Positive Day 90 Data for the First Patient in the Phase 1/2 Clinical Trial Dosed With VX-880, a Novel Investigational Stem Cell-Derived Therapy for the Treatment of Type 1 Diabetes [Press release]. Retrieved from: https://www.businesswire.com/news/home/20211018005226/en). This trial is still ongoing at multiple sites in both US and Canada to evaluate additional patients for safety and efficacy.

Figure 1: Current clinical strategies for T1D based on hESC transplantation. Schematic depiction of the design of current clinical trials for T1D making use of hESCs. The first ones to be initiated (NCT03163511 and NCT04678557) make use of partially differentiated hESCs (Pancreatic Endoderm, or PE) that are transplanted subcutaneously within a microencapsulation device. In NCT04786262 (bottom), the cells are matured in vitro into functional glucose-responsive β-like cells (sBCs), which are subsequently transplanted naked (without immunoisolation device) intraportally with general immunosuppression of the recipient. Remaining challenges common to these approaches, further discussed in the main body of the article, include long-term function and survival of the graft, efficient generation of endocrine cells, and localized (vs. systemic) immunosuppression/immune evasion. This figure was created using Biorender.
4. TUMORIGENESIS

For all the promise of these early results, caution is warranted. The possibility that hESC products may give rise to teratomas (a common observation in preclinical models during the second half of the last decade) was arguably the most prominent roadblock that the sponsors of these initial clinical trials had to overcome in order to get regulatory approval. Teratomas are benign tumors formed as a result of the uncontrolled proliferation and differentiation of pluripotent stem cells along the three germ layers (endoderm, mesoderm and ectoderm [30]). In addition to this inherent risk, like in all rapidly dividing cells, the inherent risk of mutation and cancer progression exists [31]. While these concerns have somewhat abated with ever more refined versions of differentiation protocols and a better understanding of the transcriptional and regulatory changes occurring throughout targeted differentiation [25], the risk for uncontrolled differentiation remains [32]. Ultimately, it would take only one case to set back the entire field for years. If the product is given to fully immunocompetent patients encapsulated within a rigid, closed device, it can be argued that potential teratomas would be, first, contained; and, second, amenable to destruction by the immune system of the host. These considerations do not apply to the Vertex trial, where cells are administered naked into immunosuppressed patients. Likewise, future genetic modification strategies aimed at making the transplantable cell products invisible to the immune system (see below) would run into the same problem.

Various approaches are being explored to tackle this issue. One of these involves the purification and subsequent reaggregation of sBCs to form islet-like organoids, thereby reducing the potential for carry-over from undifferentiated and pluripotent cells and thus the risk of teratogenesis [22]. Another alternative actively explored by several groups involves the genetic modification of pluripotent cells with fail-safe mechanisms based on the concept of gene-directed enzyme prodrug therapy (GDEPT). In short, cells equipped with “suicide genes” convert harmless pro-drugs into cytotoxic metabolites. Recent examples of this technology include the use of inducible Caspase9 under the control of the pluripotent cell gene Nanog and Thymidine Kinase driven by β-actin promoters, which have been reported to eliminate undifferentiated/dividing cells or all cells in the graft [33]. Similarly, another study has described the use of inducible Caspase9 driven by Sox2 (another pluripotency gene), allowing for the targeted destruction of all undifferentiated and pluripotent cells in the graft [34]. However, these two approaches lack the ability to remove unwanted cells without compromising the entire graft. By modulating the expression of these kill switches depending on the differentiation outcome, one could remove all but select cells from a tissue graft. We have previously employed this method to generate a double fail-safe selection mechanism (based on Thymidine Kinase and Nitroreductase) that allows for the targeted ablation of both tumorigenic and non-insulin-producing cells, while selectively preserving β-cells [35].

All these approaches involve the permanent introduction of synthetically modified/optimized genes, whose long-term influence on host and graft physiology remains to be ascertained. Also, loss-of-function mutations in enzyme genes could result in reduced efficacy [36], a shortcoming that could be largely circumvented by implementing dual systems such as that where we and others have reported [33,35], or coupling of suicide genes with cell cycle [37] or pluripotency genes [33–35]. Ongoing research focused on the integration of these suicide genes into “safe harbor” regions of the genome aims at ensuring their stable and non-interfering expression. Furthermore, these kill switches will have to be incorporated into clinical grade stem cells to assess their therapeutic potential.

5. PROTECTION FROM THE IMMUNE SYSTEM

To circumvent the need for systemic immune suppression, different strategies have been the subject of intensive research efforts. The concept of islet encapsulation is based on the provision of a selectively permeable barrier between host and therapeutic tissues. Successful microencapsulation of sBCs using alginate has been associated with reversal of diabetes using preclinical animal models [38,39]. Additional bioengineering solutions aim at providing localized immune suppression by functionalizing biomaterials that can also improve cell engraftment during the peri-transplant period [40,41]. Considerable ischemia-induced cell death during the first days of transplant has been reported for both cadaveric islets and sBC grafts [42], but no effective strategies to circumvent this challenge have yet been developed. Prevascularization of transplantation sites have been shown to enhance functional engraftment of islets [43]. However, these approaches have not advanced into the clinic.

Using genomic engineering [44–47] or epigenetic modification [20], several groups have reported the successful generation of hypomune PSCs and cells derived therefrom [42]. These “immune cloaking” strategies usually work either by removing receptors important for immune cell recognition or by artificially elevating immune suppressive protein (e.g., immune checkpoint inhibitors) levels on the surface of the cells. This is an exciting and rapidly developing field, as evidenced by Viacyte’s plans to adopt this strategy in clinical trials set to start in 2022.

6. PHYSIOLOGIC PROLIFERATION OF β-CELLS

Among the alternatives to the transplantation of sBCs, interventions aimed at increasing β-cell numbers endogenously, be it by protecting those still alive after the onset of the disease or by inducing their replication, transdifferentiation or regeneration from resident progenitor-like cells, have been the subject of active research over the last decade.

The goal of expanding pancreatic β-cell mass is based on the premise that T1D patients with long-standing disease exhibit a significant number of residual β-cells, with evidence of persistent replication [48–50]. However, proliferation does not come naturally to them. β-cells control glucose homeostasis by producing large amounts of insulin and finely regulating its secretion [51]. Although they can expand in specific physiological/pathophysiological states [52], adult β-cells manifest refractoriness towards mitogenic stimuli, as is generally the case for such specialized cell types [53]. They reach the peak of their proliferative capacity during the embryonic and neonatal stages (2–4%), a figure that gradually declines during childhood, when the functional β-cell mass of an individual is fully established [54,55]. Their proliferation rates are reported to be extremely low in adult individuals (<0.5%).

The observation that new β-cells mostly originate in the postnatal period supports the concept that signaling molecules abundant either in the intrauterine environment or circulating in young individuals could promote their expansion —with the caveat that functional differences between fetal and fully developed β-cells could represent an obstacle to translate such findings in diabetic patients [56]. In this context, some key molecular pathways have already been found to regulate β-cell proliferation in both early and late stages of life. For example, the TGF-
β-cell pathway has been linked to the regulation of endocrine cell mass during pancreatic development [57]. In particular, silencing of Smad2, Smad3, and Smad 7 in endocrine progenitor cells resulted in higher numbers of insulin- and glucagon-positive cells compared to controls [58–60]. Interestingly, the inhibition of the TGF-β receptor pathway also promoted growth in adult β-cells (more on this below). Furthermore, several studies carried out in animal models have led to the identification of factors circulating in the serum of mice during pre-natal and post-natal stages. Among them, Wisp1 has been recently identified as a bloodborne factor in young mice with the capacity of stimulating expansion of adult murine and human β-cells [61]. Notwithstanding their low capacity to re-enter the cell cycle, adult β-cells are plastic enough to respond to physiological and pathophysiological increases in insulin demand, including those associated with obesity and pregnancy [52]. (Figure 2). The latter is probably the most potent phenomenon triggering β-cell expansion, as mothers increase their reserve of β-cells to face the elevated insulin needs to maintain their own glucose homeostasis and that of the fetus, as well as to provide insulin to assist in organogenesis [62]. Studies performed in animal models have identified several regulatory molecules and pathways that trigger the expansion of β-cell populations in the maternal pancreas. Indeed, the maternal β-cell pool gradually expands.

Figure 2: Molecular pathways controlling human β-cell proliferation. (Left, blue) Molecular mechanisms regulating β-cell growth to compensate for physiological (e.g. pregnancy) or pathophysiological (e.g. obesity, early onset T2D) increased insulin demand. During pregnancy, lactogenic hormones, such as prolactin are sensed by the prolactin receptor (PLR). PLR transduces the signaling downstream via the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt serine/threonine kinase (AKT) and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) axis, leading to the translocation of STAT proteins into the nucleus to initiate transcription of proliferative factors, including osteoprotegerin (OPG) and serumotin (5-HT). These molecules are then secreted in the islet milieu to sustain mitogenic signaling in an autocrine or paracrine fashion. OPG is known to increase survival of β-cells by blocking the interaction between the receptor activator of nuclear factor kappa-β (RANK) and its ligand (RANKL), avoiding the activation of glycogen synthase kinase-3β (GSK3β)-mediated intrinsic apoptotic pathways. In insulin resistance and pregnant states, circulating insulin and insulin-like growth factor 1 (IGF1) levels are elevated. Both growth factors regulate further expansion of β-cells binding the insulin (IR) and the IGF1 (IGFIR) receptors, resulting in the activation of the PI3K-AKT signaling. This, one the one hand, inhibits GSK3β; and, on the other, activates the extracellular signal-regulated kinase 1/2 (ERK1/2), which translocates into the nucleus to regulate gene expression. Overt insulin resistance leads to the increase of serum protease inhibitor B1 (SerpinB1) in the liver and other tissues with metabolic regulation properties. SerpinB1 has been identified as a bloodborne factor in young mice with the capacity of stimulating expansion of adult murine and human β-cells [61]. Notwithstanding their low capacity to re-enter the cell cycle, adult β-cells are plastic enough to respond to physiological and pathophysiological increases in insulin demand, including those associated with obesity and pregnancy [52]. (Figure 2). The latter is probably the most potent phenomenon triggering β-cell expansion, as mothers increase their reserve of β-cells to face the elevated insulin needs to maintain their own glucose homeostasis and that of the fetus, as well as to provide insulin to assist in organogenesis [62]. Studies performed in animal models have identified several regulatory molecules and pathways that trigger the expansion of β-cell populations in the maternal pancreas. Indeed, the maternal β-cell pool gradually expands.

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from G0 and peaks at 1.5-fold around G15.5, after which their number starts to decline [63]. Interestingly, the timing of this peak has been linked to an increase in placental lactogen levels, which suggests a trophic role for this pregnancy hormone [64]. Subsequent experiments have demonstrated that prolactin and PL promote β-cell growth, mainly by activating the PI3K–AKT axis and MAPK signaling [65]. In addition, lactogenic hormones induce the β-cell-specific upregulation of non-hormonal peptides, including osteoprotegerin (OPG) and serotonin (5-HT), likely via activation of STAT5. Upon secretion, these molecules have been hypothesized to stimulate growth in an autocrine/paracrine fashion [66–69].

As for obesity, the reduction of insulin action in peripheral tissues leads to insulin resistance. To counteract this effect, β-cell mass expands to increase the circulating levels of insulin [70]. This compensation has been quantified in non-diabetic obese patients [71,72] as well as in mouse models of diet-induced obesity (HFD) [73] and genetically-induced obesity (Ob/Ob) [74] or insulin resistance (LIRKO) [75,76]. Although the endogenous factors responsible for such regulation are yet to be identified, the potential mediation of circulating signaling molecules from other insulin-responsive tissues is an intriguing scenario. One of these may be leptin, a hormone produced by adipocytes that is mainly involved in the regulation of food intake. Serum leptin levels are increased in obese subjects [77,78] and in rodent β-cells it has either a proliferative or an antiapoptotic effect. However, they translate poorly in human islet samples. Likewise, SerpinB1, a protease inhibitor which is upregulated in hepatocytes of insulin-resistant mice (e.g., LIRKO) via a FoxO1-dependent mechanism, stimulates β-cell proliferation in both murine and human islets [79,80]. In fact, SerpinB1 serum levels directly correlate with insulin resistance in non-diabetic subjects, and loss-of-function variants are associated with multigenerational diabetes in humans [79]. Conversely, inhibition of SerpinB1 results in enhanced β-cell development and could also have effects on adult human β-cell expansion [81].

While the use of murine models has aided in the identification of a plethora of agents able to boost β-cell duplication (including glucose, insulin, IGF-1, incretins and their analogues, placental hormones, platelet-derived growth factor, leptin, serotonin, growth hormone, and parathyroid hormone-related protein [82–88]), many of them have failed to act as β-cell mitogens in human samples. Such discrepancies are partially attributable to the structural and biological differences between rodent and human islets. Indeed, the composition and expression levels of cell cycle proteins are different between β-cells from both species. For example, the expression of Cyclin D3 and CDK6 is critical for the onset of the cell cycle in humans but not in mice, whereas Cyclin D2 is expressed in mouse β-cells yet is virtually absent in their human counterparts [85,89,90]. In this context, worldwide initiatives such as the NIH’s Human Islet Research Network (HIRN) or the Network for Pancreatic Donors with Diabetes (nPOD) mark a progressively diminished reliance on murine models and hail a renewed and square focus on the human pancreas. Among these human-centric models, islets still represent the gold standard to study β-cell proliferation [91]. The preservation of the 3D structure maintains the in vivo organization, including cell-ECM and cell-to-cell interactions, allowing for a better translation to in vivo studies [92,93]. However, the variability inherent to the donors and isolation methods, as well as their shipment from islet procurement centers, present challenges that often affect the reproducibility of the data from lab to lab. Human β-cell lines circumvent this problem and can be readily used, cultured as monolayers, to study transcriptomic and proteomic changes following treatments with mitogens, or to investigate the causes of β-cell death in T1D and T2D [94,95]. In particular, the EndoC-βH3 cell line mirrors with the basal proliferation rate of primary human β-cells, following drug-mediated excision of the immortalizing genes [96]. This feature allows proof-of-concept experiments to proceed before validation in human islets. Furthermore, they can be transfected for silencing or overexpressing candidate genes involved in β-cell regeneration and therefore employed in large-scale studies for compound or CRISPR/Cas-9 library screenings [97]. As described in earlier sections, advances in stem cell differentiation [14,15,19,22,98] have enabled the generation of sBCs with islet-like properties. These endocrine cells further aggregate into 3D islet organoids or “pseudoislets” that can be employed in studies aimed at the identification of agents and molecular signaling pathways regulating β-cell growth and survival [26,99]. The relatively short time needed to generate these cells can compensate for the restricted availability of primary human islets, especially as sBC generation is progressively becoming mainstream. Finally, human pancreatic slice (HPS)-based technologies have recently stormed the field as a powerful model that preserves the integrity of the human pancreas, including native endocrine–exocrine interactions, for extended periods [100–103]. This model affords the study of β-cell regeneration in an in vitro context that is even truer to nature than isolated islets, the standard bearer up to this point.

7. β-CELL MITOGENS: THE ROAD TO CLINICAL TRANSLATION

The natural ability of β-cells to proliferate through physiological stimuli, described in the previous section, has prompted a search for therapeutic leads that could be used in a clinical setting. Over the last several years, numerous targets and compounds have shown that human β-cell proliferation is possible (Figure 2). Among the most notable examples is the discovery of the dual-specificity tyrosine-regulated kinase1a (DYRK1A) inhibitors harmine and 5-iodotubercidin (5-IT), as well as a series of aminopyrazines [104], as potent inducers of human β-cell proliferation [104–106]. The aminopyrazines showed inhibitory effects not only towards DYRK1A but also to glycocon synthase kinase 3β (GSK3β). GSK3β is a well-known inhibitor of β- (but also general cell) proliferation [107,108], and the simultaneous inhibition of DYRK1A and GSK3β proved more effective than inhibition of either alone [104]. This finding is not so surprising, given the fact that both interact directly, and DYRK1A has been suggested as a priming kinase for GSK3β. More recent studies have shown that the simultaneous inhibition of DYRK1A and DYRK1B is necessary for proliferative activity [109]. These observations demonstrate that poly-pharmacology may be a productive avenue to induce β-cell proliferation. Consistent with these findings, a recent report showed that the simultaneous pharmacological inhibition of DYRK1A and either TGFβR or GLP-1R agonists [109], has synergistic effects on β-cell proliferation in human cells, as inhibition of TGFβ also promotes it [110].

Although important as a proof of concept, targeting DYRK1A may have some pharmacological disadvantages. First, DYRK1A has a broad tissue expression, and therefore may induce undesirable proliferation events in peripheral tissues. Second, most DYRK1A inhibitors described thus far show a lack of specificity within their kinase families. In fact, both 5-IT and harmine target not only DYRK1A but also a subset of other kinases [111], whose inhibition may have yet unknown effects in humans.

On balance, these discoveries are paving the way to the identification of compounds with novel mechanisms of action and high selectivity for β-cells. For example, GNF-9228 was discovered to promote DYRK1A-independent human β-cell proliferation [112]. Further, heterozygous inactivation of the Na+/Ca2+ exchanger isoform 1 (Ncx1) increased...
their proliferation in mice [113]. Based on these findings, the same group found that chemical inhibition of NCX1 had the same effect [114].

Glucocorticoids have also been found to promote β-cell replication in rat and human islets [115]. While glucocorticoids induce insulin resistance on their own [116], in this case the authors aimed to expand β-cells ex vivo for transplantation, so this systemic effect may not be as much of an issue.

Finally, a high-throughput screening assay based on lentivirus-mediated silencing of genes coding for G-protein coupled receptors (GPCRome) in islet cells led to the identification of the G-protein coupled receptor 3 (GPR3)-salt induced kinase 2 (SIK2) axis as a regulatory pathway of adult human β-cell quiescence [117]. These findings may suggest new targets to promote human β-cell proliferation.

8. INDUCTION OF β-CELL REGENERATION: PROGENITOR-LIKE CELLS

The traditional hypothesis that ductal progenitor cells contribute to β-cell regeneration in adulthood, as they do to islet formation during embryonic development [118,119], was significantly challenged nearly two decades ago, when it received a blow from which it is only now starting to recover. In 2004, lineage-tracing experiments in mice led to the conclusion that post-natal generation of β-cells occurred only through the proliferation of pre-existing ones, rather than by neogenesis [120]. Although this study did not disprove the existence of facultative multipotent cells [121], it shaped the now commonly held view that pancreatic regeneration does not rely at all on progenitor cells [122,123].

However, a closer examination of these and other similar studies reveals that most of the evidence cited to support the self-replication model is based on mouse models. We have discussed in a previous section the prominent differences between murine and human islets, especially those related to their natural β-cell turnover (several orders of magnitude higher in mice) [124] and the very fabric of their proliferation machinery [13,58,59]. As for the use of lineage tracing as the primary tool to draw conclusions about cellular fate, the emergence of newer paradigms about cell identity (i.e., that there are developmental gradients and dynamic de-differentiation/re-differentiation forces at play in adult tissues, rather than immutable cell fates) puts in question the reliability of most lineage tracing inferences. Inaccurate techniques conducted on suboptimal models are unlikely to yield sound biological conclusions (reviewed in [125]). This notion, coupled with a recent renaissance of the ductal progenitor cell hypothesis (now under the light of cell fate heterogeneity and facultative stress-induced de-differentiation) is bringing back a lively debate that many had considered to be settled by now.

The reality is probably more nuanced than any of the two camps is likely to admit. Revisiting the available data shows that self-duplication and progenitor cell-based replenishment hypotheses could potentially be reconciled into a unified model. Self-replication and low-level local transdifferentiation could be the primary mechanisms controlling natural turnover, but more extensive damage may activate de-differentiation and redifferentification processes that include ductal-based endocrine regeneration [125]. Our group has recently identified a subpopulation of human ductal resident cells characterized by their ability to respond to the stimulation of the bone morphogenetic protein (BMP) pathway [126,127]. These cells, whose bona fide progenitor features have been confirmed by single cell transcriptomics [128], proliferate in response to BMP agonists and differentiate into all pancreatic lineages (including functional insulin-producing β-cells) upon withdrawal of the stimulus. When sorted and transplanted into immune-deficient mice, BMP-responsive progenitors not only differentiate spontaneously but, strikingly, are able to rearrange their differentiated progeny into micro-pancreata of sorts, with well-formed acini, draining ducts and interspersed functional islets [129]. These effects, also reported in human pancreatic slices from healthy and diabetic donors, were significantly magnified when the hosts received systemic treatment with THR-123, a BMP receptor agonist [129]. The pharmacological activation of resident progenitor-like cells within the pancreas could be a viable therapeutic strategy, especially for those longstanding patients who may no longer have a meaningful residual mass of β-cells amenable to replication-based interventions. Of course, any islet regenerative drug may have to be given alongside others to stop T1D autoimmunity, a consideration common to all endogenous regeneration approaches.

9. β-CELL PRESERVATION

Another approach to prevent the loss of β-cell mass during T1D is to protect them from cell death in the first place. A model frequently used to mimic the autoimmune attack in T1D involves treatment of cultured islets with inflammatory cytokines such as interleukin-1β (IL-1β), interferon-β (IFN-β), and tumor necrosis factor-α (TNF-α) produced by macrophages and T cells [130,131]. IL-1β and TNF-α induce NF-κB expression and downstream gene expression regulated by nitric oxide (NO) signaling. This, in turn, increases endoplasmic reticulum stress response pathways and decreases β-cell specific function and survival [130,132]. NF-κB activation, along with IFN-γ-induced STAT1 signaling [133,134], abolishes insulin secretion and induces β-cell apoptosis [135]. Efforts to inhibit signaling of individual cytokines with protein-based receptor antagonists have previously progressed to clinical trials, but not led to approved therapies [136]. On the other hand, small-molecule suppressors of cytokine-induced β-cell apoptosis could lead to new targets for therapeutic intervention. Some studies have associated natural products, such as extracts from *Artemisia capillaris* [137] and St. John’s wort [138] with β-cell protective effects against cytokines. Many of these compounds were discovered because of their general antioxidant or anti-inflammatory properties, but some of them have more specific activity against JAK-STAT [139] or NF-κB signaling [140]. These findings have historically been serendipitous, but more systematic efforts have been described. For example, we identified compound BRD0476 after high-throughput screening of our Diversity-Oriented Synthesis (DOS) library as a suppressor of β-cell apoptosis [141]. More recently, TYK2 (tyrosine kinase 2) has been proposed as a susceptibility gene for T1D [142]. Early insulitis activates Interferon (IFN)-κ signaling via JAK1 and TYK2 and produces endoplasmic reticulum stress, inflammation and HLA class I overexpression, leading to β-cell apoptosis. Preclinical evaluation of TYK2 inhibitors revealed their efficacy in inhibiting IFNκs signaling in human β-cells, decreasing inflammation and apoptosis [143]. Estrogens also protect human β cell survival and function in the face of multiple diabetic insults [144]. In a model of xenotransplantation of human islets in immunodeficient diabetic mice, estrogen agonists enhanced islet oxygenation, prevented islet oxidative injury and apoptosis, and promoted islet graft revascularization, leading to prolonged normoglycemia [145]. Estrogen ligands could prove useful in the early phase of hESC-derived β-like cells transplantation to protect from pro-apoptotic stress and promote graft revascularization. Finally, the importance of senescent
β-cells to T1D has become recently appreciated [146]. The adoption of a senescence-associated secretory phenotype (SASP) by a subset of β-cells is accompanied by upregulation of the anti-apoptotic protein Bcl-2. Using the small-molecule Bcl-2 inhibitor venetoclax to eliminate senescent β-cells in the nonobese diabetic (NOD) mouse model stopped autoimmune destruction and prevented the development of diabetes [147]. The fate of the β-cell in overt T2D is also compromised. Toxicity due to high glucose and free fatty acids (glucolipotoxicity) are considered critical driving forces of β-cell death. At least two reports of systematic high-throughput screening campaigns have been described [148,149]. The first report focused more on the protection from lipotoxicity. Hit prioritization and in silico optimization ultimately identified MAP4K4 inhibitors and the endocannabinoid anandamide as effective suppressors of palmitate-induced apoptosis in INS-1 cells, rat islets, and human islets [149]. The second described a large-scale effort aimed at protecting β-cells from glucolipotoxicity. Screening hit characterization identified six scaffolds that specifically protected β-cells from glucolipotoxicity-induced apoptosis [148]. Following systematic medicinal chemistry efforts, Compound D was identified as the most potent and selective. Mechanistic studies showed that this agent works by reducing calcium influx, although definitive target proteins were not identified.

Thioredoxin-interacting protein (TXNIP) has also emerged as a promising therapeutic target to promote β-cells survival. TXNIP was discovered as the top glucose-induced gene in a human pancreatic islet gene-expression analysis [150]. Increased TXNIP expression is associated with diabetes and β-cell apoptosis, while its absence in β-cells decreases apoptosis and protects from diabetes. Screening of a ~300,000-compound library identified SRI-37330, a small molecule inhibitor of TXNIP expression, that effectively rescued mice from obesity-induced diabetes [151]. Moreover, SRI-37330 inhibited glucagon secretion, reduced hepatic glucose production, and reversed hepatic steatosis. Continued studies in this area are needed ensure that this approach is effective at protecting hESC-derived β-like cells in vivo. Importantly, approaches aimed at preserving the reserve of pre-existing β-cells to re-establish glucose homeostasis in patients with T1D require specific targeting of healthy β-cells, eliminating the risk of maintaining cells that should be eventually depleted, like senescent β-cells with a secretory phenotype, known to take part in the pathogenesis of T1D [147].

10. β-CELL TRANSDIFFERENTIATION

Cell state plasticity has become an increasingly recognized phenomenon that allows us to entertain the notion of inducing cellular transdifferentiation. Although several groups aim to direct the formation of β-cells from other cell types using long-term cultures and multiple treatments ex vivo, here we focus on studies involving single treatments to induce β-cell conversion from either exocrine or other islet cells. An intuitive starting point for generating new β-cells is the α-cell, since both cell types share a common developmental lineage trajectory [152]. Indeed, human α-cells were shown to be plastic enough for reprogramming into insulin-producing cells [153]. A few examples in the literature help lay the foundation for the promise of small molecule-induced α-to β-cell transdifferentiation. First, BRD7389 was identified from a phenotypic screen of ~30,000 compounds inducing insulin production in the murine α-cell line αT3/1 [154]. Mechanically, BRD7389 exhibited inhibitory effects on the RSK kinase family. Subsequently, we described a high-throughput screen of ~60,000 compounds to identify inducers of PDX1 (a master regulator of β-cell development and insulin expression) in the human ductal adenocarcinoma cell line PANc-1. The compound BRD7552 was identified as an inducer of PDX1 mRNA and protein expression. Prolonged BRD7552 treatment also induced insulin mRNA and protein expression, although the insulin levels were not comparable to those of normal β-cells [155]. In addition, the newly generated β-cells were not able to secrete insulin in response to glucose stimulation [156]. Finally, another approach showed that chronic GABA treatment could induce α-to β-cell transdifferentiation in vivo, leading to higher insulin secretion levels upon challenge with glucose [156]. Similar results were described independently, which also identified artemisins, molecules that act by stabilizing gephyrin to potentiate GABA signaling [157]. However, subsequent lineage-tracing studies called these results into question [158]. Although these studies may provide some promise, much work is required to reduce this idea to practice.

11. CONSIDERATIONS ABOUT SYSTEMIC VS. TARGETED ADMINISTRATION

Unwanted effects in tissues other than the target cells is a potential concern in the clinical translation of in situ β-cell preservation/regenerative strategies. For example, DYRK1A inhibitors are known to also induce the replication of α-cells, which exhibit an even higher proliferative capacity than β-cells [159,160]. In this context, identifying β-cell-specific mitogens, or developing targeting strategies specifically to β- or progenitor cells, may be desirable. Efforts in this direction include the development of zinc ion (Zn²⁺)-binding molecules to carry small molecules to β-cells based on the unusually high concentration of this element in their insulin secretory granules (more than a million-fold higher than in other cell types and plasma [161–164]). Zn²⁺ is a strong Lewis acid, a feature that makes it especially suitable to develop selective reaction-based probes to image it in tissues [165,166]. Notably, Yang and collaborators adapted a Zn²⁺-prodrug (Zn-PD) to carry a fluorophore (DA-ZP1) that is released selectively in mouse and human β-cells [162,167–169]. In our own proof-of-concept studies, we designed molecules able to release ZP1 and BODIPY selectively in β-cells for sorting and islet imaging [162,168]. We also developed second-generation conjugates of Zn²⁺ chelators with proliferating reagents (GFN4877 and harmine) [162,167]. New assays that allow rapid screening of known Zn²⁺ chelators and cargos (e.g., mitogens, immunosuppressants) will facilitate the development of this approach to delivery.

GLP-1 receptor agonists (GLP-1RAs) are currently used in the clinic to stimulate insulin secretion in T2D patients [170,171]. The GLP-1 receptor (GLP-1R) is highly expressed in β-cells compared to other surrounding tissues, an observation that has led to the consideration of chemically optimized GLP-1RAs as a backbone to deliver therapeutic cargo to β-cells. For example, to explore the preferential targeting of estrogens to β-cells, GLP-1 and estrogen dual agonists were generated to avoid estrogen release in circulation and maximize estrogen release in GLP-1R-expressing β-cells. GLP-1 and estrogen dual agonists provided additional efficacy relative to GLP-1 alone not only on β-cell survival [172] but also on insulin sensitivity and glucose homeostasis in wild-type mice [173]. However, GLP-1Rs are far from β-cell specific, as expression has also been reported in the central nervous system, the heart, gastric smooth muscle, and pancreatic ductal cells, among other tissues [106,174–176]. There are other cell-surface markers that could be used for β-cell targeting, such as VMAT2, dopamine receptor D2, and SUR1.
Cargo delivery can take a similar approach to antibody—drug conjugates, where conjugating a small molecule to specific surface-marker antibodies can help deliver the cargo specifically to the intended cell type. Alternatively, small molecules or peptides that bind those surface markers themselves can be used instead of antibodies. Probes targeting cell surface include dihydrotestrazenine (DTBZ), which targets VMAT2; (-)-4-propyl-9-hydroxynaphthoxazine (PHNO) and fallypride, which target D2; and glibenclamide, which targets the KATP channel. Other markers, such as GRP44, somatostatin receptor SSTR2/5, and NTPDase3 are less studied, but still hold promise [165,177-179].

Ultimately, one must balance whether the additional regulatory burden of including targeting provisions is truly warranted in view of the potential scope of off-target effects. The GLP-1 analogs above are a clear example of this: while they are intended to act on β-cells, they also target many other cell types. This has not impeded their widespread pharmacological use, as they have proven exceedingly safe. Reservations about potential off-target induction of proliferation may be less concerning from a clinical perspective, provided that there is a measurable therapeutic effect and proliferation occurs only during exposure to the drug. Proliferation in itself is not associated with carcinogenesis, or an impediment for initial efficacy assessment at early drug development [180]. The FDA has approved many drugs with broad proliferation effects, such as estrogens or growth hormone. Similarly, while some may frown upon the notion of using BMP agonists (i.e., growth factors) systemically for ductal-mediated β-cell regeneration, THR-184, a cyclic peptide with BMP activity, has recently completed a multicenter Phase 2 clinical trial for acute kidney injury (AKI) following cardiac surgery. Although the study was not continued after Phase 2, due to lack of efficacy for the intended application, the agent was given systemically to hundreds of patients with an excellent toxicity profile and no reported side effects [181].

Ultimately, no drug is 100% safe, but if the benefits far outweigh the risks, systemic administration would not be contraindicated.

12. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Looking back at two decades of research on pancreatic regeneration, it is difficult not to marvel at the progress the field has made. The turn of the century had just brought us the Edmonton protocol, which arguably marked the heyday of human islet transplantation [2]. Human embryonic stem cells had been isolated barely a couple of years before [182], and in those early days it was difficult to separate hype from legitimate promise.

For nearly a decade, the groundbreaking vision of generating a bona fide replacement for islets from hPSCs collided, first, with the stubborn resistance of these cells to become endoend; and then, with their inability to become functional β-cells, the undesirable occurrence of teratomas, and a hostile political climate that led to much of this pioneering research to be conducted exclusively through private funding.

One by one, all these obstacles have been successfully overcome. When the FDA greenlighted hPSC-based clinical trials at the beginning of the last decade, a door opened that will not likely close anytime soon. With several clinical trials already ongoing and a healthy pipeline in the immediate horizon, the prospects of developing an effective cell therapy available to millions of T1D patients have never looked closer to materializing. Following a road paved by three decades of IT, stem cell-based transplantation will probably be the treatment of choice in the short run. Ensuring the survival and function of the transplanted cells remains the top challenge in the field, and few would consider systemic immunosuppression to be a viable therapeutic path regardless of the constant refinement of existing protocols. Retrieveable immunosoliation devices, such as the ones currently tested clinically with hESC, might offer a temporary solution until the ultimate goal of immune tolerance is achieved. However, their effectiveness to fend off autoimmunity has not yet been demonstrated. In this context, insulin-dependent T2D may become a first target to test these interventions in the absence of autoimmune reaction to the graft. Of note, the sex of the donor hPSCs may be considered a genetic modifier of cellular function and transplantation outcomes [183]. In a retrospective analysis of T1D patients followed for 20 years after IT, recipients of islets from female donors exhibited prolonged graft survival compared to recipients of islets from male donors [184]. However, variability inherent to the donors, including lifestyle and medical treatments, may be confounding factors that need to be further analyzed in larger samples.

The unprecedented pace of hESC research has not stymied the pursuit of alternative paths aimed at inducing β-cell replacement in situ. Research on β-cell regeneration is as active now as it has ever been, stoked by a better understanding of what makes β-cells replicate and ductal progenitors activate. Remaining challenges in immunosoliating transplanted cell products have made the prospect of harnessing endogenous regeneration pathways all the more attractive, as pharmacological strategies would entirely circumvent the need for transplantation. An emerging view is taking shape where cell therapies might be able to provide glucose regulation for as long as the devices can effectively protect the cells — which may be weeks, months, or years. Since current embodiments of this approach involve subcunaneous transplantation, the prospect of replacing devices and cell cargo every few months as needed is not unreasonable. Therapies such as these are now within sight, and they are expected to improve dramatically the quality of life of patients compared to the best standards of care today. Still, a permanent cure will likely be one where new β-cells grow and stay in place, hopefully aided by interventions to stop autoimmunity. If the speed at which this research has progressed in recent years is any indication, the next decade may bring the realization of many of these groundbreaking ideas into transformative therapies for diabetes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in connection with this manuscript.
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