

Genome editing for engineering stem cell-derived pancreatic β cells: recent trends and future perspectives

Seeun Jang^{1,2,*}, Siyoon Shin^{1,2,*}, Yujin Jeong^{1,2}, Donghyun Lim^{1,2}

¹School of Biopharmaceutical and Medical Sciences, Sungshin Women's University, Seoul, Korea

²Department of Next-Generation Applied Science, Sungshin Women's University, Seoul, Korea

Received: September 10, 2023

Revised: October 15, 2023

Accepted: October 30, 2023

Correspondence to:

Donghyun Lim, PhD

Sungshin Women's University, 55

Dobong-ro 76ga-gil, Gangbuk-gu,

Seoul 01133, Korea

E-mail: dlim@sungshin.ac.kr

*These authors contributed equally to this work.

Rapid advancements in biotechnology have enabled the development of new therapeutic modalities for refractory diseases. Among these, stem cell-derived β (SC- β) cells are emerging as next-generation therapeutics for diabetes. However, several challenges must be overcome for these cell-based therapies to enter widespread use. Current SC- β cells exhibit functional immaturity, and transplanted cells face rejection by the host's immune system. The cells also undergo apoptosis shortly after transplantation due to insufficient oxygen supply. Additionally, the use of stem cell-derived live cells may raise safety concerns, including the risk of teratoma formation. To address these challenges, genome-editing tools, particularly clustered regularly interspaced short palindromic repeats/Cas9 technologies, are being extensively explored owing to their ease of use, modularity, and robustness across diverse cell types. Herein, we provide a comprehensive overview of the various issues in the development of cell-based therapies for diabetes and discuss how genome-editing tools can enhance this emerging therapeutic modality. We also discuss the use of genome editing with SC- β cells for modeling genetic variants in diabetic β cells and correcting pathogenic variants to enable personalized diabetes therapy. Furthermore, we propose future research directions for leveraging genome-editing tools to improve the performance of cell-based therapeutics for diabetes.

Keywords: Diabetes; Cell-based therapy; Stem cell-derived β cells; Genome editing; Clustered regularly interspaced short palindromic repeats/Cas9

Introduction

Type 1 diabetes is characterized by the autoimmune destruction of pancreatic β cells, necessitating lifelong insulin administration for disease management [1,2]. In contrast, type 2 diabetes occurs when β cells fail to meet increased insulin demands, which can lead to insulin administration in certain cases. In the late stages of type 2 diabetes, β -cell failure can also occur, requiring constant insulin administration [1,3]. While direct insulin injections can be life-saving, achieving tight glucose control remains difficult, often resulting in complications such as life-threatening hypogly-

cemia, as well as microvascular and macrovascular complications induced by hyperglycemia [1,4,5].

New therapeutic modalities, such as cell and gene therapies, have emerged as complementary approaches to conventional drugs for treating refractory diseases. Cell therapies, in particular, offer substantial potential for addressing type 1 and late-stage type 2 diabetes, as they allow the replacement of lost β cells through the transplantation of therapeutic cells into patients [6,7]. Given the ongoing increase in the number of diabetes patients worldwide [8], there is an urgent need to develop cell-based therapies to treat and potentially cure this refractory disease.

The original Edmonton protocol developed in the early 2000s and a recently approved cell therapy, Lantidra, both utilize pancreatic islets from organ donors to produce cell-based therapeutics [9,10]. However, the scarcity of organ donors limits the widespread adoption of this treatment approach. To address this challenge, insulin-producing β cells have been generated *in vitro*, primarily from pluripotent stem cells (PSCs) [6,7]. This process involves mimicking *in vivo* developmental pathways in cell cultures to guide PSCs toward the pancreatic lineage, ultimately yielding stem cell-derived β cells (SC- β cells) [6,7]. Currently, SC- β cells are the subject of extensive research as next-generation therapeutics for diabetes [11–13].

Despite the potential of SC- β cells, several critical challenges must be addressed to generate highly functional SC- β cells suitable for therapeutic applications. Notably, current SC- β cells exhibit functional immaturity, characterized by insufficient glucose-stimulated insulin secretion (GSIS) when compared to primary islets, although they can synthesize and store insulin in their secretory granules [14,15]. Furthermore, the transplantation of these cells may trigger immune responses in the recipient, leading to the complete elimination of the cell therapeutics. The risk of immune rejection necessitates the continuous administration of immunosuppressive drugs, which can give rise to various adverse effects [6,16]. In addition, transplanted cells could suffer from hypoxia and subsequent apoptosis due to inadequate vascularization [17]. Research has shown that about half of transplanted β cells may be lost within the first 7 days post-transplantation [18]. Given that β cells are particularly vulnerable to hypoxia due to their exceptionally high oxygen requirements [19], methods for protecting the transplanted β -cell mass from hypoxia are crucial to ensure maximal therapeutic effects. Furthermore, the transplantation of live cells raises safety concerns as they may develop into tumors or trigger severe immune responses [20–22]. To guarantee the safe administration of these novel therapeutic modalities, it is of the utmost importance to develop methods for precise control over the cells.

These challenges can be addressed through a variety of approaches, including the refinement of differentiation protocols, biomaterial engineering, and genome editing. In particular, current genome-editing tools based on the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system hold significant potential because they can permanently alter the genomic sequence and, consequently, the phenotype of cell therapeutics [23].

SC- β cells not only serve as cell therapeutics, but also as a crucial source of human β cells for investigating β -cell biology and unraveling the mechanisms of diabetes. Currently, functionally

mature human β -cell lines are difficult to obtain [24], and researchers often resort to using rodent β -cell lines as substitutes, despite the evident differences between human and rodent islets [25]. Moreover, the availability of primary human islets for basic research is severely limited. Consequently, SC- β cells are emerging as indispensable tools for exploring human β cells and advancing diabetes research. CRISPR/Cas9-based genome editing is employed as a key technology to introduce genetic variants present in diabetes into SC- β cells, enabling precise investigations into the genetic aspects of the disease.

Here, we provide an overview of recent progress in engineering SC- β cells through genome editing. First, we briefly discuss methods for generating SC- β cells and state-of-the-art genome editing tools. We then delve into how these genome editing tools are being employed to enhance the function of SC- β cells for the development of cell-based therapeutics for diabetes. Next, we explore genome editing-based modeling of diabetes-related genetic variations in SC- β cells. Furthermore, we discuss recent efforts to engineer primary islets through genome editing (Table 1). Since the application of genome editing in β -cell therapeutics is still in its infancy, we present remaining tasks and outline future directions for the application of this approach in implementing cell-based therapeutics for diabetes.

Generation of stem cell-derived β cells

Ethics statement: This study was a literature review of previously published studies and was therefore exempt from institutional review board approval.

Two seminal protocols reported in 2014 outlined the generation of insulin-producing β -like cells from embryonic stem cells (ESCs) and induced PSCs (iPSCs), collectively referred to as PSCs in this review [26,27]. Since then, many researchers have relied on these protocols to generate SC- β cells. These protocols induce the differentiation of PSCs into stem cell-derived tissues using various molecules that modulate intracellular signaling pathways, directing the PSCs through specific developmental stages (Fig. 1).

Some of these cell-directing molecules are proteins, such as activin A, keratinocyte growth factor, and betacellulin, which act on membrane receptors [26,27]. The majority of cell-directing components consist of small molecules that act on diverse intracellular targets and modulate corresponding signaling pathways. For instance, in the first stage of the differentiation, CHIR99021 inhibits glycogen synthase kinase 3, activating the Wnt pathway

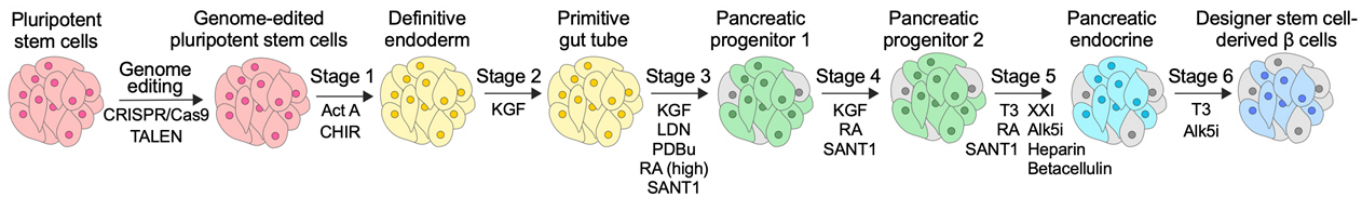


Fig. 1. A protocol for generating stem cell-derived β cells (SC- β cells) combined with genome editing. TALEN, transcription activator-like effector nuclease; Act A, activin A; KGF, keratinocyte growth factor; CHIR, CHIR99021, a glycogen synthase kinase 3 inhibitor; LDN, LDN193189, a bone morphogenetic pathway inhibitor; PDBu, phorbol 12,13-dibutyrate, a protein kinase C activator; RA, retinoic acid; SANT1, an inhibitor of the Sonic hedgehog pathway; XXI, a γ -secretase inhibitor; Alk5i, a TGF- β type I receptor kinase inhibitor; T3, L-3,3',5-triiodothyronine.

and promoting stem cell differentiation into the endoderm [26–28]. Retinoic acid, a fundamental signaling molecule in vertebrate endoderm development, is employed to direct differentiation into pancreatic progenitors [26,27,29]. SANT1, an inhibitor of the Sonic hedgehog pathway, enhances pancreatic specification [26,27,30], while LDN193189, an inhibitor of the bone morphogenetic pathway, facilitates conversion into pancreatic endocrine precursors [26,27,31]. Alk5i, an inhibitor of the transforming growth factor- β type I receptor kinase (ALK5), and the small thyroid hormone T3 (L-3,3',5-triiodothyronine) are employed in the late stage of differentiation to generate insulin-positive β -like cells (Fig. 1) [26,27].

These universal protocols demonstrated the feasibility of generating SC- β cells for future therapeutic applications. However, these protocols have limitations, such as low yields of insulin-producing cells and insufficient GSIS by the resulting SC- β cells. To address these challenges, researchers have developed numerous improved protocols based on the universal protocols. For example, new small molecules have been employed to generate more mature SC- β cells by modulating alternative intracellular signaling pathways [32,33].

Hogrebe et al. [32] conducted RNA sequencing analysis to identify factors affecting the differentiation of PSCs into endodermal lineage. They discovered that polymerized cytoskeleton inhibited neurogenin 3 (NEUROG3)-induced endocrine differentiation. NEUROG3 expression should be initiated at a specific differentiation step to guide the cells into becoming SC- β cells, while its early expression results in the production of polyhormonal cells. Building on this insight, they investigated small-molecule modulators of actin polymerization and found that treating cells with latrunculin A, an actin filament destabilizer, at stage 5 resulted in increased NEUROG3 expression and the subsequent generation of SC- β cells with enhanced GSIS. Notably, the improved protocol allowed β -cell production using

a 2-dimensional culture platform, simplifying production methods and reducing costs for lab-scale processes, thereby enabling the widespread use of SC- β cells [32,34].

β -cell maturation is linked to reduced proliferation, and aurora kinase is known to induce the proliferation of primary β cells [35]. Therefore, Balboa et al. [33] treated cells at the final stage of differentiation with ZM447439, an aurora kinase inhibitor, to promote the maturation of SC- β cells. This treatment reduced cell proliferation without affecting the fraction of insulin-expressing cells. Importantly, the compound-treated cells displayed substantially improved GSIS, underscoring the importance of suppressing cell proliferation at the final stage [33].

In another approach, the differentiation process was modified to recapitulate endocrine cell clustering, resulting in islet-like clusters that exhibited physiological properties analogous to those of primary human β cells [15].

Another promising approach for improving the quality of SC- β cells is genome editing. Recent advances in genome-editing technologies have enabled the introduction of any genetic material into the genome. This capability allows cells to be readily modified to harbor functional genes or correct pathogenic variants for use in therapeutics. In the next section, we briefly discuss recent genome-editing technologies widely used for basic research and therapeutics development.

Genome-editing technologies

Rapid advancements in genome-editing technologies, particularly the CRISPR/Cas9 system, have enabled the facile modification of specific genes for elucidating biological phenomena and developing new therapeutic modalities. Cas9 generates a double-strand break (DSB) at the desired genomic locus, directed by specific guide RNAs (gRNAs). The resulting DSB is then repaired by cell's own DNA repair pathways to join the broken

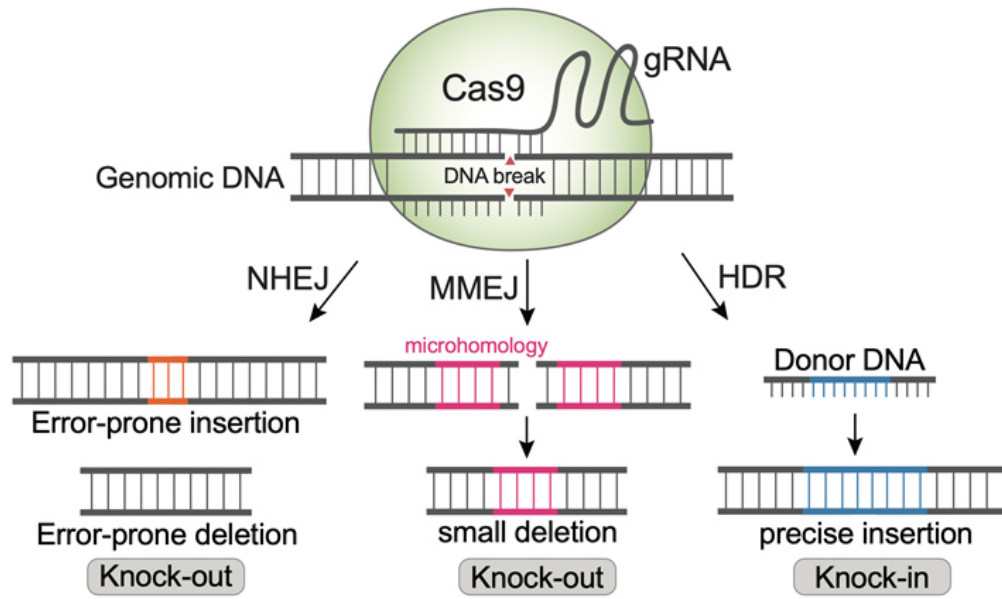


Fig. 2. Endogenous DNA repair pathways following Cas9-induced DNA breaking. The non-homologous end-joining (NHEJ) pathway and the microhomology-mediated end-joining (MMEJ) pathway result in gene knock-out, while the homology-directed repair (HDR) pathway allows precise knock-in when donor DNA is present. gRNA, guide RNA.

ends (Fig. 2).

In the error-prone non-homologous end-joining (NHEJ) pathway, a small insertion or deletion is introduced into the DNA, ultimately leading to the knock-out of the target genes. When short homologous sequences are present on both sides of the DSB, the microhomology-mediated end-joining (MMEJ) pathway can lead to small deletions and consequent gene knock-out. For knock-in of specific sequences at the DSB, a donor DNA that contains homology arms and the desired sequence is delivered along with Cas9 and gRNA, which leads to homology-directed repair (HDR), facilitating the insertion of DNA sequences into the genome (Fig. 2). Typically, the knock-out efficiency by NHEJ and MMEJ is high, but the HDR efficiency is low. Consequently, a significant amount of research has been focused on enhancing HDR efficiency to enable precise editing in various cell types, including stem cells [36–38].

Different genomic loci can be targeted simply by changing the 20-nt gRNA sequence; therefore, CRISPR/Cas9 has been extensively applied for generating genetic modifications in various cells. This modular feature has allowed many researchers, including non-specialists, to use the CRISPR/Cas9 system effectively. Furthermore, CRISPR/Cas9 exhibits robustness across diverse biological systems. For instance, it can be utilized with various cell types, including cell lines and primary cells, and even across different organisms *in vivo* [23,39]. It can be delivered into cells through various forms, such as plasmids,

mRNA, ribonucleoproteins, and viral vectors. This versatility allows researchers to choose the most suitable tools for their specific genome-editing scenarios.

The extensive application of CRISPR/Cas9 to genome editing in PSCs holds the promise of dramatic improvements in the performance of stem cell-derived therapies. Notably, the HDR pathway, which is essential for gene knock-in, is active only in dividing cells. This enables gene knock-in to be performed in PSCs, which can proliferate through cell division. However, SC- β cells, which rarely proliferate, do not allow gene knock-in via HDR [40,41]. As a result, a common approach involves initially editing PSCs to introduce genetic materials through the HDR pathway. The edited PSCs are then converted into SC- β cells (Fig. 1). In contrast, knock-out using the NHEJ pathway can be performed in both dividing and non-dividing cells. Therefore, target genes can be efficiently knocked out in PSCs, and the resulting cells can be converted into knock-out SC- β cells. In some cases, mature SC- β cells or even primary pancreatic islets can be directly edited for knock-out.

Genome editing to overcome challenges of current SC- β cells

In this section, we briefly discuss the major challenges encountered in the production of SC- β cells and introduce how genome-editing tools are being employed to overcome the issues.

A significant challenge with current protocols for generating insulin-producing β cells is that they often yield a mixture of diverse cell types, resulting in a low yield of pure insulin-producing β cells [42]. Therefore, it is crucial to purify the β cells from this heterogeneous cell mixture for therapeutic applications.

Lee et al. [43] employed genome editing to address this challenge by generating an iPSC line expressing the pancreas/duodenum homeobox protein 1 (PDX1)-enhanced green fluorescent protein (EGFP) fusion protein. PDX1 is a critical pancreatic β -cell marker essential for β -cell development and maturation [43]. Thus, functionally mature SC- β cells would express the PDX1-EGFP fusion protein. They used CRISPR/Cas9-initiated HDR to knock in the *EGFP* sequence at the C-terminus of *PDX1* in an iPSC line. When these engineered iPSCs were differentiated into SC- β cells, EGFP expression was specifically detected in insulin-producing cells, but not in the definitive endoderm stage. This strategy holds promise for purifying mature β -cell therapeutics [43]. Yoshihara et al. [44] employed CRISPR/Cas9 to knock in the *GFP* sequence downstream of the *INS* promoter, enabling the facile observation of insulin-producing cells in iPSC-derived islet-like organoids.

Many recent reports on generating SC- β cells rely on engineered ESC lines where the *INS* locus was edited through homologous recombination without the aid of Cas9 [15,45]. In this case, the ESCs were engineered only in one allele of the *INS* locus by inserting the *GFP* sequence. This approach allows easy identification, purification, and subsequent conversion of insulin-producing cells into highly functional 3-dimensional β -cell clusters [15,45]. While the absolute level of insulin production may decrease because only one allele produces insulin while the other produces GFP, these edited ESCs have been used in a range of research endeavors for generating functional SC- β cells due to their ease of use [46–50].

Genome editing has also been used to address the issues of immaturity in current SC- β cells. For example, Ma et al. [49] identified ZnT8, a zinc transporter predominantly expressed in β cells, as a potential target for enhancing the functional maturity of SC- β cells, on the basis of previous studies conducted in rodent and human cells. To investigate the role of ZnT8 in SC- β cells, CRISPR/Cas9-based genome editing was performed in ESCs to knock out the *SLC30A8* gene, which encodes ZnT8. Subsequently, these modified ESCs were differentiated into SC- β cells. This knock-out yielded promising results, as GSIS significantly improved. The enhancement is attributed to the alleviation of Zn-mediated inhibition of insulin secretion upon stimulation by glucose. Furthermore, the knock-out SC- β cells exhibited increased resistance to cell death triggered by gluco-

toxicity and lipotoxicity, achieved by a reduction in endoplasmic reticulum (ER) stress through zinc level modulation. Single-cell RNA-seq revealed that ZnT8 loss-of-function altered the gene expression profile toward a functionally mature state in SC- β cells. Moreover, the edited SC- β cells demonstrated their therapeutic potential by successfully restoring normoglycemia in streptozotocin-induced diabetic mice.

Building upon the observation that ZNF148, a zinc finger transcription factor, acts as a negative regulator of insulin secretion in rodent cells [51], de Klerk et al. [48] employed a knock-out strategy to target the *ZNF148* gene in ESCs. Subsequently, these knock-out cells were differentiated into SC- β cells. Remarkably, the loss of ZNF148 function did not impair the process of β -cell specification during differentiation, as evidenced by the similar fraction of C-PEP⁺, NKX6.1⁺, PDX1⁺ cells in the knock-out cells and their wild-type counterparts. However, when these knock-out cells were subjected to high glucose challenges, they exhibited notably higher insulin secretion than the wild-type cells. This enhancement in insulin secretion was attributed to upregulation of annexin and S100 proteins, which are involved in the regulation of insulin trafficking and exocytosis.

In summary, these studies underscore the potential of simple knock-out strategies to functionally enhance β -cell therapeutics.

Another significant challenge in current cell-based therapeutics arises from graft rejection triggered by immune responses. To circumvent this issue, cells can be delivered to patients using encapsulation devices. Alginate is a commonly used material for microencapsulation devices, while artificial polymeric materials find application in macroencapsulation devices [52]. Although these encapsulation devices effectively shield the transplanted cells from host's immune system, they can introduce a new challenge—namely, a foreign body reaction to the implanted materials, which hampers the practical application of encapsulation strategies. For example, recent clinical trials involving macroencapsulated SC- β cells have observed foreign body responses and subsequent fibrosis around the devices in most cases [11,13]. To solve these problems, researchers are turning to genome editing of PSCs to create hypoimmunogenic cells. This approach aims to minimize allograft rejection by the recipient's immune system without the need for cell encapsulation or the administration of immunosuppressive drugs (Fig. 3A) [53].

As allograft rejection is primarily mediated by the recognition of human leukocyte antigens (HLAs) by T cells, one of the key strategies in cell engineering focuses on the ablation of these molecules. In this context, Hu et al. [54] conducted CRISPR/Cas9-mediated genome editing in iPSCs to knock out the *B2M*

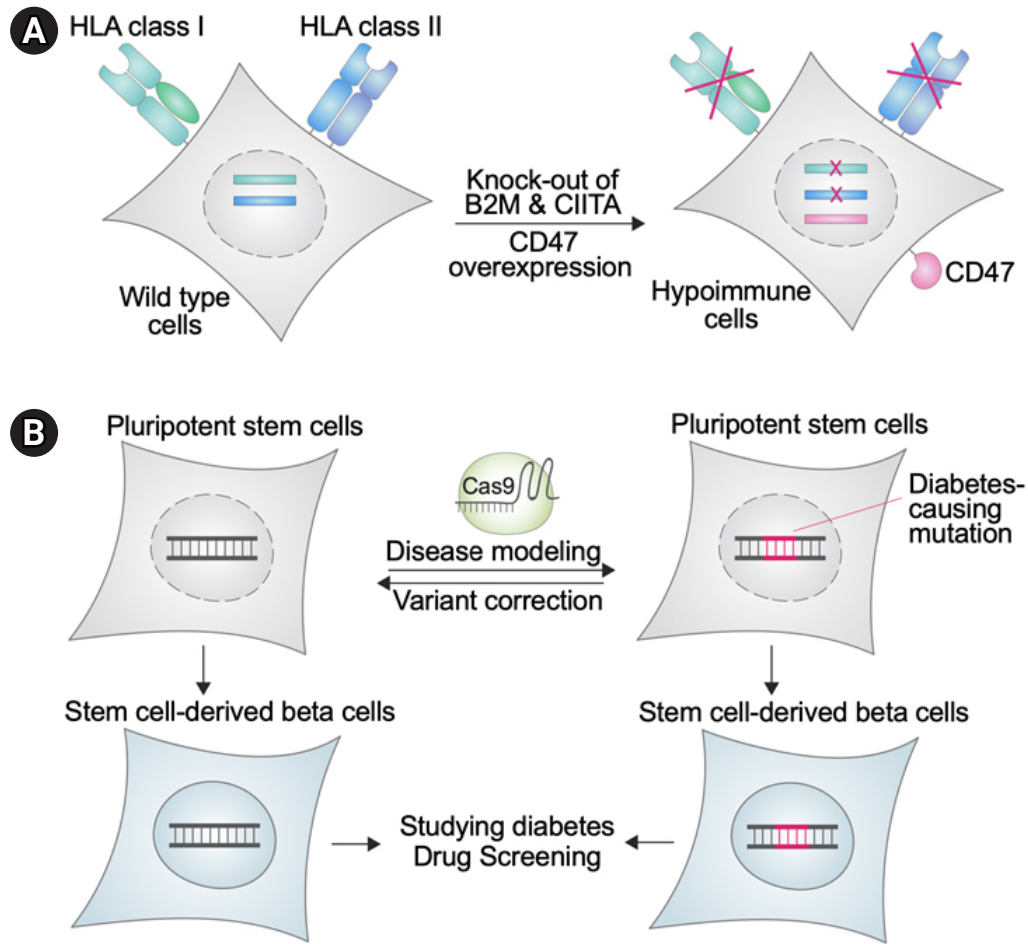


Fig. 3. Representative applications of genome editing in SC- β cells. (A) Cells are engineered to be hypoimmunogenic by knocking out *B2M* and *CIITA*, and overexpressing CD47. (B) Diabetes-causing genetic variants can be introduced or corrected by genome editing for studying the genetic basis of diabetes and identifying variant-specific therapies.

and *CIITA* genes for ablating HLA class I molecules and HLA class II molecules, respectively. As the depletion of *B2M* can lead to the “missing self” killing response by natural killer (NK) cells [54,55], they conducted screening to identify additional factors capable of inhibiting the NK-cell response. Among several transgenes tested, virally expressed CD47 was found to be highly effective as an immune checkpoint inhibitor for NK cells. The resulting hypoimmune iPSCs, characterized by *B2M* and *CIITA* knock-out, along with CD47 overexpression, were then differentiated into SC- β cells. Importantly, the hypoimmune editing did not compromise the differentiation capacity of the iPSCs. These hypoimmune SC- β cells exhibited remarkable survival and successfully restored normal glucose levels in immunocompetent allogeneic humanized mice. Notably, the team applied the same hypoimmune engineering to primary macaque islets and transplanted them into an allogeneic rhesus macaque

model. In this context, the engineered islets demonstrated long-term survival, while the wild-type islets faced rejection within a week. This groundbreaking study highlights the potential of hypoimmune engineering as a viable approach for developing therapeutic SC- β cells [54].

Similarly, Gerace et al. [56] employed the *B2M* knock-out strategy in human ESCs (hESCs) to generate HLA-deficient SC- β cells. These engineered cells demonstrated resistance to allogeneic immune destruction by peripheral blood mononuclear cells and NK cells *in vitro*. Although these cells were able to restore normoglycemia in a diabetic mouse model, they were eventually destroyed, albeit with a reduced rejection rate. As a complementary strategy, the researchers developed another genome editing approach to engineer cells to secrete 3 cytokines (interleukin [IL]-2 N88D mutein, transforming growth factor [TGF]- β , and IL-10) that promote localized immune toler-

ance by recruiting regulatory T cells (T_{reg} s) to the grafts. The CRISPR/Cas9-based HDR pathway was employed in hESCs to knock in the genes for these cytokines at the C-terminal locus of *GAPDH*. Since *GAPDH* is a constitutively expressed gene, the knock-in at the *GAPDH* locus allowed persistent transgene expression. Indeed, the resulting engineered hESCs were differentiated into SC- β cells that constitutively secreted these cytokines. This engineering enabled the survival of the transplanted cells and the reversal of diabetes *in vivo*, with T_{reg} s localized within the graft [56]. This study demonstrates the feasibility of local immune modulation for the long-term survival of allografts.

Another hypoimmune strategy was developed by Gravina et al. [57] to protect cell therapeutics from antibody-mediated rejection. Their approach involved the overexpression of CD64, a high-affinity receptor for IgG F_c, on cell membranes. This overexpression led to the capture of monomeric IgG F_c on CD64, rendering it inaccessible to effector cells or complement systems. When this CD64 overexpression strategy is combined with the previously mentioned hypoimmune engineering (*B2M* and *CIITA* knock-out, CD47 overexpression), the iPSC-derived endothelial cells became entirely immune-evasive to prevent anti-Rh(D)-mediated cytotoxicity. In contrast, cells without CD64 overexpression were susceptible to cell death. This CD64 overexpression has the potential to extend to SC- β cells that express HLA-A2 and have not undergone prior genome editing. Upon transduction with viral vectors to overexpress an intracellularly truncated analog of CD64 (CD64t), these cells demonstrated protection against killing by anti-HLA-A2 IgG1, while the unmodified SC- β cells faced rapid loss both *in vitro* and *in vivo* [57]. Looking ahead, we envision the possibility of obtaining completely immune-evasive SC- β cells through multiplex editing (*B2M* and *CIITA* knock-out, CD47 and CD64 overexpression) in the near future.

Hypoimmune engineering has found application in several other studies, each with slightly different strategies. For instance, Parent et al. [50] conducted multi-step genome editing in ESCs to knock-out all classical HLAs except HLA-A2, because HLA-A2 enables the retention of HLA-E, which is capable of inhibiting NK cell-mediated killing. Importantly, this hypoimmune engineering did not disrupt the differentiation potential of ESCs. The resulting SC- β cells were effectively protected from rejection mediated by both T cells and NK cells, as demonstrated in a humanized mouse model. In addition to hypoimmune editing, another genome editing technology was employed to introduce a gene of interest at the *AAVS1* genomic safe harbor locus. The researchers utilized transcription activator-like effector nucleases (TALENs) as a genome editor to insert a lucifer-

ase gene at *AAVS1*, facilitating noninvasive monitoring of cell survival *in vivo* through bioluminescence imaging [50]. Since the *AAVS1* locus is transcriptionally active and amenable to the expression of transgenes without compromising cellular fitness [58], the *AAVS1* editing strategy offers a versatile means of introducing diverse functional genes into cell therapeutics. For example, Castro-Gutierrez et al. [47] generated immune-evasive ESCs by knocking out *B2M* with CRISPR/Cas9 and knocking in a doxycycline-inducible overexpression cassette (Tet-On system) for programmed death ligand 1 (PD-L1) at the *AAVS1* locus using TALENs. Their engineered ESCs were efficiently differentiated into SC- β cells with a similar efficiency as unedited cells. Notably, the engineered SC- β cells displayed reduced stimulation of CD8 T cells *in vitro* upon doxycycline-induced PD-L1 expression [47]. Similarly, Leite et al. [59] employed *B2M* knock-out in iPSCs to decrease the expression of HLA class I molecules in differentiated SC- β cells. The resulting cells displayed reduced T-cell activation, confirming that T-cell activation is mediated by direct engagement of T-cell receptors with HLA-peptide complexes in SC- β cells [59].

These HLA knock-out strategies have the potential for widespread use in the development of cell-based therapeutics due to their effectiveness in reducing immune responses post-transplantation. Technically, knock-out cells can be readily isolated by flow cytometry, since HLA molecules are located on the plasma membrane. This feature allows many researchers to employ this hypoimmune engineering technique to create a wide range of cell-based therapeutics.

To identify mediators of immunogenicity against transplanted SC- β cells, Sintov et al. [60] conducted single-cell RNA sequencing and genome-wide CRISPR screening under inflammatory environments, revealing that genes in the interferon pathways were upregulated under inflammatory conditions. A detailed examination of the screen's findings highlighted that chemokine ligand 10 (*CXCL10*) was particularly implicated in immune-graft interactions. Therefore, *CXCL10* was ablated by knocking in an *EGFP* gene into the middle of the *CXCL10* locus in ESCs, and the edited ESCs were differentiated into SC- β cells. As anticipated, the *CXCL10* knock-out improved the survival of SC- β cells in a humanized mouse model [60].

Cai et al. [61] also conducted genome-scale CRISPR screening in a mouse β -cell line (NIT-1) and found that the knock-out of *Rnls*, a candidate gene for type 1 diabetes identified through genome-wide association studies (GWASs), rendered the β cells resistant to autoimmune destruction. Further mechanistic investigation revealed that *Rnls* knock-out conferred cell resistance to ER stress and apoptosis induced by inflammatory

cytokines such as IL-1 β and IFN- γ . In line with these observations, *Rnls* overexpression had the opposite effect, promoting the autoimmune destruction of NIT-1 cells. Inspired by these results, *RNLS* was knocked out in human iPSCs and the edited cells were differentiated into SC- β cells. While this genetic modification did not affect the differentiation efficiency, it conferred resistance to thapsigargin-induced ER stress [61].

Several studies employed viral transduction to introduce additional genetic materials into SC- β cells due to its high efficiency in gene delivery. For instance, lentiviral delivery was employed to induce the overexpression of the immune checkpoint protein PD-L1, a strategy aimed at protecting stem cell-derived islet-like organoids from immune-mediated destruction [44]. The examples previously discussed also utilized viral vectors for the overexpression of CD47 and CD64 [54,57]. While viral transgene expression is highly efficient, it comes with potential safety concerns. These include the possibility of an immune response to viral factors and the unpredictable random insertion of DNA fragments in the cell's genome, which could lead to unforeseen adverse effects such as oncogenicity [62–64]. Alternatively, a more precise approach involves knocking in transgenes at specific genomic safe harbor loci, such as *AAVS1* or *CLYBL*, through genome editing. This strategy offers several advantages over viral vector-mediated gene integration. It ensures that resulting cells undergo predictable and precise genomic changes [58], which can significantly facilitate the clinical translation of the engineered cell therapeutics.

Genome editing for diabetes modeling and patient-specific therapies

Basic investigations into β -cell biology have traditionally relied on the use of rodent β -cell lines due to the lack of suitable human β -cell models that accurately display mature β -cell characteristics. However, it is crucial to note that human and rodent β cells exhibit significant differences [25]. For example, human β cells possess a single insulin gene (*INS*), while rodent β cells have dual insulin genes (*Ins1* and *Ins2*). Indeed, many findings derived from rodent β cells do not necessarily translate to human β cells. The rapid development in technologies for generating SC- β cells has opened the door to using them as reliable sources of human β -cell models. When combined with genome editing that can introduce any genetic variant into the genome, SC- β cells could be a superior choice for studying genetic aspects of diabetes (Fig. 3B).

For example, neonatal diabetes arising from the mutations in the *INS* gene can be modeled using SC- β cells edited through

CRISPR/Cas9. In a study by Balboa et al. [65], patient-derived iPSCs were initially generated to study the effect of *INS* mutations that led to proinsulin misfolding, specifically mutations at cysteine residues critical for disulfide bond formation (C96R or C109Y). As precise investigations into the role of mutations can be conducted only in isogenic cell lines, they employed CRISPR/Cas9-based genome editing on the patient-derived iPSCs to correct the mutations via the HDR pathway. The resulting mutants and corrected iPSCs were then differentiated into SC- β cells. In-depth investigations revealed that these *INS* mutations induced ER stress in β cells, leading to impaired proliferation. When transplanted *in vivo*, the mutant cells exhibited reduced insulin secretion, increased ER stress, decreased cell size, and diminished mTOR signaling. Interestingly, however, β -cell apoptosis was not increased by these mutations [65]. This study exemplifies how genome editing technologies can be harnessed to precisely study the functional consequences of diabetes-causing mutations within an isogenic background.

Similarly, in a study by Panova et al. [66], genome editing was employed to investigate the role of a mutation located in the second intron of *INS* (c.188-31G>A). Patient-derived iPSCs were corrected using CRISPR/Cas9 and subsequently differentiated into SC- β cells with an isogenic background. This investigation revealed that the intronic mutation created a new splicing site, resulting in the generation of a novel splicing variant (with a 29-nt insertion compared to the wild type). Consequently, a reduction in insulin production was observed in the mutant cells [66].

Mutations in other genes can also be studied using a similar approach. For example, an iPSC line was derived from a congenital hyperinsulinism patient who carried a mutation (V187D) in the *ABCC8* gene, which encodes the sulfonylurea receptor 1 (SUR1) subunit of the K_{ATP} -channel [67]. An isogenic wild-type iPSC line was then generated via CRISPR/Cas9. When these iPSCs were differentiated into SC- β cells, the SUR1-mutant cells displayed a significantly higher rate of proliferation than their wild-type counterparts. In addition, the mutant cells secreted more than threefold higher levels of insulin even under low-glucose conditions. When transplanted *in vivo*, these cells continued to secrete insulin even under hypoglycemic conditions, consistent with the *in vitro* findings. This study exemplifies how genome editing techniques facilitate the exploration of a previously unexplored role for the K_{ATP} -channel during human islet development [67].

PDX1 is a crucial transcription factor that serves as a master regulator of pancreas development and β -cell function [68]. In a study by Wang et al. [68], iPSC lines carrying known *PDX1* mutations were generated using CRISPR/Cas9 technology.

These mutations included homozygous P33T or C18R variants, as well as a *PDX1*-haploinsufficient model. Subsequently, the mutant iPSCs were then differentiated into SC- β cells. All the mutant cells displayed reduced GSIS in comparison to their wild-type counterparts. Notably, the P33T mutation had more pronounced detrimental effects on the expression of β -cell markers. Specifically, the P33T mutation downregulated genes bound by PDX1, revealing the mechanism underlying its adverse impact on β cells.

Genome editing was also employed to investigate variants of *GATA6*, a critical regulator during pancreas development, in patients with pancreatic agenesis (PA). To study the effects of a heterozygous 4-bp duplication in *GATA6* under an isogenic background, a patient-derived iPSC line was corrected to restore wild-type sequence. Conversely, a wild-type ESC line was edited to introduce the heterozygous *GATA6* mutations. These 2 systems were used to explore the role of the *GATA6* variants during the pancreatic differentiation toward SC- β cells. The findings revealed that *GATA6* haploinsufficiency led to defects in pancreas progenitors and a shift in cell fate, as evidenced by a decrease in pancreas-specific gene expression and an upregulation of genes associated with stomach development [69]. Additionally, the study identified a single-nucleotide polymorphism (SNP) located downstream of the *GATA6* coding sequence in PA patients. To model this variant, CRISPR/Cas9-based knock-in was performed in ESCs. The differentiation of the resulting cells indicated that the SNP decreased *GATA6* expression, negatively affecting the pancreas development and resulting in a significantly reduced fraction of SC- β cells [69].

While precise HDR-based knock-in technologies were utilized in the studies mentioned earlier, NHEJ or MMEJ-based knock-out is a simpler approach and can be employed to model gene disruptions in diabetes (Fig. 1). Shi et al. [70] generated *GATA6* knock-out cells using CRISPR/Cas9. This genome editing allowed the creation of both heterozygous and homozygous knock-out ESC lines. Upon differentiation of these edited ESCs, it was observed that the loss of one *GATA6* allele led to impaired generation of β -like cells. Notably, this *GATA6* haploinsufficiency-related phenotype is not evident in mouse β cells, highlighting the significance of SC- β cells in modeling diabetes when combined with genome editing.

In an early study aimed to model type 2 diabetes-associated genetic variants identified through GWASs, Zeng et al. [71] used CRISPR/Cas9 to knock out the *CDKAL1*, *KCNQ1*, or *KCNJ11* genes in ESCs. These specific genes were chosen since they are known to be associated with β -cell function rather than insulin resistance in peripheral tissues, making them suitable

candidates for investigation using SC- β cells. The ablation of *CDKAL1*, *KCNQ1*, or *KCNJ11* did not impair the differentiation process toward β -like cells, as evidenced by the expression of mature β -cell markers and the fraction of insulin-positive cells. However, all mutant cells exhibited an inability to increase insulin secretion in response to high glucose concentrations *in vitro*, and they failed to maintain glucose homeostasis when transplanted into streptozotocin-induced diabetic mice. Notably, *CDKAL1* knock-out SC- β cells displayed hypersensitivity to glucolipotoxicity, while the knock-out of *KCNQ1* or *KCNJ11* did not impact cell death induced by glucolipotoxicity. A remarkable feature of genome-edited SC- β cells is their potential for developing personalized medicines targeting specific gene variants. To demonstrate this potential, the authors conducted phenotype-based high-throughput chemical screening and identified a small molecule (T5224) that selectively rescued *CDKAL1*-deficient SC- β cells from glucolipotoxicity. Furthermore, this compound specifically improved GSIS in these cells. As T5224 is an inhibitor of the FOS/JUN activator protein-1, they further investigated the role of the FOS/JUN pathway in β -cell dysfunction. Interestingly, the CRISPR-based knock-out of FOS was found to enhance GSIS *in vivo* [71].

Cardenas-Diaz also harnessed CRISPR/Cas9-based knock-out to study the most common monogenic form of diabetes arising from mutations in *HNF1A* [72]. In this study, heterozygous or homozygous *HNF1A* knock-out ESCs were differentiated into SC- β cells. The analysis of endocrine hormone expression patterns revealed a decrease in insulin-positive cells and an increase in glucagon-positive cells among the stem cell-derived cells. In-depth investigation revealed that *HNF1A* deficiency drove endocrine differentiation toward a more alpha cell-like gene expression signature. Furthermore, the study demonstrated that *HNF1A* is necessary for optimal insulin secretion in SC- β cells. These findings offer a perspective distinct from observations in rodent models, emphasizing the significance of genome editing and SC- β cells in advancing diabetes research [72].

GWASs have been extensively conducted to identify risk factors associated with type 1 diabetes and type 2 diabetes. Since type 1 diabetes results from β -cell failure, modeling this condition using SC- β cells has emerged as an essential tool for in-depth research. Intriguingly, many genetic variants associated with type 2 diabetes are linked to β -cell development or function [73], highlighting the need for investigations utilizing SC- β cells in conjunction with genome editing.

Patient-derived iPSCs represent invaluable research assets for studying the mechanisms of genetic diabetes as discussed

earlier. Beyond mechanistic investigations, these cells hold great promise for advancing the development of patient-specific autologous cell-based therapeutics for diabetes. Particularly, single pathogenic variants can be corrected by one-step genome editing, making them an ideal starting point for the creation of patient-specific cell therapies.

To demonstrate the feasibility of autologous cell therapies for neonatal diabetes caused by *INS* mutations, Ma et al. [74] obtained iPSCs from a neonatal diabetes patient who had an *INS* mutation at the translation start site (ATG to ATA). Using a CRISPR/Cas9-based knock-in approach, they successfully restored the start site to produce corrected iPSCs. Subsequently, these corrected iPSCs were differentiated into SC- β cells. While the uncorrected iPSCs could be converted into endocrine cells, as evidenced by the expression of several endocrine markers, they failed to produce insulin. In contrast, the corrected iPSCs were capable of differentiating into insulin-producing β -like cells. Remarkably, those cells exhibited the ability to restore normal glucose homeostasis *in vivo*, including the precise regulation of insulin secretion in response to metabolic changes [74]. Even though there were various issues encountered in this study, such as teratoma formation from transplants and off-target effects of genome editing, the value of the gene-correction strategy was clearly demonstrated, highlighting its potential for use in autologous cell therapies.

Maxwell et al. [75] derived iPSC lines from fibroblasts of individuals with Wolfram syndrome (WS). WS is an autosomal recessive disorder caused by variants in the *WFS1* gene, resulting in ER stress, unfolded protein response, and subsequent β -cell death. Since there is currently no standard therapy for WS or ER stress-related disease, the authors aimed to generate gene-corrected, patient-derived SC- β cells. First, a CRISPR/Cas9-based knock-in approach in iPSCs was employed to correct the pathogenic variants. Subsequently, these edited cells were differentiated into SC- β cells. Remarkably, the corrected SC- β cells exhibited significantly increased insulin secretion coupled with an appropriate response to glucose challenges. Consistent with these findings, there was a significant increase in the fraction of cells displaying β -cell markers. Importantly, when transplanted into mice with preexisting diabetes, the corrected SC- β cells successfully restored normoglycemia. Mechanistic investigations into the corrected SC- β cells revealed that the relief of ER stress was attributed to the gene correction [75].

Autologous cell therapies hold significant promise as they do not require the administration of immunosuppressants. This approach is particularly attractive for diabetes caused by single mutations, which account for approximately 1% to 5% of all di-

abetes cases [74]. Correcting these monogenic variants through genome editing offers a straightforward solution to treat the disease. For other types of diabetes arising from mutations in multiple genes, the rapid advancement of genome editing technologies would enable multiplex genome editing. This approach would allow the simultaneous correction of multiple genes, thereby enhancing the efficiency and potentially reducing the cost of autologous cell therapies.

Genome editing in primary pancreatic islets

Genome editing has been primarily performed in PSCs due to their amenability to editing, rapid proliferation, and the ability to develop into clones. The edited PSCs are then differentiated into gene-corrected β -like cells. While these SC- β cells hold promise as an abundant source for therapeutic development, primary pancreatic islets have historically been used for developing cell-based therapeutics for diabetes because of their mature β -cell function. Indeed, a primary islet-derived cell therapy was recently approved by the Food and Drug Administration for treating diabetes [9,10]. However, the use of primary islets presents challenges, such as the need for immunosuppressants administration to prevent allograft rejection. Thanks to rapid advances in CRISPR technologies, genome editing can now be applied to primary islets, addressing several issues associated with current islet-based cell therapies.

Bevacqua et al. [76] demonstrated the feasibility of CRISPR/Cas9-based knock-out in primary islets. The study began by successfully disrupting 2 critical genes for β -cell functions, *PDX1* and *KIR6.2*. Indeed, the knock-out impaired β -cell regulation and function. To further validate their approach, the researchers explored the role of non-coding DNA sequences through genome editing. Specifically, they identified a diabetes risk SNP located at the *KCNJ11-ABCC8* or *SIX2-SIX3* non-coding locus. Then, they introduced small deletions at the SNP locus by delivering Cas9 along with 2 gRNAs. This editing impaired β -cell function, revealing the significance of non-coding regulatory elements in type 2 diabetes [76].

For developing primary islet-derived cell therapeutics, hypo-immune engineering was performed in primary islets, so that the transplant could survive without immunosuppression. Hu et al. [54,77] employed CRISPR/Cas9 to knock out *B2M* and *CIITA*, while simultaneously overexpressing CD47 through viral delivery. The resulting hypoimmune islets survived and ameliorated diabetes in immunocompetent allogeneic humanized mice. Moreover, the engineered islets could be protected from

Table 1. Examples of genome editing in stem cell-derived β cells and primary islets

Genome-editing strategies and target cells	Editing machinery	Notes	Ref.
Genome editing for improving stem cell-derived β-cell therapeutics			
Knock-in of an <i>EGFP</i> reporter into the C-terminus of <i>PDX1</i> in iPSCs	SpCas9-mediated knock-in by HDR	The editing method allowed the facile isolation of insulin-positive β cells after differentiation.	[43]
Knock-in of a <i>GFP</i> sequence downstream of the <i>INS</i> promoter in iPSCs	SpCas9-mediated knock-in by HDR	Insulin-positive cells could be spotted from a cell mixture.	[44]
Knock-out of <i>SLC30A8</i> in ESCs	SpCas9-mediated knock-out	ZnT8 loss-of-function improved GSIS in SC- β cells. It also endowed the cells with resistance to lipotoxicity- and glucotoxicity-induced cell death.	[49]
Knock-out of <i>ZNF148</i> in ESCs	SpCas9-mediated knock-out	ZnF148 loss-of-function improved insulin secretion by modulating proteins involved in insulin trafficking and exocytosis.	[48]
Knock-out of <i>B2M</i> and <i>CIITA</i> , overexpression of CD47 in iPSCs	SpCas9-mediated knock-out, viral delivery of <i>CD47</i>	The hypoimmune SC- β cells survived long-term in immunocompetent mice and non-human primates.	[54]
Knock-out of <i>CIITA</i> and all classical HLA class I genes except <i>HLA-A2</i> , knock-in of luciferase at <i>AAVS1</i> in ESCs	SpCas9-mediated multi-step gene knock-out, TALEN-mediated knock-in by HDR	The resulting SC- β cells were less immunogenic, allowing long-term survival in a mouse model. Luciferase transgene allowed facile <i>in vivo</i> monitoring of the transplant.	[50]
Knock-out of <i>B2M</i> , knock-in of 3 cytokines (IL-2 N88D mutein, TGF- β , and IL-10) at the <i>GAPDH</i> locus of hESCs	SpCas9-mediated knock-out, SpCas9-mediated knock-in by HDR	The <i>B2M</i> knock-out SC- β cells displayed varying success of immune evasion <i>in vivo</i> . Cytokine knock-in was a complementary strategy for allograft survival.	[56]
Knock-out of <i>B2M</i> , and knock-in of doxycycline-inducible <i>PD-L1</i> at <i>AAVS1</i> locus of ESCs	SpCas9-mediated knock-out, TALEN-mediated knock-in by HDR	The resulting SC- β cells displayed reduced stimulation of diabetogenic CD8 T cells.	[47]
Knock-out of <i>B2M</i> in iPSCs	SpCas9-mediated knock-out	Decreased expression of HLA class I on SC- β cells resulted in reduced T-cell activation.	[59]
Knock-in of a <i>GFP</i> reporter in the middle of <i>CXCL10</i> to disrupt the gene in ESCs	SpCas9-mediated knock-in by HDR	Knock-out improved SC- β cell survival against allo-rejection in a mouse model.	[60]
Knock-out of <i>RNLS</i> in iPSCs	SpCas9-mediated small deletion by simultaneously delivering 2 gRNAs	Knock-out protected SC- β cells from ER stress and apoptosis.	[61]
Genome editing of stem cell-derived β cells for diabetes modeling and patient-specific therapies			
Correction of diabetes-inducing <i>INS</i> point mutations (C96R and C109Y) in patient-derived iPSCs	SpCas9-mediated knock-in by HDR	The mutant SC- β cells displayed reduced insulin secretion, increased ER stress and reduced proliferation, although apoptosis was not promoted.	[65]
Correction of diabetes-inducing <i>INS</i> intronic mutation in patient-derived iPSCs	SpCas9-mediated knock-in by HDR	The mutation resulted in the production of splicing variant of insulin mRNA, leading to decreased insulin production.	[66]
Correction of a congenital hyperinsulinism-inducing mutation (V187D) of <i>ABCC8</i> in patient-derived iPSCs	SpCas9-mediated knock-in by HDR	SC- β cells mutated in the SUR1 subunit of K_{ATP} -channel recapitulated the phenotype observed in the disease.	[67]
Introduction of mutations at <i>PDX1</i> , or generation of <i>PDX1</i> haploinsufficient models in iPSCs	SpCas9-mediated knock-in by HDR, SpCas9-mediated knock-out	<i>PDX1</i> mutations impaired β -cell differentiation and functions.	[68]
Introduction of heterozygous 4 bp duplication in <i>GATA6</i> and <i>GATA6</i> -related non-coding SNP in ESCs	SpCas9-mediated knock-in by HDR	<i>GATA6</i> haploinsufficiency impaired β -cell development. The non-coding SNP lowered <i>GATA6</i> expression to prevent β -cell development.	[69]
Heterozygous and homozygous knock-out of <i>GATA6</i> in ESCs	SpCas9-mediated knock-out	<i>GATA6</i> haploinsufficiency led to impaired formation of glucose-responsive SC- β cells.	[70]
Knock-out of <i>CDKAL1</i> , <i>KCNJ11</i> , or <i>KCNQ1</i> in ESCs	SpCas9-mediated knock-out	GWAS-identified gene variants were studied. Disrupting each of them led to impaired GSIS in SC- β cells. <i>CDKAL1</i> -defective cells were hypersensitive to glucolipotoxicity. Drug screening for <i>CDKAL1</i> knock-out SC- β cells identified a small-molecule enhancer of the cell function.	[71]
Knock-out of <i>HNF1A</i> in ESCs	SpCas9-mediated knock-out	Disruption of <i>HNF1A</i> led to the abnormal expression of genes related to β -cell function and development. SC- β cells allowed the study of human β -cell biology distinct from rodent cells.	[72]
Correction of diabetes-causing <i>INS</i> variants in patient-derived iPSCs	SpCas9-mediated knock-in by HDR	Gene correction restored insulin production and secretion in SC- β cells. The corrected cells restored blood glucose homeostasis in a diabetic mouse model.	[74]
Correction of diabetes-inducing <i>WFS1</i> variants in patient-derived iPSCs	SpCas9-mediated knock-in by HDR	The corrected SC- β cells exhibited robust insulin secretion and reversed diabetes in a mouse model.	[75]

(Continued to the next page)

Table 1. Continued

Genome-editing strategies and target cells	Editing machinery	Notes	Ref.
Genome editing in primary islets			
Knock-out of <i>PDX1</i> and <i>KCNJ11</i> in primary human islets	SpCas9-mediated knock-out	Regulation and function of primary β cells were impaired by knock-out.	[76]
Small deletion at the non-coding <i>KCNJ11-ABCC8</i> or <i>SIX2-SIX3</i> locus in primary human islets	SpCas9-mediated small deletion by simultaneously delivering 2 gRNAs	β -cell function was impaired by editing. This study demonstrated the roles of variants located at non-coding regulatory elements.	[76]
Knock-out of <i>B2M</i> and <i>CIITA</i> , overexpression of <i>CD47</i> in primary human islets	SpCas9-mediated knock-out, viral delivery of <i>CD47</i>	The engineered cells survived in an immunocompetent mice model and reversed hyperglycemia.	[54,77]

Ref., reference; EGFP, enhanced green fluorescent protein; PDX1, pancreas/duodenum homeobox protein 1; iPSC, induced pluripotent stem cell; HDR, homology-directed repair; ESC, embryonic stem cell; GSIS, glucose-stimulated insulin secretion; SC- β cell, stem cell-derived β cell; HLA, human leukocyte antigen; TALEN, transcription activator-like effector nuclease; IL, interleukin; TGF, transforming growth factor; hESC, human embryonic stem cell; PD-L1, programmed death ligand 1; CXCL10, chemokine ligand 10; gRNA, guide RNA; ER, endoplasmic reticulum; SUR1, sulfonylurea receptor 1; SNP, single-nucleotide polymorphism; GWAS, genome-wide association study.

autoimmune destruction in autologous, diabetic humanized mice.

One of the major limitations in genome editing of primary islets is that the editing method is restricted to knock-out. HDR-based knock-in is not feasible in primary islets because the HDR pathway is active only in dividing cells, but not in primary β cells that usually do not proliferate. Nevertheless, CRISPR-based knock-out coupled with transgene delivery through viral vectors could offer a viable approach to broaden the application of gene-edited primary islets in therapeutics.

Limitations of CRISPR/Cas9-based genome editing and potential solutions

While genome editing is an extraordinary tool for creating designer cell therapeutics, there always exist concerns about off-target gene editing [36,78–80]. Unintended genomic changes at non-targets can potentially affect cell fitness leading to cell death. The cells may be converted into non-functional cells or even into cancerous cells. As such, precautions must be taken to avoid off-target editing risks. For example, bioinformatics tools should be employed to design gRNAs with minimal off-target potential. In cases where only a limited set of gRNAs can be used, and the chances of off-target effects are high (e.g., when correcting a specific gene variant through HDR), recently developed anti-CRISPR tools may be employed to mitigate off-target effects [36,81]. Whenever possible, whole-genome sequencing of edited stem cell clones should be conducted to assess the potential for off-target modifications. If whole-genome sequencing is unavailable, it is advisable to examine several high-confidence off-target loci to ensure the safety of the cell therapeutics.

The genome-editing examples mentioned above primar-

ily employed CRISPR/Cas9-mediated knock-out or donor DNA-assisted knock-in techniques (Fig. 1). In addition to these conventional tools, emerging genome editing methods, such as base editors or prime editors, could be employed for more precise gene editing. Base editors can introduce single-base exchanges, while prime editors can make small insertions, deletions, and base exchanges without causing DNA DSBs, unlike Cas9 [82]. Consequently, base editors and prime editors display significantly reduced off-target effects, making genome editing safer. Although their editing efficiencies may not match that of Cas9, rapid advancements in genome editing technologies are paving the way for their widespread application in PSCs, particularly for introducing small genomic changes (e.g., modeling disease-related gene variants and correcting disease-causing variants).

CRISPR/Cas9-mediated HDR should be employed when introducing large gene fragments into PSCs (Fig. 2). However, HDR efficiency is typically low, necessitating the use of additional techniques for generating gene-edited cells. These techniques include the use of small-molecule inhibitors of the NHEJ pathway, which competes with the HDR pathway [83], chemical modification of donor DNA [84], and conjugating donor DNA to Cas9 [62], among others. A combination of multiple methods can be employed to achieve maximal HDR efficiency. Nonetheless, it is important to exercise caution when applying these techniques. For example, small-molecule inhibitors of the NHEJ pathway could have detrimental effects on certain cell types, as NHEJ is a constitutively active pathway that is important for cellular fitness.

Alternatively, methods for isolating knock-in clones can be employed. Genes for antibiotic resistance proteins or fluorescent proteins are commonly introduced into the genome along

with the desired knock-in fragment to facilitate the isolation of gene-edited PSCs. However, the introduction of these reporter genes is inevitably accompanied by the introduction of additional regulatory factors (e.g., promoters, terminators, or polycistronic expression cassette). Since these elements often originate from viral sequences, it is essential to consider whether the new sequences in the genome might provoke adverse effects *in vivo* [63,64].

Regardless of the type of editing, undesirable genomic changes at the on-target site should also be investigated. These on-target effects may involve large deletions or insertions at one allele, while the other allele carries the correct edits. As these effects cannot be detected through standard Sanger sequencing, it is recommended to perform quantitative polymerase chain reaction-based copy number quantification when isolating correct PSC clones [85]. Another on-target effect to consider is the loss of heterozygosity, which can be identified by genotyping a neighboring SNP [85]. These approaches can ensure the safe use of gene-edited PSCs.

Future perspectives

With rapid advancements in genome editing, researchers can now precisely modify therapeutic cells to confer them with new functionalities. The most notable achievements in this field include enhancing GSIS and developing strategies for evading immune destruction. Since there still exist diverse issues to be solved for the practical application of β -cell therapeutics, genome editing is poised to play an even more prominent role in future research.

For example, β -cell therapeutics often face challenges related to hypoxia and subsequent cell death due to inadequate vascularization to support the survival of transplants, especially in the early stages after transplantation [17]. Genome editing can be employed to enhance the resistance of these cells to hypoxia-induced apoptosis, maximizing their therapeutic potential. Recent clinical trials have utilized macroencapsulation devices for protecting SC- β cells from immune attacks. However, foreign body responses against the transplanted device have led to device fibrosis and failure [11,13]. Genome editing could also offer a solution to this issue, for instance, by engineering cells to locally secrete anti-fibrosis factors.

Nonetheless, transplanted cell therapies carry inherent safety risks, as stem cell-derived cells may potentially develop into teratomas [21,74]. Therefore, it is crucial to develop safety switches for selectively eliminating transplants when adverse effects arise. The need for safety switches becomes even more apparent when

transplants are engineered to be hypoinmunogenic, as these cells cannot be naturally eliminated by the patient's immune system even in the presence of adverse effects. Hence, there is a critical requirement for precise control over these therapeutic modalities. In the conventional Edmonton protocol and recent clinical trials, primary islets or SC- β cells are transplanted directly into the portal vein, in combination with immunosuppressive drug administration, without encapsulation [9,10]. This direct infusion of cells necessitates additional safeguards to mitigate potential adverse effects.

These requirements can be addressed through genome editing techniques designed to incorporate safety switches into cell therapeutics. For instance, the CRISPR/Cas9-based gene knock-in method was employed to introduce a drug-inducible caspase-9 (iCasp9) safety switch into PSCs [86]. This switch was integrated at the C-terminus of *NANOG*, along with a 2A self-cleaving peptide [87]. Thus, the safety switch is specifically expressed in PSCs since *NANOG* is a stem cell-specific transcription factor not expressed in differentiated cells. Upon treatment with a specific small molecule, the caspase-9 protein dimerizes and induces the selective death of undifferentiated stem cells, effectively addressing concerns related to teratoma formation [87]. When the iCasp9 switch is knocked in at the C-terminus of *ACTB*, a housekeeping gene expressed in all cell types, both undifferentiated and differentiated cells were eliminated upon treatment with a small molecule. Notably, all these switches efficiently worked in mouse models to remove the transplanted cells when the corresponding small molecules were administered [87]. Similar genome-editing strategies could be employed in β -cell therapeutics to ensure their safe use in future research.

In summary, SC- β cells can play crucial roles as sources of cell-based therapeutics for diabetes. They also serve as valuable models for human β cells, as obtaining mature human β -cell lines is challenging. When combined with genome editing techniques, SC- β cells will offer a powerful platform for both treating diabetes and conducting in-depth studies on the genetic aspects of diabetes.

Notes

Conflict of interest

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

Funding

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT)

(grant number: NRF-2021R1F1A1062027 and RS-2023-00209725), and by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HV22C0235).

Author contributions

Conceptualization: all authors; Funding acquisition: DL; Investigation: SJ, SS, YJ; Project administration: DL; Supervision: DL; Writing—original draft: all authors; Writing—review & editing: all authors.

Data availability

Not applicable - no new data were generated in this study.

ORCID

Seeun Jang, <https://orcid.org/0000-0002-6759-0864>

Siyoon Shin, <https://orcid.org/0000-0002-0325-2700>

Yujin Jeong, <https://orcid.org/0009-0001-6669-5285>

Donghyun Lim, <https://orcid.org/0000-0002-6070-198X>

References

1. Nair GG, Tzanakakis ES, Hebrok M. Emerging routes to the generation of functional β -cells for diabetes mellitus cell therapy. *Nat Rev Endocrinol* 2020;16:506–18.
2. Quizon MJ, García AJ. Engineering β cell replacement therapies for type 1 diabetes: biomaterial advances and considerations for macroscale constructs. *Annu Rev Pathol* 2022;17:485–513.
3. Fonseca VA. Defining and characterizing the progression of type 2 diabetes. *Diabetes Care* 2009;32(Suppl 2):S151–6.
4. Lin YK, Fisher SJ, Pop-Busui R. Hypoglycemia unawareness and autonomic dysfunction in diabetes: Lessons learned and roles of diabetes technologies. *J Diabetes Investig* 2020;11:1388–402.
5. An J, Nichols GA, Qian L, Munis MA, Harrison TN, Li Z, et al. Prevalence and incidence of microvascular and macrovascular complications over 15 years among patients with incident type 2 diabetes. *BMJ Open Diabetes Res Care* 2021;9:e001847.
6. Hogrebe NJ, Ishahak M, Millman JR. Developments in stem cell-derived islet replacement therapy for treating type 1 diabetes. *Cell Stem Cell* 2023;30:530–48.
7. Siehler J, Blöching AK, Meier M, Lickert H. Engineering islets from stem cells for advanced therapies of diabetes. *Nat Rev Drug Discov* 2021;20:920–40.
8. Lin X, Xu Y, Pan X, Xu J, Ding Y, Sun X, et al. Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. *Sci Rep* 2020;10:14790.
9. Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000;343:230–8.
10. First cell therapy for diabetes approved. *Nat Biotechnol* 2023;41:1036.
11. Ramzy A, Thompson DM, Ward-Hartstonge KA, Ivison S, Cook L, Garcia RV, et al. Implanted pluripotent stem-cell-derived pancreatic endoderm cells secrete glucose-responsive C-peptide in patients with type 1 diabetes. *Cell Stem Cell* 2021;28:2047–61.
12. Shapiro AM, Verhoeff K. A spectacular year for islet and stem cell transplantation. *Nat Rev Endocrinol* 2023;19:68–9.
13. Shapiro AM, Thompson D, Donner TW, Bellin MD, Hsueh W, Pettus J, et al. Insulin expression and C-peptide in type 1 diabetes subjects implanted with stem cell-derived pancreatic endoderm cells in an encapsulation device. *Cell Rep Med* 2021;2:100466.
14. Veres A, Faust AL, Bushnell HL, Engquist EN, Kenty JH, Harb G, et al. Charting cellular identity during human in vitro β -cell differentiation. *Nature* 2019;569:368–73.
15. Nair GG, Liu JS, Russ HA, Tran S, Saxton MS, Chen R, et al. Recapitulating endocrine cell clustering in culture promotes maturation of human stem-cell-derived β cells. *Nat Cell Biol* 2019;21:263–74.
16. Tahbaz M, Yoshihara E. Immune protection of stem cell-derived islet cell therapy for treating diabetes. *Front Endocrinol (Lausanne)* 2021;12:716625.
17. Yang K, O’Cearbhaill ED, Liu SS, Zhou A, Chitnis GD, Hamilos AE, et al. A therapeutic convection-enhanced macroencapsulation device for enhancing β cell viability and insulin secretion. *Proc Natl Acad Sci U S A* 2021;118:e2101258118.
18. Shilleh AH, Russ HA. Cell replacement therapy for type 1 diabetes patients: potential mechanisms leading to stem-cell-derived pancreatic β -cell loss upon transplant. *Cells* 2023;12:698.
19. Sato Y, Endo H, Okuyama H, Takeda T, Iwahashi H, Imagawa A, et al. Cellular hypoxia of pancreatic beta-cells due to high levels of oxygen consumption for insulin secretion in vitro. *J Biol Chem* 2011;286:12524–32.
20. Pellegrini S, Zamarian V, Sordi V. Strategies to improve the

- safety of iPSC-derived β cells for β cell replacement in diabetes. *Transpl Int* 2022;35:10575.
21. Nam SA, Seo E, Kim JW, Kim HW, Kim HL, Kim K, et al. Graft immaturity and safety concerns in transplanted human kidney organoids. *Exp Mol Med* 2019;51:1–13.
 22. Hoang DM, Pham PT, Bach TQ, Ngo AT, Nguyen QT, Phan TT, et al. Stem cell-based therapy for human diseases. *Signal Transduct Target Ther* 2022;7:272.
 23. Modell AE, Lim D, Nguyen TM, Sreekanth V, Choudhary A. CRISPR-based therapeutics: current challenges and future applications. *Trends Pharmacol Sci* 2022;43:151–61.
 24. Balboa D, Prasad RB, Groop L, Otonkoski T. Genome editing of human pancreatic beta cell models: problems, possibilities and outlook. *Diabetologia* 2019;62:1329–36.
 25. Benner C, van der Meulen T, Cacères E, Tigyi K, Donaldson CJ, Huising MO. The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. *BMC Genomics* 2014;15:620.
 26. Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic β cells in vitro. *Cell* 2014;159:428–39.
 27. Rezaia A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nat Biotechnol* 2014;32:1121–33.
 28. Naujok O, Diekmann U, Lenzen S. The generation of definitive endoderm from human embryonic stem cells is initially independent from activin A but requires canonical Wnt-signaling. *Stem Cell Rev Rep* 2014;10:480–93.
 29. Oström M, Löffler KA, Edfalk S, Selander L, Dahl U, Ricordi C, et al. Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. *PLoS One* 2008;3:e2841.
 30. Champeris Tsaniras S, Jones PM. Generating pancreatic beta-cells from embryonic stem cells by manipulating signaling pathways. *J Endocrinol* 2010;206:13–26.
 31. Huang H, Bader TN, Jin S. Signaling molecules regulating pancreatic endocrine development from pluripotent stem cell differentiation. *Int J Mol Sci* 2020;21:5867.
 32. Hogrebe NJ, Augsornworawat P, Maxwell KG, Velazco-Cruz L, Millman JR. Targeting the cytoskeleton to direct pancreatic differentiation of human pluripotent stem cells. *Nat Biotechnol* 2020;38:460–70.
 33. Balboa D, Barsby T, Lithovius V, Saarimäki-Vire J, Omar-Hmeadi M, Dyachok O, et al. Functional, metabolic and transcriptional maturation of human pancreatic islets derived from stem cells. *Nat Biotechnol* 2022;40:1042–55.
 34. Hogrebe NJ, Maxwell KG, Augsornworawat P, Millman JR. Generation of insulin-producing pancreatic β cells from multiple human stem cell lines. *Nat Protoc* 2021;16:4109–43.
 35. Hobson A, Draney C, Stratford A, Becker TC, Lu D, Arlotto M, et al. Aurora Kinase A is critical for the Nkx6.1 mediated β -cell proliferation pathway. *Islets* 2015;7:e1027854.
 36. Shin S, Jang S, Lim D. Small molecules for enhancing the precision and safety of genome editing. *Molecules* 2022;27:6266.
 37. Riesenberger S, Kanis P, Macak D, Wollny D, Düsterhöft D, Kowalewski J, et al. Efficient high-precision homology-directed repair-dependent genome editing by HDRobust. *Nat Methods* 2023;20:1388–99.
 38. Fu YW, Dai XY, Wang WT, Yang ZX, Zhao JJ, Zhang JP, et al. Dynamics and competition of CRISPR-Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing. *Nucleic Acids Res* 2021;49:969–85.
 39. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;8:2281–308.
 40. Acekifi C, Wang P, Karakose E, Manning Fox JE, González BJ, Liu H, et al. GLP-1 receptor agonists synergize with DYRK1A inhibitors to potentiate functional human β cell regeneration. *Sci Transl Med* 2020;12:eaaw9996.
 41. Orthwein A, Noordermeer SM, Wilson MD, Landry S, Enchev RI, Sherker A, et al. A mechanism for the suppression of homologous recombination in G1 cells. *Nature* 2015;528:422–6.
 42. Kahraman S, Manna D, Dirice E, Maji B, Small J, Wagner BK, et al. Harnessing reaction-based probes to preferentially target pancreatic β -cells and β -like cells. *Life Sci Alliance* 2021;4:e202000840.
 43. Lee Y, Choi HY, Kwon A, Park H, Park MH, Kim JW, et al. Generation of a PDX1-EGFP reporter human induced pluripotent stem cell line, KSCBi005-A-3, using the CRISPR/Cas9 system. *Stem Cell Res* 2019;41:101632.
 44. Yoshihara E, O'Connor C, Gasser E, Wei Z, Oh TG, Tseng TW, et al. Immune-evasive human islet-like organoids ameliorate diabetes. *Nature* 2020;586:606–11.
 45. Micallef SJ, Li X, Schiesser JV, Hirst CE, Yu QC, Lim SM, et al. INS(GFP/w) human embryonic stem cells facilitate isolation of in vitro derived insulin-producing cells. *Diabetologia* 2012;55:694–706.
 46. Liang S, Zhao J, Baker RK, Tran E, Zhan L, Kieffer TJ. Differentiation of stem cell-derived pancreatic progenitors into insulin-secreting islet clusters in a multiwell-based static 3D

- culture system. *Cell Rep Methods* 2023;3:100466.
47. Castro-Gutierrez R, Alkanani A, Mathews CE, Michels A, Russ HA. Protecting stem cell derived pancreatic beta-like cells from diabetogenic T cell recognition. *Front Endocrinol (Lausanne)* 2021;12:707881.
 48. de Klerk E, Xiao Y, Emfinger CH, Keller MP, Berrios DI, Loconte V, et al. Loss of ZNF148 enhances insulin secretion in human pancreatic β cells. *JCI Insight* 2023;8:e157572.
 49. Ma Q, Xiao Y, Xu W, Wang M, Li S, Yang Z, et al. ZnT8 loss-of-function accelerates functional maturation of hESC-derived β cells and resists metabolic stress in diabetes. *Nat Commun* 2022;13:4142.
 50. Parent AV, Faleo G, Chavez J, Saxton M, Berrios DI, Kerper NR, et al. Selective deletion of human leukocyte antigens protects stem cell-derived islets from immune rejection. *Cell Rep* 2021;36:109538.
 51. Keller MP, Rabaglia ME, Schueler KL, Stapleton DS, Gatti DM, Vincent M, et al. Gene loci associated with insulin secretion in islets from non-diabetic mice. *J Clin Invest* 2019;129:4419–32.
 52. Bose S, Volpatti LR, Thiono D, Yesilyurt V, McGladrigan C, Tang Y, et al. A retrievable implant for the long-term encapsulation and survival of therapeutic xenogeneic cells. *Nat Biomed Eng* 2020;4:814–26.
 53. Deuse T, Hu X, Gravina A, Wang D, Tediashvili G, De C, et al. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nat Biotechnol* 2019;37:252–8.
 54. Hu X, White K, Olroyd AG, DeJesus R, Dominguez AA, Dowdle WE, et al. Hypoimmune induced pluripotent stem cells survive long term in fully immunocompetent, allogeneic rhesus macaques. *Nat Biotechnol* 2023 May 8 [Epub]. <https://doi.org/10.1038/s41587-023-01784-x>.
 55. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986;319:675–8.
 56. Gerace D, Zhou Q, Kenty JH, Veres A, Sintov E, Wang X, et al. Engineering human stem cell-derived islets to evade immune rejection and promote localized immune tolerance. *Cell Rep Med* 2023;4:100879.
 57. Gravina A, Tediashvili G, Rajalingam R, Quandt Z, Deisenroth C, Schrepfer S, et al. Protection of cell therapeutics from antibody-mediated killing by CD64 overexpression. *Nat Biotechnol* 2023;41:717–27.
 58. Kelly JJ, Saeed-Marand M, Nyström NN, Evans MM, Chen Y, Martinez FM, et al. Safe harbor-targeted CRISPR-Cas9 homology-independent targeted integration for multimodality reporter gene-based cell tracking. *Sci Adv* 2021;7:eabc3791.
 59. Leite NC, Sintov E, Meissner TB, Brehm MA, Greiner DL, Harlan DM, et al. Modeling type 1 diabetes in vitro using human pluripotent stem cells. *Cell Rep* 2020;32:107894.
 60. Sintov E, Nikolskiy I, Barrera V, Hyoje-Ryu Kenty J, Atkin AS, Gerace D, et al. Whole-genome CRISPR screening identifies genetic manipulations to reduce immune rejection of stem cell-derived islets. *Stem Cell Reports* 2022;17:1976–90.
 61. Cai EP, Ishikawa Y, Zhang W, Leite NC, Li J, Hou S, et al. Genome-scale in vivo CRISPR screen identifies RNLS as a target for beta cell protection in type 1 diabetes. *Nat Metab* 2020;2:934–45.
 62. Lim D, Sreekanth V, Cox KJ, Law BK, Wagner BK, Karp JM, et al. Engineering designer beta cells with a CRISPR-Cas9 conjugation platform. *Nat Commun* 2020;11:4043.
 63. David RM, Doherty AT. Viral vectors: the road to reducing genotoxicity. *Toxicol Sci* 2017;155:315–25.
 64. Sheridan C. Why gene therapies must go virus-free. *Nat Biotechnol* 2023;41:737–9.
 65. Balboa D, Saarimäki-Vire J, Borshagovski D, Survila M, Lindholm P, Galli E, et al. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. *Elife* 2018;7:e38519.
 66. Panova AV, Klementieva NV, Sycheva AV, Korobko EV, Sosnovtseva AO, Krasnova TS, et al. Aberrant splicing of INS impairs beta-cell differentiation and proliferation by ER stress in the isogenic iPSC model of neonatal diabetes. *Int J Mol Sci* 2022;23:8824.
 67. Lithovius V, Saarimäki-Vire J, Balboa D, Ibrahim H, Montaser H, Barsby T, et al. SUR1-mutant iPS cell-derived islets recapitulate the pathophysiology of congenital hyperinsulinism. *Diabetologia* 2021;64:630–40.
 68. Wang X, Sterr M, Burtscher I, Böttcher A, Beckenbauer J, et al. Point mutations in the PDX1 transactivation domain impair human β -cell development and function. *Mol Metab* 2019;24:80–97.
 69. Kishore S, De Franco E, Cardenas-Diaz FL, Letourneau-Freiberg LR, Sanyoura M, Osorio-Quintero C, et al. A non-coding disease modifier of pancreatic agenesis identified by genetic correction in a patient-derived iPSC line. *Cell Stem Cell* 2020;27:137–46.
 70. Shi ZD, Lee K, Yang D, Amin S, Verma N, Li QV, et al. Genome editing in hPSCs reveals GATA6 haploinsufficiency and a genetic interaction with GATA4 in human pancreatic development. *Cell Stem Cell* 2017;20:675–88.
 71. Zeng H, Guo M, Zhou T, Tan L, Chong CN, Zhang T, et al.

- An isogenic human ESC platform for functional evaluation of genome-wide-association-study-identified diabetes genes and drug discovery. *Cell Stem Cell* 2016;19:326–40.
72. Cardenas-Diaz FL, Osorio-Quintero C, Diaz-Miranda MA, Kishore S, Leavens K, Jobaliya C, et al. Modeling monogenic diabetes using human ESCs reveals developmental and metabolic deficiencies caused by mutations in HNF1A. *Cell Stem Cell* 2019;25:273–89.
73. Xue A, Wu Y, Zhu Z, Zhang F, Kemper KE, Zheng Z, et al. Genome-wide association analyses identify 143 risk variants and putative regulatory mechanisms for type 2 diabetes. *Nat Commun* 2018;9:2941.
74. Ma S, Viola R, Sui L, Cherubini V, Barbetti F, Egli D. β cell replacement after gene editing of a neonatal diabetes-causing mutation at the insulin locus. *Stem Cell Reports* 2018;11:1407–15.
75. Maxwell KG, Augsornworawat P, Velazco-Cruz L, Kim MH, Asada R, Hogrebe NJ, et al. Gene-edited human stem cell-derived β cells from a patient with monogenic diabetes reverse preexisting diabetes in mice. *Sci Transl Med* 2020;12:eaax9106.
76. Bevacqua RJ, Dai X, Lam JY, Gu X, Friedlander MS, Tellez K, et al. CRISPR-based genome editing in primary human pancreatic islet cells. *Nat Commun* 2021;12:2397.
77. Hu X, Gattis C, Olroyd AG, Frieria AM, White K, Young C, et al. Human hypimmune primary pancreatic islets avoid rejection and autoimmunity and alleviate diabetes in allogeneic humanized mice. *Sci Transl Med* 2023;15:eadg5794.
78. Lim D, Zhou Q, Cox KJ, Law BK, Lee M, Kokkonda P, et al. A general approach to identify cell-permeable and synthetic anti-CRISPR small molecules. *Nat Cell Biol* 2022;24:1766–75.
79. Maji B, Gangopadhyay SA, Lee M, Shi M, Wu P, Heler R, et al. A high-throughput platform to identify small-molecule inhibitors of CRISPR-Cas9. *Cell* 2019;177:1067–79.
80. Gangopadhyay SA, Cox KJ, Manna D, Lim D, Maji B, Zhou Q, et al. Precision control of CRISPR-Cas9 using small molecules and light. *Biochemistry* 2019;58:234–44.
81. Jia N, Patel DJ. Structure-based functional mechanisms and biotechnology applications of anti-CRISPR proteins. *Nat Rev Mol Cell Biol* 2021;22:563–79.
82. Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol* 2020;38:824–44.
83. Chen S, Chen D, Liu B, Haisma HJ. Modulating CRISPR/Cas9 genome-editing activity by small molecules. *Drug Discov Today* 2022;27:951–66.
84. Yu Y, Guo Y, Tian Q, Lan Y, Yeh H, Zhang M, et al. An efficient gene knock-in strategy using 5'-modified double-stranded DNA donors with short homology arms. *Nat Chem Biol* 2020;16:387–90.
85. Weisheit I, Kroeger JA, Malik R, Wefers B, Lichtner P, Wurst W, et al. Simple and reliable detection of CRISPR-induced on-target effects by qPCR and SNP genotyping. *Nat Protoc* 2021;16:1714–39.
86. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011;365:1673–83.
87. Martin RM, Fowler JL, Cromer MK, Lesch BJ, Ponce E, Uchida N, et al. Improving the safety of human pluripotent stem cell therapies using genome-edited orthogonal safeguards. *Nat Commun* 2020;11:2713.