Scaling Insulin-Producing Cells by Multiple Strategies

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In the quest to combat insulin-dependent diabetes mellitus (IDDM), allogenic pancreatic islet cell therapy sourced from deceased donors represents a significant therapeutic advance. However, the applicability of this approach is hampered by donor scarcity and the demand for sustained immunosuppression. Human induced pluripotent stem cells are a game-changing resource for generating synthetic functional insulin-producing β cells. In addition, novel methodologies allow the direct expansion of pancreatic progenitors and mature β cells, thereby circumventing prolonged differentiation. Nevertheless, achieving practical reproducibility and scalability presents a substantial challenge for this technology. As these innovative approaches become more prominent, it is crucial to thoroughly evaluate existing expansion techniques with an emphasis on their optimization and scalability. This manuscript delineates these cutting-edge advancements, offers a critical analysis of the prevailing strategies, and underscores pivotal challenges, including cost-efficiency and logistical issues. Our insights provide a roadmap, elucidating both the promises and the imperatives in harnessing the potential of these cellular therapies for IDDM.

Keywords: Diabetes mellitus; Islet transplantation; Stem cells; Cell expansion; Mitogens; Cryopreservation

INTRODUCTION

Diabetes is a heterogeneous metabolic disorder characterized by chronic hyperglycemia, principally due to the loss of β cell mass or a decline in β cell function [1-3]. Although current therapeutic strategies mitigate symptoms and temporarily improve glycemic control, they do not prevent or significantly forestall the progression to associated comorbidities. True remediation of diabetes necessitates the replenishment of β cells capable of precise blood glucose regulation. This can be accomplished primarily through two approaches: increasing the number of endogenous β cells or transplanting β cells from external sources. Stem cell-based islet replacement therapy has emerged as a curative approach for insulin-dependent diabetes mellitus (IDDM) across several preclinical animal studies [4-12] and human trials [13-15]. In 2000, the capabilities of islet replacement therapy were demonstrated by a hallmark islet transplantation study, wherein all seven patients with type 1 diabetes (T1D) who underwent transplantation with islets from cadaveric donors became insulin-independent for a minimum duration of 1 year [16]. Advancements in the islet isolation protocol, along with improvements in immunosuppressive regimen, known as the Edmonton Protocol, have set a new benchmark in diabetes treatment. However, the limited availability of cadaveric donor islets in comparison to the demand by T1D patients presents a substantial challenge. Over the past decades, efforts have been made to establish...
a reliable islet supply chain through the use of advanced islet storage and pooling of islets from multiple donors. Another renewable source for the generation of β cells using human pluripotent stem cells (hPSCs) has been attracting attention as an alternative strategy to overcome the shortage of healthy donor pancreases [17,18]. Clinical trials typically employ hPSC-derived pancreatic and endocrine progenitor cells for transplantation. Viacyte’s 2014 phase 1/2 clinical trial (NCT02239354) used pancreatic progenitor cells with a macroencapsulation device to treat T1D, followed by a 2016 study (NCT02939118) to assess adverse effects. Although minor adverse effects were reported, graft longevity requires further study. Recently, a phase 1/2 clinical trial (NCT04786262) by Vertex Pharmaceuticals demonstrated that all six T1D patients receiving transplants of hPSC-derived islets (VX-880) showed improved glycemic control through endogenous insulin production. Moving beyond the scope of islet transplantation from external sources, there is a growing focus on therapeutic strategies aimed at increasing the body’s own production of insulin-producing cells, a paradigm shift that offers the potential for more integrated and sustainable diabetes management. To increase the number of endogenous β cells, one strategy is to stimulate the proliferation of existing β cells, while another involves inducing the transdifferentiation of non-insulin-producing cells into β cells (Fig. 1). β cell proliferation has shown promise in preclinical animal models, as well as in select human cases, such as during pregnancy, offering potential therapeutic avenues for IDDM [19,20]. However, human β cells exhibit a lower proliferative capacity than those in preclinical animal models, achieving an average proliferation rate of less than 1% in human adults and diminishing further with age [21]. Current research efforts are directed toward identifying small molecules, biologics, and pathways that can enhance the proliferative capacity of human β cells. Compounds targeting glucagon-like peptide 1 receptor (GLP-1R) [22] and dual-specific tyrosine-phosphorylation regulated kinase 1A (DYRK1A) [23] are emerging as front-runners, with evidence from in vivo...
diabetic mouse models transplanted with human β cells, which have shown that even a 1% to 2% proliferation increase can lead to significant glycemic control improvements, reaching a level of control similar to that in normal mice [24-26]. Nevertheless, the clinical viability of these findings for enhancing adult human β cell proliferation remains uncertain. Alternative β cell replication strategies include the transdifferentiation of liver cells [27,28], stomach and intestinal cells [29], and other pancreatic origin cells such as ductal cells, yet no Food and Drug Administration-approved strategy for this purpose has been applied clinically [30,31]. Despite these advances, current production methods for creating homogeneous, high-quality hPSC-derived insulin-producing β-like cells (hPSC-derived insulin-producing cells hereinafter referred to as “β-like” cells) face challenges. Large-scale clinical application is impeded by complex differentiation processes, batch variability, cost inefficiency, and underdeveloped cryostorage and delivery methods (Fig. 1). This review discusses progressive strategies to surmount hurdles in islet transplantation, including islet availability and the critical aspects of islet preservation.

**Sources of Functional Human β Cells**

While hPSC technology holds promise for addressing various challenges in cell replacement therapies, including the generation of β cells, there are significant scalability issues that must be addressed. The indefinite replication potential of PSCs and their ability to differentiate into any cell type, including β cells, underpins the technology’s promise. hPSCs come from two main sources: embryonic pluripotent stem cells (ESC), and induced pluripotent stem cells (iPSC). The same differentiation process can be applied to both ESCs and iPSCs; however iPSCs offer an ethical advantage over ESCs. The generation of iPSCs involves reprogramming adult cells by targeting transcription factors like octamer-binding transcription factor 4 (OCT4), SRY-box transcription factor 2 (SOX2), Krüppel-like factor 4 (KLF4), and cellular myelocytomatosis oncogene (c-MYC) [32,33], which can lead to inconsistent differentiation efficiencies due to residual epigenetic memory [34]. This variability can severely limit the large-scale production of functionally homogeneous β cells. Additionally, the differentiation stage of the β cells may influence their propensity to form teratomas, a type of tumor. Less differentiated cells carry a higher tumorigenic risk, raising concerns about the safety of cell therapies [35]. Ensuring the success, purity, and homogeneity of differentiated β cells is critical and remains a significant hurdle for scaling up this technology for widespread clinical application. The differentiation of β cells from hPSCs follows a meticulous, stepwise protocol that guides cells through sequential lineage commitments: starting with induction into definitive endoderm, followed by commitment to foregut and pancreatic progenitor stages, further specializing into endocrine progenitors, and culminating towards mono-hormonal endocrine cells, including insulin-secreting β cells, glucagon-secreting α cells and somatostatin-secreting δ cells. This approach to β cell differentiation has been successfully implemented in both monolayer cultures and three-dimensional (3D) cell aggregates [36]. hPSC-derived β cells express the hallmark markers, such as pancreatic and duodenal homeobox1 (PDX1), NK6 homeobox 1 (NKX6-1), urorcortin 3 (UCN3), MAFA bZIP transcription factor A (MAFA), insulin (INS), and neurogen differentiation 1 (NEUROD1), and have the functionality of glucose-sensing and insulin-secretion at varying levels [4,5]. Despite these successes, hPSC-derived β cells still face major issues that hinder their therapeutic application.

**Identity and Purity**

Protocols for *in vitro* differentiation to generate β-like cells from iPSCs commonly emulate the developmental progression of pancreatic islets. Consequently, the resulting cultures typically comprise not only β-like cells, but also α-like cells and δ-like cells. The complex and labor-intensive nature of these protocols, along with the dynamics of morphogen signaling gradients and the susceptibility of different precursor cells to develop into different lineages, occasionally leads to the emergence of cell types atypical to cadaveric islets. Such anomalies include enterochromaffin cells, which are closer to intestinal than pancreatic endocrine lineages, as well as polyhormonal cells that exhibit a less developed state than monohormonal β, α, or δ cells [37-39]. Moreover, the differentiation process is not universally efficient throughout the culture process, with some cells stalling at progenitor stages, others deviating to alternative lineages, and yet others displaying traits of both intestinal and pancreatic types. These undesired cells can be considered contaminants that can potentially impair the functionality of the β-like cells or alter the differentiation trajectory of neighboring cells via direct contact or the secretion of soluble factors. These cells can also persist even after transplantation despite allowing the *in vivo* maturation of derived β-like cells [39,40]. Sorting and reaggregation of β-like cells with the removal of the undesired cell types has been shown to enhance the functional maturation of β-like cells clusters [41]. Variability in the differentiation efficiency of β-like
cells across different protocols and the disparate responsiveness of various iPSC lines further complicate the process. Determining the optimal ratio of β-like cells to other pancreatic endocrine cells for clinical applications remains a topic of debate. Nonetheless, controlling this ratio and reducing contaminant cell populations are critical research objectives. An emerging aspect of β-like cell differentiation that is coming into focus is the importance of epigenetics and chromatin states. Although single cell transcriptomic analyses have provided useful insights into the cellular identities of cells generated by various differentiation protocols, they fall short in explaining the emergence of non-β-like cells and their interrelations. The prevailing theory suggests a divergence from a common progenitor lineage into distinct end-branches, which precludes the possibility of transdifferentiation or a continuum of cellular identities. Thus, in the past few years, strategies to improve β-like cell differentiation efficiency have involved employing cell surface markers such as CD49a [37], CD9 [36,42], glycoprotein 2 [43-45], CD142 [45], CD24 [46], and CD63 [47], followed by purification and/or reaggregation [41]. However, these methods might be challenging to implement on a larger scale for the mass production of β-like cells. In contrast, single cell transcriptomic analysis complemented by single cell transposase-accessible chromatin sequencing on β-like cells, revealed that the presence of enterochromaffin cells may represent an intermediary transitory state of pancreatic endocrine cells and intestinal cells [48]. This finding suggests the possibility of transdifferentiating enterochromaffin cells into a β cell identity by modulating the chromatin states, timing, and expression of key transcription factors and signals, similar to the process of transdifferentiating stomach cells into β-like cells [49,50]. Nonetheless, it is essential to evaluate the purity and identity of the differentiated cells for the presence of β cell markers and the absence of stem cell and other cell type markers. Utilizing single cell multiomic assays can deepen our understanding of β cell differentiation in vitro. This approach can help analyze the different resulting cells and may pave the way to optimize the in vitro differentiation protocols to achieve a desirable proportion of mature and functional β cells, along with other pancreatic endocrine cells [46].

**Transplantation site and immune reaction**

The selection of a transplantation site for PSC-derived β-like cells is critical in mitigating graft rejection and immune responses [51]. To achieve functional efficacy, the loss of transplanted insulin-producing cells should be minimized. For this purpose, a current focus of interest is increasing graft survival by rapid vascularization to deliver nutrition and oxygen, as well as protecting from harsh allogenic and autoimmune responses. Certain transplantation sites may provide an immune-privileged or immune-tolerant environment, potentially reducing graft rejection risk. Sites including the anterior chamber of the eye and the omentum are under preclinical investigation for their efficacy in this regard [52,53]. Immune evasion via genetic engineering of β cells is also being tested. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 deletion of the human leukocyte antigen-A/B/C (HLA-A/B/C) and class II transactivator (CIITA) genes, and the introduction of the programmed death-ligand 1 (PD-L1), HLA-G, and CD47 genes allow cells to be less immunogenic [10,54-58]. The use of encapsulation devices is another innovative transplantation strategy. These devices are designed to protect the cells from immune attacks; closed-type devices prevent immune interactions, while open-type devices facilitate vascularization and nutrient exchange [59]. These approaches are in a developmental stage and have not yet achieved full protection of transplanted cells, presenting a trade-off. Previous reviews discuss these strategies in depth [51,60].

**Maturity**

Maturity in hPSC-derived β-like cells is marked by the expression of key β cell markers, including MAFA, UCN3, islet amyloid polypeptide (IAPP), SIX homeobox 2 (SIX2), and Wnt family member 4 (WNT4), proper glycolysis and mitochondrial metabolic activity, and some functional capacity for glucose sensing and insulin secretion [7,10,18]. Although it is widely acknowledged that these differentiated cells do not yet exhibit definitive β cell metabolic maturity and functionality comparable to human islets, they can attain in vivo maturity and reduce hyperglycemia when transplanted in diabetic mice. Advancement toward defining signals and pathways that can further mature hPSC-derived monohormonal β cells, mimicking in vivo maturity, is a current subject of intense research. Furthermore, it is being increasingly recognized that there is heterogeneity of mature β cells with putatively different functions. Determining whether current protocols are able to replicate these heterogeneous subtypes and how they influence β cell functionality for transplantation and clinical applications remain to be thoroughly investigated. The signal pathways involved in β cell maturation have recently been reviewed in detail [18,51].

**Expansion and scalability**

Typically, around 3,000 islet equivalents (IEQ) per mouse and
approximately 100,000 IEQ per kilogram of body weight in humans are required to observe a beneficial effect on glucose homeostasis in diabetes (Table 1) [4,5,10-12,61-75]. Consequently, a strategic expansion of fully mature and functionally homogeneous β cells is necessary to standardize these therapeutic interventions. The self-renewal capacity of hPSCs is expected to provide an infinite resource for newly synthetic β cells. However, it is known that the passage number of hPSCs influences their differentiation function. In general, hPSCs with a higher passage number show more variability and increased genomic instability, and lose the function of proper differentiation [76]. In addition, different sources of human iPSCs or human embryonic stem cells show distinct patterns of epigenetic inheritance [34], which can result in variability in their efficacy for generating β cells. The cell count tends to decrease during the lengthy differentiation process, highlighting the limitations of scalability with hPSCs. Besides relying on the self-renewal function of hPSCs for expansion, the regulation of proliferation at the advanced stages of differentiation of hPSCs to the pancreatic lineage has been explored. Distinct stages of the β cell development have different proliferative capacities; thus, in vitro culture of these cells is under investigation for intensive proliferation before differentiation to increase the scalability of β cells. We discuss these approaches in the next section.

### Table 1. Summary of Islet Quantities Required for Transplantation of Different Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Donor</th>
<th>Recipient</th>
<th>Types</th>
<th>Sites</th>
<th>Islet quantity, IEQ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Mouse, Kidney</td>
<td>Primary islets</td>
<td>Portal vein</td>
<td>350</td>
<td>Approximately 1,000</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kidney capsule</td>
<td></td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td>Dog</td>
<td>Dog</td>
<td>Primary islets</td>
<td>Kidney</td>
<td>3,000–5,000</td>
<td></td>
<td>[62]</td>
</tr>
<tr>
<td>Porcine</td>
<td>Mouse, NHPs</td>
<td>Primary islets</td>
<td>Portal vein</td>
<td>2,000</td>
<td>Approximately 2,000</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Porta vein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Mouse, NHPs</td>
<td>Primary islets</td>
<td>Portal vein</td>
<td>782,550</td>
<td>Approximately 3,000</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kidney capsule</td>
<td>Approximately 2,000</td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td>Human</td>
<td>Mouse</td>
<td>Primary islets</td>
<td>Kidney</td>
<td>85,000–100,000/kg of BW</td>
<td></td>
<td>[67]</td>
</tr>
<tr>
<td>Human</td>
<td>Mouse, NHPs</td>
<td>hPSC-β</td>
<td>Kidney capsule</td>
<td>3×10^6–7×10^6 cells</td>
<td></td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3×10^6–5×10^6 cells</td>
<td></td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3×10^5 cells</td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Approximately 1.25×10^6 cells</td>
<td></td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Approximately 1.6×10^6 cells</td>
<td></td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250–750 (diameter 100–200 μm)</td>
<td></td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.2×10^6–4.9×10^6 cells</td>
<td></td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3×10^6 cells</td>
<td></td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Approximately 17,000</td>
<td></td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30,000–40,000/kg of BW</td>
<td></td>
<td>[75]</td>
</tr>
</tbody>
</table>

IEQ, islet equivalent; NHP, non-human primate; BW, body weight; hPSC-β, human pluripotent stem cell-derived β cell.
3% of human β cells are observed to divide during the infancy-childhood β cell expansion period, a rate that gradually declines to less than 0.5% in adulthood [77]. While mitogens that induce β cell proliferation in rodents have been identified, the majority of those mitogens have not been as effective in human β cells. This discrepancy may stem from differences in cell cycle regulation mechanisms between species. For example, human β cells have high expression of cyclin-dependent kinase 6 (CDK6), which is important for cell division in these cells, but not in rodent β cells [78-80]. Additionally, rodent β cells express all three D-cyclins, and the genetic deletion of cyclin D2 leads to β cell hypoplasia and diabetes [81]. In contrast, human β cells express very little or no cyclin D2 [80,82].

Inhibition of DYRK1A activity is known as a representative pathway that induces human β cell division; therefore, DYRK1A inhibitors such as harmine, INDY, leuketaine-41, GNF4877, 5-idotubericidin, TG003, AZ191, and CC-401 have been used to promote human β cell proliferation [24,83]. DYRK1A phosphorylates nuclear factor of activated T-cell transcription factors (NFATs) and prevents their translocation to the nucleus, thereby preventing their activation. Activation of NFATs through inhibition of DYRK1A induces cell cycle regulator expression and increases human β cell proliferation and mass [24]. Additionally, DYRK1A inhibition induces human β cell proliferation through reduced expression of the cell cycle inhibitor p27Kip1 and conversion of the repressive DREAM (DP, RB-like, E2F, and MuvB) complex to the pro-proliferative MMB (MYB, MuvB, and FOXM1) conformation [84,85]. Harmine, a prominent DYRK1A inhibitor, can enhance adult human β cell proliferation by up to approximately 3% in vitro and in vivo [24]. In a study, streptozotocin (STZ)-induced diabetic nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice that received transplanted human islets and were treated with harmine exhibited a significant reduction in blood glucose levels—measuring approximately 200 mg/dL within 21 days, compared to around 300 mg/dL in the control group. Interestingly, harmine treatment not only induced human β cell proliferation, but also increased the expression of important β cell transcription factors, such as PDX1, NKX6.1, and MAFA [24]. While transforming growth factor β (TGFβ) signaling has been shown to suppress β cell proliferation by increasing the expression of CDK inhibitors, including P15, P16, P21, and P57 [86,87], its suppression through inhibitors like LY364947, ALK5, and GW788388 did not markedly increase human β cell proliferation [25]. However, it has recently been reported that leukemia inhibitory factor (LIF) signaling stimulated the expression of cyclins and CDKs via the signal transducer and activator of transcription 3 and CCAAT Enhancer Binding Protein Delta (CEBPΔ) pathways, facilitating human β cell cycle progression [26]. Treatment with recombinant LIF (rLIF) led to a modest 1.5% rise in human β cell proliferation in vitro [26]. rLIF treatment for 14 days improved glycemic regulation by up to approximately 200 mg/dL (phosphate-buffered saline treatment group: approximately 350 mg/dL) before a single nephrectomy in STZ-induced diabetic NOD-SCID mice with human islet transplantation. In particular, in an in vivo glucose-stimulated insulin secretion test, the rLIF-treated group showed a twofold increase in insulin secretion compared to controls [26]. Additionally, it has been reported that γ-aminobutyric acid (GABA) signaling, an inhibitory neurotransmitter, induces protein kinase B (PKB or AKT) and cAMP-response element binding protein (CREB) pathway activity to increase β cell proliferation, and glycogen synthase kinase 3β (GSK3β) inhibitors such as 1-azakenpaullone, CHIR99021, or 6-bromoindirubin-30-oxime (BIO) also induces rat β cell survival and proliferation [88,89]. Contrary to previous findings, it was recently reported that GABA did not restore islet capacity and function in diet-induced obese mice, and GSK3β inhibitors alone were unable to increase human β cell proliferation [83,90]. Synergistic effects—where two or more drugs, when used in combination, produce an amplified effect—have been harnessed in recent studies to enhance human β cell proliferation. Research has demonstrated that combined treatment of harmine (DYRK1A inhibitor)+LY364947 (TGFβ inhibitor) or CC-401 (DYRK1A inhibitor)+ALK5 inhibitor II (TGFβ inhibitor) can increase human β cell proliferation by 4% to 8% in vitro [25,91]. Similarly, the combined treatment of harmine and GW788388 (TGFβ inhibitor) showed a synergistic effect, increasing human β cell proliferation by 1.5% (harmine treatment group: approximately 1.2%) in NOD-SCID mice [25]. In addition, harmine also synergized with GLP-1 agonists to induce human β cell proliferation in vitro and in vivo. The combination treatment improved normoglycemic levels in STZ-induced diabetic NOD-SCID gamma (NSG) mice transplanted with 500 IEQ of human islets, and it increased human β cell proliferation approximately two-fold over the harmine treatment group. However, a single treatment with harmine failed to reduce blood glucose levels [92]. Moreover, in hPSC-β cells exhibiting a 1% cell division rate, a triple combination of LIF+harmine+LY364947 was able to boost cell division to approximately 5% in vitro [26]. Moreover, efforts to increase the mass of insulin-producing cells have targeted not only human β cell proliferation, but also the expansion of pancreatic progenitors [93-95]. The recent success in cultivating expandable protein C receptor positive pancreatic progenitors
### Table 2. Human β Cell Proliferation by Mitogenic Factors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Molecules</th>
<th>Experiment types</th>
<th>Cell types</th>
<th>Proliferation index (vs. CON)</th>
<th>Mechanism of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single DYRK1A</td>
<td></td>
<td>Harmine</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>1%–3% –2% –0.3%</td>
<td>NFAT signaling</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>INDY</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>1.5% –2% –0.2%</td>
<td></td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucettine-41</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–2% –4% –2%</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-IT</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–3% –2% –0.4%</td>
<td></td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG003</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–3% –2% –0%</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC-401</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–3% –2% –0%</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GNF7156</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>3%–6%</td>
<td></td>
<td>[97]</td>
</tr>
<tr>
<td></td>
<td>inhibitor+GSK3β</td>
<td>GNF4877</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>3%–6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibitor</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>SB431542</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–2.5% –1%</td>
<td>CDKIs (P15, P16, P21, P57) ↓</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LY364947</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>ALK5</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
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<tr>
<td></td>
<td></td>
<td>GW788388</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>A83-01</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K02288</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>LDN193189</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–1% –2%</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>LIF</td>
<td>Recombinant LIF</td>
<td>Human β cells</td>
<td>–2% –1.5%</td>
<td>STAT3 &amp; CEBPD</td>
<td>[26]</td>
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<td></td>
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<td>singaling</td>
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<td></td>
<td></td>
<td>GABA</td>
<td>Recombinant GABA</td>
<td>Human β cells</td>
<td>–2% –0.3%</td>
<td>PKA-CREB</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>signaling</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GSK3β</td>
<td>inhibitor</td>
<td>Human β cells</td>
<td>NS NS</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Tideglusib</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>NS NS</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CHIR99021</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>NS NS</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GLP-1</td>
<td>recombinant GLP-1</td>
<td>Human β cells</td>
<td>NS NS</td>
<td></td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expедин-4</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>NS NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination</td>
<td>DYRK1A</td>
<td>Harmine+SB431542</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–4%</td>
<td></td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>inhibitor+TGFβ</td>
<td>Harmine+LY364947</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibitor</td>
<td>Harmine+ALK5</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Harmine+GW788388</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–5%</td>
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<tr>
<td></td>
<td></td>
<td>Harmine+A83-01</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–6%</td>
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<tr>
<td></td>
<td></td>
<td>Harmine+K02288</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Harmine+LDN193189</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>–4%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued to the next page)
Fig. 2. Updated methodologies for β-like cell expansion at each step during stem cell-derived β cell differentiation. To date, methodologies have been reported for cell expansion at each stage of human pancreatic stem cell (hPSC)-derived β-like cell differentiation using optimized culture media. These methods report that cells retain their identity and capacity to differentiate through multiple passages. Exceptionally, a methodology to expand endocrine progenitor (EP) cells has not been reported. The Hippo signaling pathway is essential for regulating pancreatic development, as well as β cell proliferation, differentiation and survival. Overexpression of YAP-S6A in pancreatic progenitor cells reduces the differentiation efficiency into β-like cells, but increases the number of proliferating β-like cells [26,93,99-102]. FGF10, fibroblast growth factor 10; EGF, epidermal growth factor; TGFβ, transforming growth factor β; Inh, inhibitor; P, passage; Ha, harmine; LY, LY364947; LIF, leukemia inhibitory factor; P, protein; ALK5, activin like kinase 5; LIF, leukemia inhibitory factor; hPSC-β, human pluripotent stem cell-derived β cell; STAT3, signal transducer and activator of transcription 3; CEBPD, CCAAT/enhancer-binding protein delta; GABA, γ-aminobutyric acid; PKA-CREB, protein kinase A-cAMP response element-binding protein; NS, not significant; GLP-1, glucagon-like peptide-1.

Table 2. Continued

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Molecules</th>
<th>Experiment types</th>
<th>Cell types</th>
<th>Proliferation index (vs. CON)</th>
<th>Mechanism of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYRK1A inhibitor+GSK3β inhibitor</td>
<td>Harmine+Tiglusiub</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>−4%</td>
<td></td>
<td>[83]</td>
<td></td>
</tr>
<tr>
<td>DYRK1A inhibitor+TGFβ inhibitor+LIF</td>
<td>Harmine+LY364947</td>
<td>In vitro</td>
<td>hPSC-β cells</td>
<td>−5%</td>
<td></td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>DYRK1A inhibitor+GLP-1 inhibitor+GLP-1</td>
<td>Harmine+Expandin-4</td>
<td>In vitro</td>
<td>Human β cells</td>
<td>−5%</td>
<td>−3.5%</td>
<td>−1.5%</td>
<td>[92]</td>
</tr>
</tbody>
</table>

CON, control; KI67, antigen Ki6 67; BrdU, 5-bromo-2'-deoxyuridine; EdU, 5-ethynyl-2'-deoxyuridine; P-HH3, phospho-histone H3; DYRK1A, dual-specific tyrosine-phosphorylation regulated kinase 1A; NFAT, nuclear factor of activated T-cell transcription factor; hPSC, human pluripotent stem cell; INDY, 1Z-(3-ethyl-5-hydroxy-2(3H)-benzothiazolylidene)-2-propanone; 5-IT, 5-iodotubericidin; GSK3β, glycogen synthase kinase 3β; GNF, Genomics Institute of the Novartis Research Foundation; TGFβ, transforming growth factor β; CDK1, cyclin-dependent kinase inhibitors; P, protein; ALK5, activin like kinase 5; LIF, leukemia inhibitory factor; hPSC-β, human pluripotent stem cell-derived β cell; STAT3, signal transducer and activator of transcription 3; CEBPD, CCAAT/enhancer-binding protein delta; GABA, γ-aminobutyric acid; PKA-CREB, protein kinase A-cAMP response element-binding protein; NS, not significant; GLP-1, glucagon-like peptide-1.
can generate functional islet organoids in a mouse model has further encouraged these approaches [96]. The synergistic effect of several drugs based on the new understanding of the mechanism of pancreatic β cell replication has significantly increased human β cell proliferation (Table 2, Fig. 2) [24-26,83,89,92,93,97-102]. These findings hold promise for the potential clinical application of these drug combinations in the future.

**ISLET CRYOPRESERVATION**

Cryopreservation of islets is a crucial component in scaling the delivery of insulin-producing cells. This method has been extensively studied, as it offers a solution to challenges in the islet supply chain by enabling high-quality storage and the pooling of islets from multiple donors. However, islets are highly susceptible to cellular stress and damage during the freeze-thaw cycle, which may lead to impaired function or cell death. Over the past decades, various conditions have been studied for islet cryopreservation in order to improve islet survival and functional recovery after thawing. In particular, major factors affecting successful islet cryopreservation include the use of cryoprotective agents (CPAs), the management of cellular stress, and the maintenance of the islets’ 3D structure.

Rapid freezing can cause the formation of intracellular and extracellular ice crystals, which are detrimental to cell viability [103]. In 1949, the discovery of glycerol’s cryoprotective properties paved the way for the use of CPAs such as dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, and propylene, which are introduced prior to freezing to prevent ice crystallization [104,105]. Effective cryopreservation thus requires sufficient time to equilibrate with CPAs within and around the cells. Furthermore, it is important to set the optimal temperature and concentration of CPAs, as different CPAs have varying rates of diffusion and levels of cytotoxicity [106].

In 1977, cryopreserved rat islets were transplanted into the livers of diabetic rats through the portal vein [107]. The diabetic rats transplanted with cryopreserved rat islets showed hyperglycemia for 6 weeks after transplantation but thereafter maintained normal blood glucose levels until 13 weeks. These findings have spurred the development of various islet cryopreservation protocols [108]. Despite success in small rodents, only 20% of pigs with transplanted cryopreserved porcine islets achieved normal glucose regulation, highlighting the need for improved cryopreservation methods for larger mammals, including humans [109]. In 2001, it was shown that islet survival improved with a protocol incorporating slow cooling at a rate of 0.25°C per minute and rapid thawing, facilitated by the addition of 2 moles (M) DMSO to the University of Wisconsin organ preservation solution or to a hypothermosol preservation solution [110].

Although CPAs are effective in preventing ice crystal formation, they do not alleviate the cellular stress associated with freezing and thawing. In particular, the oxidative stress that occurs during this process poses a threat to islet survival due to the inherently low antioxidant defense of islet cells [111]. Over past decades, research has focused on various CPA additives to reduce oxidative stress in islets, including taurine (an antioxidant), metformin (an anti-diabetic drug), GABA, eicosapentaenoic acid (a polyunsaturated fatty acid), or docosahexanoic acid (a polyunsaturated fatty acid). When combined with CPAs, these additives have been shown to significantly lower reactive oxygen species levels in islets, thereby enhancing their function and survival after cryopreservation [112,113].

Human islets are aggregates of approximately 1,500 to 2,000 cells with an average diameter of 100 to 150 μm [114]. The 3D structure of islets prevents CPAs from spreading uniformly as the temperature of each cell within the islets changes [115]. The differential temperature pattern formed in this way can generate intracellular ice crystals and eventually lead to cell death. To overcome this problem, a method was proposed to separate islets into single cells, freeze them, and reassemble them into their original spheroid form after thawing. In fact, islets reconstituted after cryopreservation showed higher cell survival and functional recovery than native islets, both in vitro and in vivo [116].

In addition, improved islet survival rate and functional recovery after cryopreservation have been achieved by reducing the amount of CPAs using hollow fiber vitrification, encapsulating islets with 1.75% alginate, or combining CPAs with ethylene glycol and DMSO [117,118]. In particular, a recent islet cryopreservation protocol using cryomesh has achieved survival rates exceeding 89% and an islet recovery rate of more than 95% in 2,500 islets after thawing. In addition, it was suggested that clinically meaningful throughput could be achieved if a larger-sized cryomesh and cryomesh overlapping method were used [119]. In the near future, high-quality islet banking through the establishment of successful islet cryopreservation methods holds the potential to substantially reduce the geographical and temporal barriers between donors and recipients. This advancement is anticipated to markedly improve the success rates of islet translation by increasing the opportunity for high-dose transplantation and more precise HLA matching through the pooling of islet resources.
CONCLUSIONS

Taken together, this review highlights recent findings on novel methodologies that provide game-changing resources for generating synthetic functional insulin-producing β cells and directly expanding human β cells using small molecules or pooling via islet cryopreservation. Although these developments are promising, significant hurdles remain. For instance, the production of uniform hPSC-derived β-like cells in quantities sufficient for clinical applications is still a challenge. Current differentiation protocols are labor-intensive and struggle with the heterogeneity and nonuniformity of the resulting β cells, which are critical issues to resolve for industrial-scale production. Despite these challenges, the various cutting-edge methods for obtaining human β cells are paving the way toward making islet transplantation a clinically viable and more successful treatment in the foreseeable future.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

This work was supported by grants from Beatson Foundation (2022-006), Tobacco-Related Disease Research Program (TRDRP) research award (T33IR6551) and National Institute of Diabetes And Digestive And Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) under Award Number R01DK136888. Eiji Yoshihara is supported by the Juvenile Diabetes Research Foundation (JDRF) Career Development Award (5-CDA-2022-1178-A-N). Jinhyuk Choi and Harvey Perez are supported by postdoctoral fellowships from the California Institute for Regenerative Medicine (CIRM)-training grant (EDUC4-12837).

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