ADVANCES IN STEM CELL TECHNOLOGY TO MODEL AND TREAT DIABETES

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MINI REVIEW
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Epigenetic Memory: Lessons From iPS Cells Derived From Human β Cells

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Incomplete reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) may be responsible for the heterogeneity in differentiation capacity observed among iPSC lines. It remains unclear whether it results from stochastic reprogramming events, or reflects consistent genetic or cell-of-origin differences. Some evidence suggests that epigenetic memory predisposes iPSCs to enhanced differentiation into the parental cell type. We investigated iPSCs reprogrammed from human pancreatic islet β cells (BiPSCs), as a step in development of a robust differentiation protocol for generation of β-like cells. BiPSCs derived from multiple human donors manifested enhanced and reproducible spontaneous and induced differentiation towards insulin-producing cells, compared with iPSCs derived from isogenic non-β-cell types and fibroblast-derived iPSCs (FiPSCs). Genome-wide analyses of open chromatin in BiPSCs and FiPSCs identified thousands of differential open chromatin sites (DOCs) between the two iPSC types. DOCs more open in BiPSCs (Bi-DOCs) were significantly enriched for known regulators of endodermal development, including bivalent and weak enhancers, and FOXA2 binding sites. Bi-DOCs were associated with genes related to pancreas development and β-cell function. These studies provide evidence for reproducible epigenetic memory in BiPSCs. Bi-DOCs may provide clues to genes and pathways involved in the differentiation process, which could be manipulated to increase the efficiency and reproducibility of differentiation of pluripotent stem cells from non-β-cell sources.

Keywords: Epigenetic memory, Islet β Cells, pluripotent stem cell differentiation, ATAC-seq, Foxa2

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INCOMPLETE REPROGRAMMING INTO IPSCS

Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) using Yamanaka's combination of four transcription factors (OCT4, SOX2, KLF4, and c-MYC, together termed OSKM) (1) has opened new avenues for in-vitro generation of multiple human differentiated cell types for disease modeling, drug screening, and regenerative medicine. Initially iPSCs derived from multiple cell types have been expected to manifest similar differentiation capacities, resembling those of human embryonic stem cells (ESCs). However, accumulated experience has shown a great heterogeneity in differentiation capacity among iPSC lines. This heterogeneity is thought to result from incomplete reprogramming.

The mechanisms activated by ectopic expression of OSKM in somatic cells, which result in reprogramming to pluripotency, are only partly understood. This prolonged process, lasting several weeks, involves multiple events, including silencing of somatic cell genes and activation of pluripotency-associated genes. The low efficiency of OSKM-mediated reprogramming is thought to reflect the stochastic nature of these complex events. Only a small fraction of cells acquire pluripotency, as judged by accepted assays (e.g. differentiation into cells from the three embryonic germ layers in embryoid bodies (EB), and teratoma formation assay). OSKM factors function by binding to chromatin regions and inducing their remodeling, thereby activating or repressing gene expression. Evidence suggests that broad epigenetic changes are among early key events of the reprogramming process (2).

The epigenetic landscape, shaped by DNA methylation and histone modifications, is critical for maintaining cell identity. Erasing cell-specific patterns of epigenetic modifications, and replacing them with pluripotency patterns, are central to cell reprogramming to pluripotency. Ample evidence supports the incomplete and varying erasure of the original epigenetic marks in both mouse and human iPSC lines. However, it remains unclear whether these variations represent consistent patterns, based on the cell type of origin, or genetic differences among donors, or reflect stochastic differences caused by low efficiency of the reprogramming mechanisms (**Table 1**). While epigenetic memory may not necessarily affect gene expression patterns in iPSCs (3), likely due to missing transcription factors, it is expected to affect differentiation capacity towards specific cell fates. This may limit some applications of iPSCs, but at the same time may predispose iPSCs to enhanced differentiation into the parental cell type, thereby facilitating generation of cells for specific uses.

IPSC DIFFERENCES RELATED TO THE CELL TYPE OF ORIGIN

Mouse iPSCS

Early comparison of mouse iPSCs derived from bone marrow progenitor cells, dermal fibroblasts, and neural progenitor cells, identified residual DNA methylation signatures characteristic of the somatic tissue of origin (4). These differences favored iPSC differentiation towards the donor cell type, while restricting alternative cell fates. These results were contrasted to the methylation and differentiation patterns of nuclear-transfer-derived pluripotent stem cells, which were more similar to those of ESCs. Similar findings were reported in early-passage iPSCs obtained from mouse fibroblasts, hematopoietic and myogenic cells, which exhibited distinct epigenetic patterns (5). These patterns were reflected in different transcriptional profiles of the iPSCs, and in their differentiation efficiency into embryoid bodies (EBs) and hematopoietic cell types. These differences were eroded with iPSC passaging, suggesting that epigenetic memory was a transient phenomenon. iPSCs derived from mouse neonatal cardiomyocytes (CMs) were also shown to differentiate toward CMs more efficiently than fibroblast-derived iPSCs (FiPSCs) or mouse ESCs (6).

TABLE 1 | Possible sources of iPSC variability.

Possible source of iPSC variability	Expected phenotype
Stochastic reprogramming events	Differences among clones from a single cell type of origin from a single donor
Donor-related	Similarity among clones from multiple cell types of origin from a single donor; differences among iPSCs from multiple donors
Cell type of origin	Similarity among iPSCs from the same cell type of origin from multiple donors

Human iPSCs

A number of reports documented similar epigenetic memory in human iPSCs. Kim et al. compared DNA methylation profiles and differentiation potential of iPSCs derived from human umbilical cord blood and neonatal keratinocytes (7). They identified distinct genome-wide DNA methylation patterns in iPSCs derived from each cell type, resulting from both incomplete erasure of tissue-specific methylation and aberrant de-novo methylation. These differences did not disappear upon extended passaging. Ohi et al. observed that human iPSCs generated from hepatocytes, skin fibroblasts, and melanocytes, retained some transcriptional characteristics of the original cells at low passages, which could be partially explained by incomplete promoter DNA methylation (8). They noticed that incompletely silenced genes tended to be isolated from other genes that were repressed during reprogramming, indicating that silencing of isolated genes may be less efficient. Global DNA methylation analyses of iPSCs reprogrammed from human cornea limbal epithelial stem cells (LESC) showed gene methylation patterns similar to those of the parental cells, and different from those of FiPSCs (9). Upon differentiation, LESC-derived iPSCs expressed higher levels of LESC markers, compared with FiPSCs.

IPSC DIFFERENCES RELATED TO DONORS AND STOCHASTIC VARIABILITY

In contrast to these findings, which associated epigenetic and differentiation differences among iPSCs with their cell type of origin, other studies supported a donor-related or stochastic basis for these differences. In one such study (10), whole-genome profiling of DNA methylation in five human iPSC lines derived from adipocytes and fibroblasts (including 3 subclones of a single line) identified over 1,000 differentially methylated sites, most of them associated with CG islands and genes, indicating stochastic interclone reprogramming variability. Two other studies (11, 12) surveyed 16–18 iPSC lines each, derived from fibroblasts and peripheral blood cells of four human donors in each study. Both studies concluded that the majority of transcriptional and DNA methylome differences among iPSCs, as well as differences in their differentiation capacity towards the hematopoietic cell

lineage, could be attributed to the donor, rather than the cell type of origin, indicating that genetic differences among donors can result in reproducible reprogramming differences. Finally, two studies, which analyzed differences in gene expression patterns in human iPSCs, concluded that genetic differences between individual donors were the major cause of transcriptional variation between lines. One of these studies (13) compared 25 iPSC lines from four donors and three tissues. The second study compared two human ESC lines with genetically matched iPSCs derived from fibroblasts differentiated from each ESC line (14). Their findings showed reproducible gene expression patterns among each ESC line and three iPSC clones derived from it, and variations compared with the other ESC line and its iPSC derivatives. The study concluded that ESCs and iPSCs exhibited similar gene expression patterns, and that the donor genetic background was responsible for transcriptional variations among pluripotent stem cell lines.

The donor genetic background may also influence the differentiation capacity of ESC lines. However, in contrast to the relatively large numbers of iPSC lines that have been generated by multiple laboratories, data on differentiation of ESC lines accumulated to date is based on a small number of established ESC lines commonly used by all research groups. This limited number, which is a result of ethical barriers to generation of new ESC lines, does not allow proper comparisons of variations among ESC lines in differentiation potential into specific cell lineages.

EPIGENETIC MEMORY IN IPSCS DERIVED FROM HUMAN β CELLS

Directed in-vitro differentiation of iPSCs into β-like cells is a promising approach for generation of abundant insulin-producing cells for cell therapy of diabetes, disease modeling and drug screening. Despite significant progress (15–21), current differentiation protocols result in cells with heterogeneous and immature phenotype, and suffer from low efficiency and variability among iPSC lines. In a step towards developing a more robust differentiation protocol, we investigated iPSCs reprogrammed from human pancreatic islet β cells (BiPSCs). This approach depended on lineage tracing of human β-cell-derived (BCD) cells within the heterogeneous cell population in cultures of isolated human islets (22). The stable genetic label allowed positive identification of the β-cell origin of individual iPSC clones, that otherwise would be difficult to distinguish from iPSCs derived from non-β-cell types present in the expanded islet cell culture. Initial studies of four BiPSC lines derived from β cells of three nondiabetic human donors established their pluripotency, as judged by standard assays (23). Nevertheless, chromatin immunoprecipitation (ChIP) analyses showed that the levels of histone H3 acetylation, a hallmark of open chromatin structure, at the *INS* and *PDX1* loci in BiPSCs were similar to those found in human islets and BCD cells, and significantly higher than those in FiPSCs, as well as in two iPSC lines derived from isogenic islet non-β cells (termed PiPSCs) from two of the donors. BiPSCs also exhibited significantly lower DNA methylation levels, characteristic of transcribed genes, in genes expressed in human islet cells, such as *PDX1*, compares with FiPSCs. Despite the open chromatin marks, β-cell genes were not expressed in BCD and BiPS cells.

The epigenetic memory of BiPSCs was associated with higher expression levels of *PDX1*, *FOXA2*, and *INS* transcripts following spontaneous differentiation into EBs and teratomas, compared with those derived from FiPSCs and PiPSCs, and an enhanced induced differentiation capacity into insulin-producing cells in mice transplanted with BiPSC-derived endocrine progenitors, following the protocol of Kroon et al. (24). The epigenetic phenotype and differentiation capacity of BiPSCs were reproducible among the four lines obtained from three human donors, and appeared stable within the passage range analyzed (passages 10–20).

To identify genes and pathways, which may be responsible for the enhanced and reproducible differentiation capacity of BiPSCs, we performed a global analysis of chromatin sites differentially open in BiPSCs, compared with FiPSCs, using an Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) (25). For this analysis we generated five new BiPSC lines from three nondiabetic donors, which were compared to five FiPSC lines from two nondiabetic donors. All these lines passed pluripotency assays, and EBs generated from BiPSCs at passages 9-12 showed enhanced spontaneous expression of *FOXA2*, *PDX1*, and *INS*, compared with those derived from FiPSCs (25), similarly to the four BiPSC lines in the initial study.

Despite high overall similarity in open chromatin between the two iPSC types, the ATAC-seq analysis identified thousands of significantly differential open chromatin (DOC) sites between BiPSCs and FiPSCs, most of which were more open in BiPSCs (Bi-DOCs). Bi-DOCS overlapped gene regulatory elements known to be involved in development, such as weak enhancers (marked by H3K4me1) and bivalent enhancers and promoters (marked by H3K27me3), especially near genes involved in endodermal development, such as *FOXA2* and its target genes, and pancreas development, such as *PDX1*, and *NKX2-2*, as well as genes expressed in mature β cells, such as *INS*. The ATAC-seq data for these four genes (*FOXA2*, *PDX1*, *NKX2-2*, and *INS*) was validated by H3K4me3 ChIP analysis, which found higher levels of this open chromatin mark in the promoter regions of all four genes in BiPSCs, compares with FiPSCs. These findings could explain the enhanced expression of these genes in EBs generated from BiPSCs, compared with those derived from FiPSCs.

The relevance of Bi-DOCs to differentiation towards islet cells was analyzed by comparing directed differentiation of BiPSCs and FiPSCs into definitive endoderm (DE) and pancreatic progenitor (PP) cells according to the protocol of Rezania et al. (16). Global transcriptome analyses by RNA-seq identified 567 protein-coding genes expressed at higher levels in BiPSC-derived DE, compared with FiPSC-derived DE, and 181 genes expressed at higher levels in BiPSC-derived PP, compared with FiPSC-derived PP (25). These genes were significantly enriched for genes mapping near Bi-DOCs. Among genes expressed at higher

levels in BiPSC at both stages, the most prominent was estrogen receptor 1 (*ESR1*; 3.5-fold and 53.4-fold higher in DE and PP, respectively) and several of its target genes. 17β-estradiol (E2) has been shown to protect mouse β cells from apoptosis by signaling through estrogen receptor (ER) α encoded by *ESR1* (26). ERα activity has been reported to increase *Neurog3* expression and β-cell proliferation in a mouse model of pancreas partial duct ligation, and during mouse islet development (27). It has been suggested to regulate endocrine lineage specification through downregulation of NOTCH signaling. Thus, inclusion of E2 in the culture medium at key stages of the in-vitro differentiation protocol may increase its efficiency and reproducibility.

CONCLUSION

Overall, the analyses of Bi-DOCs support the existence of reproducible epigenetic memory in BiPSCs. The association between Bi-DOCs and gene expression levels at early stages of the in-vitro differentiation protocol provides a plausible explanation for the enhanced differentiation capacity of BiPSCs into the β-cell lineage, compared with pluripotent stem cells from a non-β-cell source. Both chromatin structure and differentiation capacity were reproducible in a combined number of nine BiPSC lines from six human islet donors in two separate studies. Bi-DOCs may provide clues to genes and pathways involved in the differentiation process, which could be manipulated to increase the efficiency and reproducibility of differentiation of pluripotent stem cells from other sources. Such manipulations could include activation of candidate genes using CRISPR-on approaches or small-molecule compounds.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Human Pluripotent Stem Cells: A Unique Tool for Toxicity Testing in Pancreatic Progenitor and Endocrine Cells

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Diabetes prevalence is increasing worldwide, and epidemiological studies report an association between diabetes incidence and environmental pollutant exposure. There are >84,000 chemicals in commerce, many of which are released into the environment without a clear understanding of potential adverse health consequences. While *in vivo* rodent studies remain an important tool for testing chemical toxicity systemically, we urgently need high-throughput screening platforms in biologically relevant models to efficiently prioritize chemicals for in depth toxicity analysis. Given the increasing global burden of obesity and diabetes, identifying chemicals that disrupt metabolism should be a high priority. Pancreatic endocrine cells are key regulators of systemic metabolism, yet often overlooked as a target tissue in toxicology studies. Immortalized β-cell lines and primary human, porcine, and rodent islets are widely used for studying the endocrine pancreas *in vitro*, but each have important limitations in terms of scalability, lifespan, and/or biological relevance. Human pluripotent stem cell (hPSC) culture is a powerful tool for *in vitro* toxicity testing that addresses many of the limitations with other β-cell models. Current *in vitro* differentiation protocols can efficiently generate glucose-responsive insulin-secreting β-like cells that are not fully mature, but still valuable for high-throughput toxicity screening *in vitro*. Furthermore, hPSCs can be applied as a model of developing pancreatic endocrine cells to screen for chemicals that influence endocrine cell formation during critical windows of differentiation. Given their versatility, we recommend using hPSCs to identify potential β-cell toxins, which can then be prioritized as chemicals of concern for metabolic disruption.

Keywords: stem cells, diabetes, pollution, beta cells, pancreas development, toxicology

INTRODUCTION

Diabetes Pathogenesis

Diabetes is a chronic disease characterized by high blood sugar levels and devastating secondary health complications (1). In 2019, there were >460 million people with diabetes worldwide, which translates to roughly 1 in 11 adults ages 20-79 years. For those over the age of 65, diabetes rates further increase to 1 in 5 (1). Moreover, the International Diabetes Federation projects that diabetes incidence will increase by 51% over the next 25 years to exceed 700 million adults worldwide.

Glucose homeostasis is maintained by the exquisite balance of hormones secreted from pancreatic islets. The predominant islet cell type is the β-cell, which secretes insulin in a tightly regulated manner in response to glucose and other stimuli (2, 3). Type 1 diabetes (T1D), accounting for ~10% of patients with diabetes, is caused by autoimmune destruction of β-cells leading to insufficient insulin production. Type 2 diabetes (T2D) accounts for ~90% of cases and was classically thought of as a disease of insulin resistance. However, we now appreciate that β-cell dysfunction and loss of β-cell mass are also central to T2D pathogenesis (4–6). The critical role of β-cells in driving diabetes risk is further confirmed by genome-wide association studies, which find that most loci influencing T2D risk are involved in regulating insulin secretion (7–9). These studies also emphasize that rising diabetes rates cannot simply be explained by genetics, but rather must be influenced by environmental factors (9, 10). For example, there is strong epidemiological evidence linking exposure to persistent organic pollutants (POPs) with increased T2D incidence (11–29) and β-cell dysfunction (28–30) in humans. However, basic research in clinically relevant models is needed to understand the potential causal role for environmental contaminants in diabetes pathogenesis and to explore underlying tissue- and cell-specific mechanisms of toxicity.

Environmental Contaminants

Environmental pollutants are a major global concern due to their wide-ranging acute and chronic adverse effects on human health (31). With over 84,000 chemicals in commerce, there is an urgent need to develop tools for extensive chemical screening and toxicity testing (32). Environmental contaminants fall within a wide range of classes, including but not limited to POPs (e.g., pesticides, polychlorinated biphenyls (PCBs), dioxin-like compounds), estrogen analogues (e.g., bisphenol A (BPA), used in polycarbonate plastics), phthalates (used in cosmetics, paints, textiles), heavy metals, perfluorinated chemicals (e.g., perfluorooctane sulfonate (PFOS) used in food packaging and fire-fighting foams), and flame retardants (e.g., polybrominated diphenyl ethers, organohalogen compounds, organophosphates esters) (33–35). Contaminants can be further classified by their mechanism of action. For example, chemicals that impair proper hormone function are referred to as endocrine-disrupting chemicals (EDCs) (33, 36–40) and those that disrupt metabolism are classified as metabolism-disrupting chemicals (MDCs) (41–43). Despite restrictions on many environmental pollutants, these chemicals continue to persist in the environment, contaminating food and water sources, and remain detectable in human tissues (44, 45).

Biomonitoring is essential for tracking human contaminant exposure and predicting adverse health outcomes (46, 47). However, this is a reactive approach to evaluating the impact of toxins on human health. Ideally, we need to efficiently screen chemicals for toxicity in relevant model systems prior to their release into the environment. Since pollutants often accumulate in tissues, effectively creating a chemical mixture cocktail (48), we also need to consider the combined effects of complex chemical mixtures. Dose and duration of exposure add additional layers of complexity. For example, POPs have long half-lives of years to decades (49), but the shorter lifespan of other chemicals such as BPA and phthalates is also not trivial. Much like hormones, EDCs can exert their effects on the human body at low concentrations over an extended period of time (50). Despite being excreted within days, frequent consumption of these pollutants results in chronic, low dose exposure over time (51, 52). Furthermore, nonlinear dose-responses are frequently seen with EDCs, so acute high dose studies may not accurately predict adverse health outcomes of chronic or subacute low dose exposures (42, 53, 54). The need to consider chemical exposures ranging from acute high doses to chronic low doses, as well as individual chemicals and complex mixtures, further emphasizes the importance of scaling up toxicity testing capacity.

Developmental Origins of Disease

Another important consideration for toxicology studies is the timing of exposure to environmental contaminants. Gestational or early life stressors, such as undernutrition or overnutrition, are linked to a variety of adult-onset diseases - termed developmental origins of health and disease (DOHaD) (55, 56). For instance, low birth weight and early life "catch up" growth are well-established risk factors for developing metabolic disease later in life (57, 58). Maternal-fetal exposure to POPs has been linked to adverse outcomes such as reduced birth weight, disruption of hormone levels in cord blood, and changes in epigenetic markers of development (59–61). There is also mounting epidemiological evidence suggesting a possible link between early-life environmental contaminant exposure and long-term metabolic dysfunction (62–66). More epidemiology is needed and important cohort studies like the Maternal-Infant Research on Environmental Chemicals (MIREC) continue to track long-term metabolic outcomes in offspring (67–70). However, it takes decades to truly establish a link between early-life exposure and long-term adverse health outcomes. *In vitro* model systems that allow for toxicity screening in developing human cells will be a powerful starting point for studying DOHaD.

Perspective Overview

There is an urgent need to identify environmental contaminants, specifically EDCs or MDCs, that contribute to diabetes pathogenesis. To do so, we must consider non-classical toxicological endpoints in a wide variety of tissues involved in regulating metabolic homeostasis. This means thinking beyond typical hepatoxicity endpoints and considering diverse metabolic

targets such as neuroendocrine cells, enteroendocrine cells, white or brown adipocytes, skeletal muscle, thyroid gland, and pancreatic endocrine cells (38, 40, 71). While injury to any of these tissues would potentially disrupt energy homeostasis, we propose that pancreatic endocrine cells should be a high priority for toxicity testing to identify MDCs of concern for diabetes pathogenesis. In this Perspective Article, we discuss a range of endpoints that could be considered in the context of β-cell toxicity. We also discuss various model systems available for toxicity testing, including the numerous advantages of human pluripotent stem cells (hPSCs). In particular, we propose hPSCs as a unique model system for evaluating toxicity both during critical windows of β-cell development and in glucose-responsive adult β-like cells (**Figure 1**).

TOXICITY TESTING IN PANCREATIC β-CELLS

Despite mounting evidence implicating pollutants as metabolic disruptors, the pancreas has not been extensively studied in the toxicology field (40, 42). Interestingly, the occasional biodistribution studies that include pancreas tissue report a slower elimination of lipophilic pollutants in the pancreas compared to liver or adipose (72, 73). Xenobiotic metabolism enzymes, such as cytochrome P450 (Cyp) enzymes, are useful biomarkers for direct cellular exposure to pollutants. We have reported induction of *Cyp1a1* in mouse and human islets following direct exposure to TCDD/dioxin or dioxin-like pollutants *in vitro* and in mouse islets following systemic administration of TCDD *in vivo* (73). Moreover, in pregnant TCDD-exposed mice, *Cyp1a1* was induced 17-fold in pancreas compared to only 3-fold and 7-fold in liver and adipose, respectively (74). Therefore, pancreatic cells are not only directly exposed to pollutants *in vivo*, but may even act as a "sink" for long-term storage of lipophilic chemicals, similar to adipose depots.

There is mounting evidence that a wide range of environmental contaminants can directly impact β-cell function. For example, BPA, a non-persistent additive commonly used in plastic products, acutely increases insulin secretion in mouse and human islets *via* inhibition of K_{ATP} channels and increased Ca^{2+} signaling (75), whereas longer-term BPA exposure inhibits Ca^{2+} entry and reduces insulin secretion (76). Newer BPA-replacement chemicals, BPS and BPF, also disrupt mouse β-cell function (77). Exposure to POPs, including organochlorine pesticides and a PCB mixture, directly inhibited insulin secretion in a rat β-cell line (INS-1E cells) (30). A "northern contaminant mixture", containing 20 different POPs at environmentally relevant concentrations, also suppressed insulin secretion in rats *in vivo* and in a rodent β-cell line (MIN6 cells) *in vitro* (78). Additionally, we and others have shown that dioxin suppresses insulin secretion in rodent islets (73, 79, 80) and human islets (73). Interestingly, acute high-dose dioxin exposure caused β-cell apoptosis in male mice but not female mice (81), whereas prolonged low-dose dioxin exposure led to impaired β-cell adaptation to high fat diet feeding in female but not male mice (74, 82).

Given the critical role for β-cells in diabetes pathogenesis, environmental toxins that adversely impact β-cells are likely to disrupt overall glucose homeostasis or at minimum, increase diabetes risk. Importantly, there are numerous plausible avenues for toxin-induced β-cell injury that could lead to adverse metabolic health outcomes. Below, we propose key toxicity endpoints for β-cells. This is not intended to be a comprehensive list of potential adverse outcomes, but rather examples that should be considered as a starting point for identifying MDCs that act as β-cell toxins.

Insulin Secretory Defects

Pancreatic β-cells are highly specialized to synthesize, process, store, and secrete insulin rapidly and sustainably in response to numerous physiological stimuli, including glucose (2). There is a rapid first phase of insulin secretion within minutes of a glucose stimulus, followed by a sustained second phase that can last for several hours. Glucose-stimulated insulin secretion is amplified by other nutrients, such as fatty acids and amino acids, and binding of gut-derived hormones (GLP-1, GIP) to incretin receptors. Dysregulated glucose-induced insulin secretion is a well-established feature of T2D (83–87) and also reported in T1D patients prior to disease onset (88–92), suggesting a possible link between insulin secretory defects and activation of inappropriate autoimmune responses. Therefore, environmental contaminants that interfere with any aspect of the complex insulin secretory pathway (e.g., glucose sensing, mitochondrial metabolism, ion flux, exocytotic machinery, sensitivity to amplification signals) could adversely affect the fine-tuned ability of β-cells to couple insulin secretion with a nutrient secretagogue. Furthermore, defects in the timing of insulin release, either the rapid first-phase or the sustained second-phase response, could impact overall glycemic control. Importantly, β-cell dysfunction is not just insufficient or decreased insulin secretion, but also refers to overproduction of insulin. Hyperinsulinemia is not only an adaptation to insulin resistance but can also be the primary defect that drives obesity and insulin resistance (93–96). Therefore, toxins that increase insulin secretion – either inappropriate insulin release under basal glucose conditions or excessive insulin secretion following a nutrient stimulus – should also be considered potential MDCs.

Loss of β-Cell Mass

Patients with T1D display near complete loss of β-cell mass (> 80% reduction) at the time of disease onset (6). Although less extreme, individuals with T2D also have reduced β-cell mass (5, 6, 97, 98), which may be caused by a combination of increased β-cell death, insufficient β-cell proliferation, or impaired β-cell neogenesis. β-cell mass is generally increased in overweight or obese non-diabetic subjects compared to lean controls but reduced by 24%–65% in patients with T2D (5, 6, 99). Loss of β-cell mass can be detected by measuring the α-cell to β-cell ratio, which is consistently higher in patients with T2D (97). Therefore, environmental toxins that disrupt β-cell mass, for

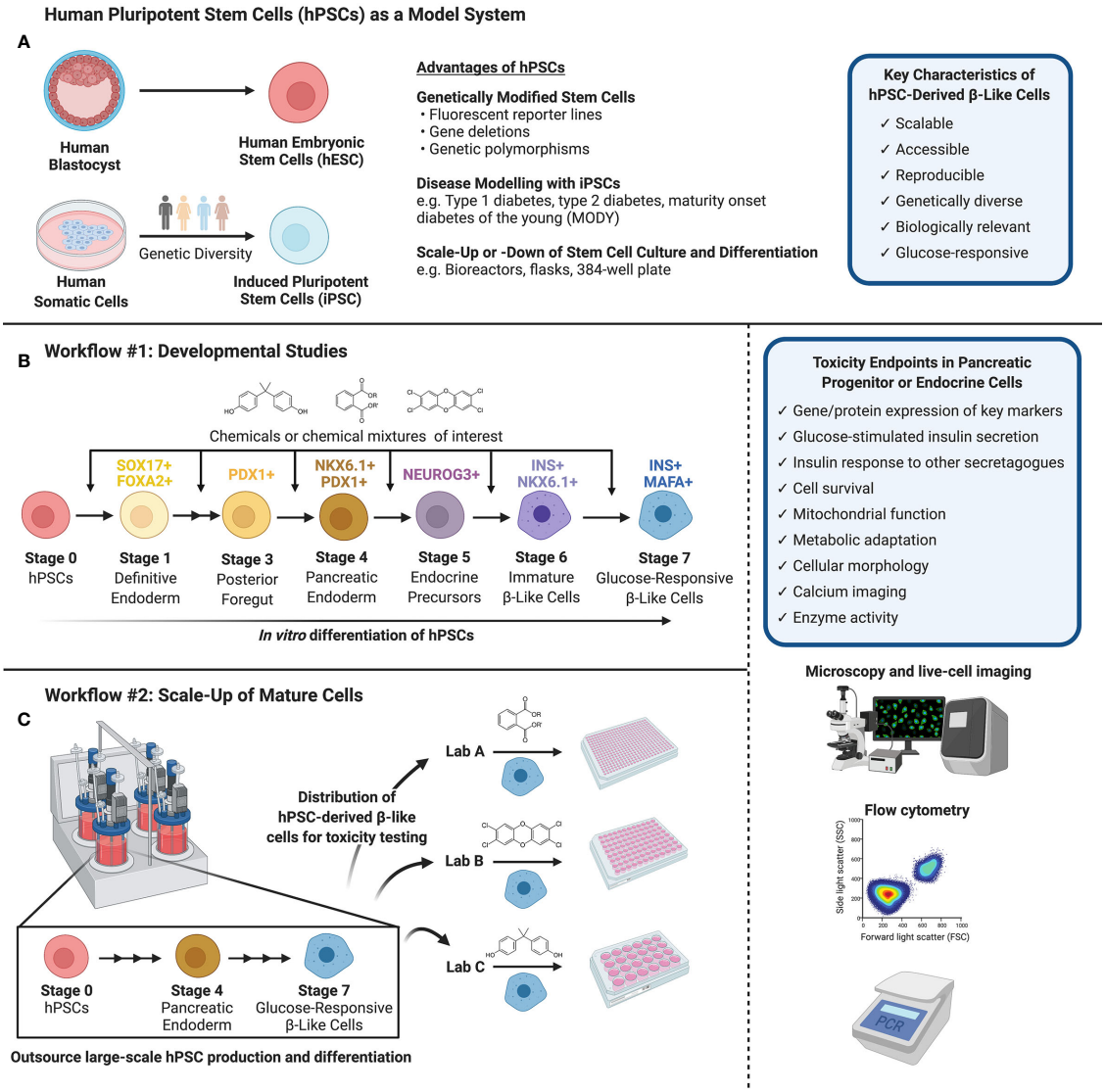

FIGURE 1 | **(A)** Human pluripotent stem cells (hPSCs) can be isolated from the inner cell mass of a human blastocyst (human embryonic stem cells, hESCs) or obtained *via* reprogramming of human somatic cells obtained from genetically diverse donors (induced pluripotent stem cells, iPSCs). hPSCs are versatile in their capacity for genetic modifications and disease modeling and may be scaled up or down to suit a variety of experimental conditions. **(B)** Workflow #1 illustrates how hPSCs may be used to screen chemicals or chemical mixtures of interest throughout pancreas development. hPSCs can be differentiated into pancreatic endoderm using published protocols or commercially available differentiation kits, and further into maturing, glucose-responsive β-like cells. Chemicals can be introduced at different days or stages of differentiation to mimic environmental exposures at different windows of pancreas development. **(C)** Workflow #2 demonstrates the capacity to outsource hPSC expansion and large-scale differentiation, allowing individual labs to conduct toxicity screening of specific chemicals/chemical mixtures using glucose-responsive β-like cells generated in a central location. **(B, C)** We suggest a number of potential toxicity endpoints, such as cell survival, insulin secretion, and mitochondrial function. Common analytical methods include but are not limited to microscopy and live-cell imaging, flow cytometry to quantify cell populations throughout differentiation, and PCR to assess gene expression (Created with BioRender.com).

example by inducing β-cell apoptosis or preventing β-cell expansion, should be flagged as potential MDCs.

Impaired β-Cell Adaptation

Healthy β-cells have fine-tuned mechanisms for adapting to fluctuations in energy supply and insulin demand to maintain appropriate glucose homeostasis (100). Examples of complex compensatory mechanisms include a) regulation of key β-cell transcription factors that control the β-cell transcriptome, b) altered activity or expression of metabolic enzymes to allow for flexibility in nutrient metabolism, and c) increased β-cell proliferation to expand functional mass of β-cells. Failed

compensatory insulin secretion and expansion of β-cell mass during insulin resistance are important predictors of diabetes susceptibility (99, 101, 102). Therefore, toxicology studies should consider the ability of β-cells to appropriately compensate under conditions of fasting or insulin resistance. If only direct cellular toxicity is tested without considering interactions with other metabolic challenges, potential MDCs will be overlooked.

Impaired β-Cell Development

The number of pancreatic progenitors present throughout embryonic development is a critical determinant of β-cell mass and pancreas size in adulthood, unlike the closely related endoderm-derived liver which can fully compensate following partial progenitor cell ablation (103). Therefore, an infant born with reduced β-cell mass may have a compromised ability to adapt to metabolic stressors later in life. Additionally, overproduction of insulin at birth caused by inappropriate β-cell expansion or accelerated maturation could lead to the development of insulin resistance and obesity (as described above) (94–96, 104). Therefore, a starting point for predicting long-term adverse metabolic health outcomes following intrauterine chemical exposure would be screening for chemicals that alter β-cell development. We propose that "developmental MDCs" could be prioritized, in part, based on whether they influence the formation of β-cells – either decreasing or increasing numbers – during critical windows of development.

TRADITIONAL MODELS FOR TOXICITY TESTING IN PANCREATIC ENDOCRINE CELLS

In Vivo Rodent Models

In vivo rodent models are an important tool for toxicity testing, but pose a significant technical barrier to high throughput screening (105) and are limited in their ability to predict human outcome. In a largescale study of pharmaceutical toxicity testing, rodents were predictive of human toxicity for only 43% of tested compounds, and demonstrated poor concordance for liver and endocrine toxicity (106). Further, human populations are genetically diverse and exist amongst variable exogenous factors, whereas laboratory animals are genetically uniform and housed within controlled environments to support reproducibility. While *in vivo* testing is necessary for assessing the impact of chemicals on a whole organism rather than just a single cell or tissue type in isolation, there has been a shift towards first prioritizing chemicals using *in vitro* model systems with higher throughput capacity (107, 108).

Immortalized β-Cells

Rodent Cell Lines

Immortalized rodent β-cell lines are robust in culture and highly proliferative, making them a useful tool for large-scale studies. Unfortunately, their replication capacity limits their applicability as a model for human β-cells, which have minimal ability to proliferate (99, 109). In addition, commonly used insulin-secreting rodent β-cell lines, including β-TC-6 (mouse), MIN6 (mouse), and INS-1 (rat) cells, have varying degrees of glucose-responsiveness (110, 111). Immortalized cell lines also tend to be genetically unstable in culture for extended periods of time. Clonal INS-1E cells have higher stability than the INS-1 parental line and maintain their insulin content in passages >40 (112), but both INS-1/INS-1E cells are cultured with the toxic reducing agent, 2-mercaptoethanol, which further limits their biological relevance for toxicity testing.

Despite these limitations, INS-1 823/13 cells were comprehensively evaluated as a potential pollutant-screening system. This cell line was found to be adequately glucose-responsive, but the insulin secretory responses to key control compounds and pollutants deviated substantially from previous reports (113). The authors concluded that INS-1 823/13 cells were lacking key β-cell characteristics and deemed inadequate as a diabetogenic pollutant screening system (113). We reached a similar conclusion after testing immortalized pancreatic endocrine cells for their response to TCDD/dioxin, a POP that acts *via* the aryl hydrocarbon receptor (AhR). In primary mouse and human islets, TCDD significantly upregulated CYP1A1 gene expression and enzyme activity, whereas immortalized β-cell (INS-1, MIN6, β-TC6) and α-cell (α-TC1 and α-TC3) lines showed no evidence of AhR activation by TCDD (73). Our findings confirm that important discrepancies exist in the cellular machinery between primary and immortalized cell lines.

Human Cell Lines

Novel engineered human β-cell lines, EndoC-βH1 and EndoC-βH2, are a useful tool for studying β-cell physiology and drug responses (114, 115). EndoC-βH1 cells were engineered from human fetal pancreatic buds transduced with an SV40LT-expressing lentiviral vector under the control of an insulin promoter. These cell lines are glucose-responsive *in vitro* and have minimal expression of other pancreatic genes (114–116). Their main drawback is the limited capacity for expansion *in vitro*, which is more biologically appropriate than rodent β-cell lines, but less practical. With a doubling time of approximately 7 days, their potential for high-throughput toxicity studies is limited. While proliferation can be stimulated with SerpinA6, STC1, and APOH (114), using compounds that alter cellular physiology is not ideal for toxicity testing. Additionally, as EndoC β-cells are a product of oncogenesis, there is undoubtedly alterations to normal pathways (117).

Primary Isolated Islets

Non-Human Islets

Primary rodent islets are more biologically relevant than immortalized rodent β-cell lines, but there are important distinctions between human and rodent islets that must be considered. For example, the distribution and composition of endocrine cells, vasculature, innervation, proliferation, and insulin secretion all differ between human and rodent islets (118, 119). Pig islets are more similar to human islets (120). Interestingly, islets isolated from juvenile porcine pancreata have

greater expression of β-cell-related genes compared to those isolated from adult pigs (121), but are functionally immature and require *in vitro* maturation following isolation (122–124). Further, it is possible to isolate up to 5,000 islets/g juvenile porcine pancreas (122), compared to a typical yield of ~200–400 islets total per mouse pancreas and 600–800 islets per rat pancreas depending on the strain (125–127). Thus, pig islets may be a useful tool for toxicity screening, although species differences will always remain a concern for translation.

Human Islets

Human islets are currently the gold standard for a physiologically relevant model to study the endocrine pancreas *in vitro* due to their cellular composition, human origin, and genetic diversity. Human islets are harvested from deceased organ donors and great strides have been made to ensure that high quality donor islets are broadly available for research (128, 129). However, even with the highest quality isolation procedures, the pancreas begins to autodigest after death, resulting in decreased cell viability and sample quality (119). Human islets have a limited functional *in vitro* lifespan with current tissue culture protocols, although advances in the field are ongoing. For example, islets cultured on specific matrices maintain glucose-stimulated insulin secretion for at least 7 days in culture (130). The number of purified islets per donor also varies; while an average healthy adult pancreas houses over 3 million islets (118), between 200,000 and 500,000 islets typically remain post-purification (131). This, coupled with the limited proliferation of human β-cells, presents a critical barrier to scalability and longevity for toxicology testing. Therefore, human islets are an excellent resource for *in vitro* chemical testing at a smaller scale, wherein endpoints such as glucose-stimulated insulin secretion, islet morphology, mitochondrial function, and gene expression can be assessed in biologically diverse organ donors. Numerous factors, such as donor sex, age, and body mass index, will influence islet function *ex vivo* and thus impact biological reproducibility. However, the genetic and environmental diversity of human organ donors (132, 133) also offer a unique opportunity for toxicity testing.

STEM CELLS ARE A UNIQUE TOOL FOR PANCREAS TOXICOLOGY STUDIES

HPSC culture offers a unique *in vitro* solution to address the need for high-throughput screening of environmental toxins in a variety of biologically relevant mature cell types, as well as in differentiating or "developing" immature progenitor cells. HPSCs can be obtained from either the inner cell mass of a human blastocyst, termed human embryonic stem cells (hESCs), or from human somatic cells that have been reprogrammed to a pluripotent state, termed induced pluripotent stem cells (iPSCs) (**Figure 1A**).

Remarkable progress has been made over the past decade unraveling the developmental cues involved in islet cell formation. We now have robust step-wise differentiation protocols that mimic the key fate decisions for directing hPSCs into pancreatic endocrine cells using small molecules and growth factors *in vitro* (134–139). These differentiation protocols efficiently guide hPSCs towards pancreatic endoderm cells ($PDX1^+/NKX6.1^+$) in four "stages", followed by commitment to the pancreatic endocrine lineage ($NEUROG3^+$), then insulin-secreting endocrine cells ($INS^+/NKX6.1^+$), and finally to β-like cells capable of glucose-induced insulin secretion ($INS^+/MAFA^+/UCN3^+$) (3) (**Figure 1B**). The challenge in recent years has been understanding the final stages of human β-cell maturation so we can generate fully mature β-cells with a rapid and robust insulin secretory response to various secretagogues. Despite these limitations, we believe that hPSCs are an excellent tool for studying adverse effects of environmental contaminants both during pancreas development and in adult pancreatic endocrine cells. Indeed, a recent study by Zhou et al. in *Nature Communications* beautifully demonstrated the diverse and powerful applicability of hPSCs for high-content screening of potential β-cell toxins, exploring gene-environment interactions, and comparing toxicity in diverse cell types (140). The authors differentiated hESCs into INS^+ cells in a 384-well plate format and screened a U.S. Environmental Protection Agency (EPA) ToxCast library of ~ 2,000 compounds for "hits" that impaired survival of INS^+ cells (140). Using this study as an example, we highlight the numerous benefits of using hPSCs, whether hESCs or iPSCs, for exploring MDC toxicity.

Flexibility to Model Developing or Adult Cells

HPSCs offer a flexible model to test for MDCs that impact either the early formation of β-cells during fetal development or the function and survival of adult insulin-secreting β-like cells. Depending on the research question, we propose two different workflow approaches. For developmental studies (**Figure 1B**, Workflow #1), environmental toxin(s) can be introduced to differentiating hPSCs at critical days or "stages" of differentiation. Thus, a critical aspect of this workflow is establishing hPSC differentiation protocols within the toxicology lab conducting chemical testing. The impact of toxins can be assessed by measuring key pancreatic cell markers by flow cytometry, image-based analysis, qPCR, or other techniques that are amenable to high throughput analysis. For example, the proportion of cells expressing markers of pancreatic commitment (% $PDX1^+$), pancreatic endoderm (% $PDX1^+/NKX6.1^+$), and induction of the endocrine program (% $NEUROG3^+$) are excellent benchmarks for early stages of differentiation. At later stages, the proportion of cells that acquire insulin (% $INS^+/NKX6.1^+$) is as an indicator of commitment to the β-cell lineage, and subsequently the proportion of INS^+ cells co-expressing critical β-cell markers such as MAFA is an important indicator of β-cell maturity. Fluorescent reporter hPSC lines generated by genome editing – for example, NEUROG3-EGFP or INS-GFP hESCs – will be particularly useful for efficient image-based screening or high-content flow cytometry applications to identify MDCs that disrupt the formation of key pancreatic cell populations (141–144).

The workflow and endpoints for toxicity studies in adult cells could differ considerably from developmental studies (**Figure 1C**, Workflow #2). First, it is feasible for hPSC-derived β-like cells to be mass-produced in large quantities at a central location to generate a reproducible starting point for toxicology screening studies. Once hPSC-derived β-like cells are validated, they can be distributed to toxicology laboratories for testing of individual chemicals or complex chemical mixtures. This is important because it separates the need for toxicity testing capacity and stem cell differentiation expertise to be housed within the same lab. As with the progenitor cell model, there are numerous potential outcomes that could be assessed in a high-throughput screening platform, such as the expression of key β-cell markers using live-cell imaging or flow cytometry, β-cell survival as in Zhou et al. (140), and basal or glucose-induced insulin secretion. Any of the outcome measures described in the section on "Toxicity Testing in Pancreatic β-cells" could be assessed in hPSC-derived β-like cells, although not necessarily in a high-content format.

It is important to recognize that current differentiation protocols generate human β-like cells with a blunted insulin secretory response to glucose compared to primary human islets (134–139). For the purpose of identifying MDCs that cause β-cell dysfunction or apoptosis, we propose that generating fully mature human β-cells *in vitro* may not be a necessary milestone. Instead, the benefits of a large-scale source of expandable stem cells that can generate large quantities of moderately glucose-responsive insulin-secreting cells outweighs the downside of working with a slightly immature β-like cell. This has certainly proven true for toxicity studies in other cell types, such as cardiomyocytes, where differentiation protocols currently generate immature cardiomyocytes, but recapitulate sufficient features of adult cells to study adverse drug reactions in specific aspects of cardiotoxicity (145–148).

Scalability and Reproducibility

The scalability of hPSCs is a significant advantage for high-content screening. Importantly, hPSCs share the proliferative advantage of immortalized β-cells, but subsequently lose this capacity as they differentiate into pancreatic lineage cells (139). The highly proliferative nature of hPSCs allows them to be substantially expanded before differentiation, 50–100 fold per week, particularly when grown in suspension format (149). Large batches of hPSCs can then be differentiated into a mass-produced cell product, which can be carefully validated with well-defined QA/QC protocols before being frozen down and distributed for toxicity testing (**Figure 1C**, Workflow #2). This is similar to the model proposed by the diabetes cell therapy field for mass-production of a GMP-grade cell product for transplantation (3, 149). Alternatively, more modest scale-up approaches can be established within the same lab that will perform toxicity endpoint assessments (**Figure 1B**, Workflow #1). For example, Zhou and colleagues expanded hESCs in a more traditional adherent format with Matrigel-coated plates before seeding dissociated cells into 384-well plates for pancreatic differentiation and chemical screening (140). The incredible flexibility to both scale-up hPSC production and differentiation or to miniaturize pancreatic differentiation is an important benefit of using hPSCs for toxicity studies.

Unique Capacity for Disease Modeling

Stem cells offer remarkable capacity for disease modeling through both the natural genetic diversity of iPSCs (150) and the ability to create isogenic hPSC lines using genome editing (151). The use of hPSCs for disease modeling in diabetes has been reviewed elsewhere (152), but here, we briefly discuss the benefits of toxicity testing in human β-cells with diverse genetic backgrounds. There is much to be learned from comparing the impact of environmental contaminants on β-like cells generated using iPSCs from a spectrum of patients with different types of diabetes (T1D, T2D, maturity onset diabetes of the young (MODY), or neonatal diabetes) or known genetic risk factors for diabetes (153–160), relative to iPSCs derived from control subjects. One particularly exciting avenue to explore in the context of T1D is how environmental toxins influence immune interactions between iPSC-derived β-cells and autologous immune cells from the same donor (159). Zhou and colleagues also demonstrated the potential for using iPSCs to explore mechanisms of toxicity (140). They used 10 different iPSC lines with heterogeneous expression of a phase 2 xenobiotic metabolism enzyme, *GSTT1*, and found that pesticide-induced INS^+ cell death was significantly higher in lines lacking *GSTT1* compared to those with at least one copy of *GSTT1 (*140). Their results were also validated in isogenic hESC lines with *GSTT1* deletion by CRISPR-based genome editing; INS^+ cells generated from $GSTT1^{-/-}$ hESCs were more susceptible to pesticide-induced cell death than INS^+ cells from wildtype hESCs. Importantly, with the advent of CRISPR-Cas9 technology, modifying the genome of hPSCs has become broadly accessible and the number of gene-edited hPSC lines that effectively recapitulate different aspects of diabetes-related phenotypes is increasing rapidly (140, 151, 155, 161–164).

One final consideration for disease modeling is that despite being reprogrammed back to their embryonic/pluripotent state, iPSCs retain DNA methylation marks, lineage bias, and other memory of previous environmental exposures (165). For this reason, there is a strong argument for developmental models of pancreatic toxicity being limited to hESCs rather than iPSCs. On the other hand, the genetic variability of iPSCs, combined with the ability to create targeted genome-edited hPSC lines with isogenic wildtype controls, should be harnessed to explore the biological diversity of gene-environment interactions in adult β-like cells.

Diversity of Human Cell Types

Another unique advantage of hPSCs is their ability to be directed into diverse cell types. For example, the toxicology field is already using hPSCs to test for adverse drug reactions in iPSC-derived hepatocyte-like cells to model hepatoxicity (166–168) and iPSC-derived cardiomyocytes to model cardiotoxicity (145–147, 168–172). While our Perspective focused on the application of hPSCs for toxicity testing in pancreatic lineage cells specifically, there is

immense value in a more integrated approach to screen for MDCs that adversely impact different metabolic target tissues, all derived from the same hPSC source. For example, Zhou and colleagues differentiated hESCs into $CD29^+/CD73^+$ mesenchymal stem cells, $CTNT^+$ cardiomyocytes, $A1AT^+$ hepatocytes, and HuC/D^+ neurons (140). They found that much like hESC-derived INS^+ cells, HuC/D^+ neurons were also highly susceptible to pesticide-induced cell death, suggesting that the pesticide flagged in their high-content screening could be involved in the pathogenesis of both diabetes and Parkinson's disease. An even more complex, but intriguing application of hPSCs is the potential to develop multi-organ systems in a microfluidic device (173) or other platform containing numerous hPSC-derived metabolic tissues such as liver, adipose, and β-cells to determine how environmental contaminants influence metabolic tissue cross-talk.

CONCLUSION

Despite the critical importance of pancreatic endocrine cells for maintaining metabolic homeostasis, the pancreas has not traditionally been studied as a key target tissue of chemical toxicity. Given the metabolic-disrupting nature of many environmental pollutants, we propose that islet toxicity should be considered a key toxicological endpoint. With the staggering number of poorly studied chemicals in commerce, physiologically relevant models that can be scaled up for efficient chemical screening are urgently needed. Human stem cells offer a unique solution to many of the limitations posed by other *in vitro* model systems of pancreatic endocrine cells. Most importantly, hPSCs are scalable and amenable to high-throughput screening for assessing the impact of environmental contaminants on either adult β-like cells or critical windows of pancreas development.

AUTHOR CONTRIBUTIONS

EM and JB conceived the review topic and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Modeling Maturity Onset Diabetes of the Young in Pluripotent Stem Cells: Challenges and Achievements

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Maturity onset diabetes of the young (MODY), is a group of monogenic diabetes disorders. Rodent models for MODY do not fully recapitulate the human phenotypes, calling for models generated in human cells. Human pluripotent stem cells (hPSCs), capable of differentiation towards pancreatic cells, possess a great opportunity to model MODY disorders *in vitro*. Here, we review the models for MODY diseases in hPSCs to date and the molecular lessons learnt from them. We also discuss the limitations and challenges that these types of models are still facing.

Keywords: maturity onset diabetes of the young, human embryonic stem cells, induced pluripotent stem cells, disease modeling, genetic and epigenetic aberrations

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INTRODUCTION

Monogenic diabetes refers to a group of disorders caused by mutations in a single gene resulting in diabetes. To date, more than 40 subtypes of monogenic diabetes have been identified, most of them results in β cell loss or function impairment. In rare cases, diabetes is caused by mutations leading to insulin resistance or associated with other features of genetic syndromes affecting multiple organs. The typical classification of monogenic diabetes includes two main subgroups; neonatal diabetes, usually presenting before 6 months of age, and maturity onset diabetes of the young (MODY), usually presenting in youth and adults. While neonatal diabetic cases are rare (1 in 1,000,000 birth), MODY disorders are more common, accounting for 1–5% of all diabetic cases (1–4). The acronym MODY was first used in 1975 by Fajans and Tattersall, to distinguish a hereditary form of diabetes presented in juvenile patients from classical type 1 diabetes patients (5). Today, the term MODY is used to describe a group of clinically heterogeneous metabolic disorders that are characterized by pancreatic β cell functional impairment. The clinical features of MODY are varied and depend on the causal gene. Some of the common features of MODY include hyperglycemia, diagnosed usually in childhood or adolescence (under 25), family history (autosomal dominant inheritance) and lack of pancreatic auto-antibodies (2, 6).

MATURITY ONSET DIABETES OF THE YOUNG GENETICS AND PATHOGENESIS

Fourteen distinct subtypes of MODY have been identified to date, all caused by mutations in genes important for pancreatic β cell development, regulation, and function. Most of these genes encode for transcription factors (TFs), *i.e.*, MODY1, MODY3, MODY4, MODY5, MODY6, MODY7, and MODY9 (caused by mutations in the genes *HNF4A*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, and *PAX4*, respectively). Some of them encode for enzymes, *i.e.*, MODY2, MODY8, and MODY11 (caused by mutation in the genes *GCK*, *CEL*, and *BLK*, respectively), and some involve other pancreatic genes, *i.e.*, MODY10, MODY12, MODY13 and MODY14 (caused by mutations in *INS*, *SUR1*, *KCNJ11*, and *APPL1* genes, respectively) (7–9).

Clinical diagnosis of MODY is still suboptimal, mainly due to the variability of clinical presentations and their similarity to symptoms of other types of diabetes, leading to misdiagnosis of MODY as type 1 or type 2 diabetes (10, 11). However, with the increasing availability and price reduction of genetic tests, MODY diagnosis is rising. An accurate and timely diagnosis of MODY can dramatically affect the medical treatment given as treatment is tailored to the specific mutation. This treatment is often dramatically different from that of type 1 or type 2 diabetes (12). Precise diagnosis is also important for early identification of asymptomatic or undiagnosed family members, in order to minimize the disorder's impact on multiple organs (1).

Some of the MODY genes are specifically involved in β cells' function, while others are related to different stages of the endocrine pancreatic development. Studying the effect of perturbation in these latter genes may also help understand the developmental processes and pathogenesis of other pancreatic diseases. Furthermore, understanding the mechanisms underlying β cell formation could improve *in vitro* differentiation protocols of β cells from human stem cells, enhancing the feasibility of pancreatic islet transplantation in type 1 diabetes patients and other pancreatic disorders.

MODELING MATURITY ONSET DIABETES OF THE YOUNG

A great part of the current understanding of pancreatic β cell development and function was achieved using rodent models. However, as pancreatic development and architecture, as well as glucose response, differ substantially between mice and humans, rodent models do not always accurately represent the human phenotypes. Such are the cases of heterozygous mutations in the genes *ABCC8*, *HNF1B*, *HNF4A*, *HNF1A*, *GATA4*, and *GATA6* that cause neonatal diabetes or MODY in humans, but do not present any diabetic pathophysiology in mice (13–15) (**Figure 1**).

Human studies of diabetes mechanisms can also be done by using cadaveric islets, which are human primary islets harvested *post mortem* from pancreatic donors (15, 16),. Recent studies that used human islets from donors diagnosed with type 1 diabetes revealed mutations in genes causing monogenic diabetes, including MODY, that are the primary cause of diabetic symptoms (17, 18). This approach is limited due to high variability between islets, the short life span of the cells composing them and mainly due to low donor accessibility. The latter is especially challenging when modeling MODY, given the low prevalence and diagnosis of this disease (17, 18). Immortalized β cells, or β cell lines, are another human cell type that are used in the field of diabetes research. To date, no cell lines were established from MODY patients, calling for gene editing to model these diseases. Although CRISPR/Cas9 editing has been previously used in EndoC-BH lines (19), these lines have low clonal efficiency which makes editing at clonal level challenging (15, 20). In addition, both cadaveric islets and immortalized β cells enable the study of mature pancreatic cells and are less suitable for studying genes that have a role during pancreatic development (**Figure 1**).

This calls for suitable monogenic diabetes models that can be fulfilled by human pluripotent stem cells (hPSCs). Both induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) can be differentiated from their pluripotent state to pancreatic β-like cells. Over the last decade, such differentiation protocols were developed and improved, enabling the generation of functional human β cells (21–25). Glucose-responsive and insulin secreting β cells can be generated *in vitro*, and were even proved to reduce blood glucose levels when transplanted in Streptozotocin-induced diabetic mice. Differentiation protocols are performed in a stepwise manner, typically mimicking *in vivo* development of β cells (26). By using PSC-derived cells carrying a mutation in MODY-causing gene, one can follow the natural course of development and elucidate the relevant stage or stages that are interrupted by the mutation (**Figure 1**).

Since MODY genes vary in their function and in the developmental stages in which they are expressed and act, there may be different mechanisms underlying each of the MODY disorders. These mechanisms can be studied using PSCs.

iPSC MODELS FOR MATURITY ONSET DIABETES OF THE YOUNG

hPSC models for several MODY types were established in the past decade (**Table 1**), mainly for the manifestations caused by mutant transcription factors. The vast majority of these models were based on iPSCs. iPSCs are generated by reprogramming patients' somatic cells, harboring the mutated gene. These reprogrammed cells are capable of self-renewal and can be maintained *in vitro*. They can be further differentiated into pancreatic cells, enabling the study of the effects of particular mutations.

Such models were generated for MODY1 (27–30), MODY2 (27), MODY3 (27, 31–33), MODY4 (34, 35), MODY5 (27, 37, 38), MODY8 (27), and MODY13 (39) (**Table 1**).

These studies modeled different mutations and used different differentiation protocols, leading to complex conclusions in some cases. Nevertheless, these studies contribute to our understanding of

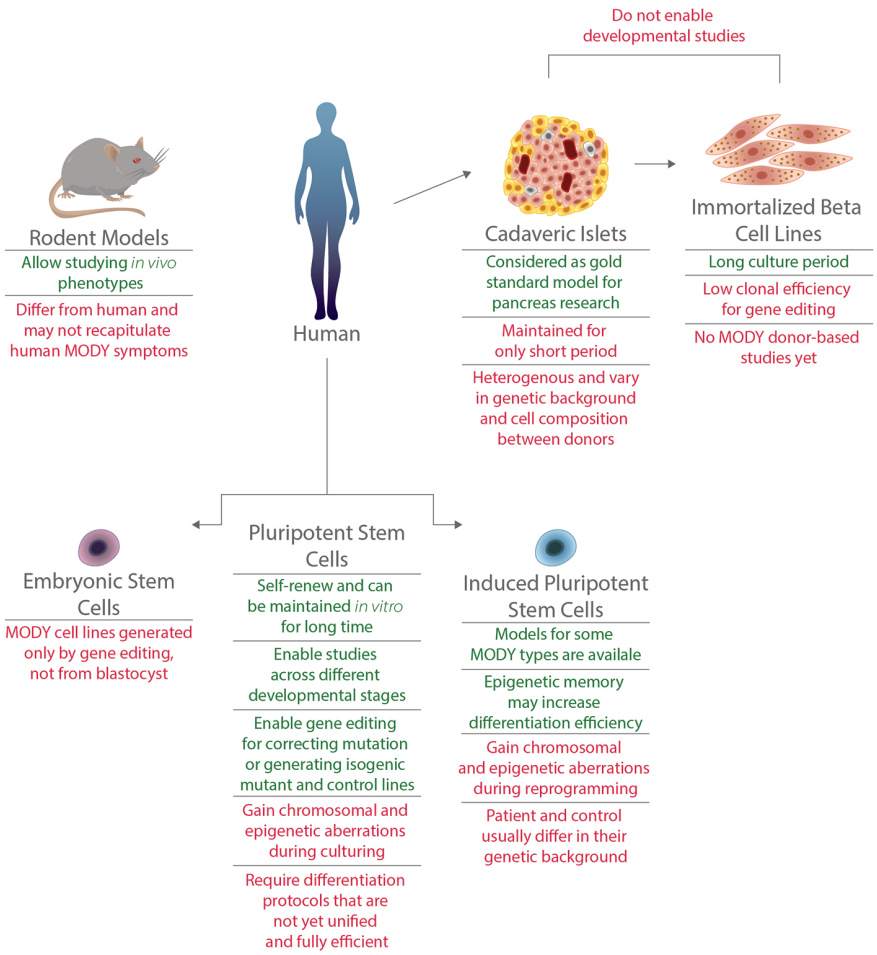

FIGURE 1 | Advantages and limitations of various models for MODY disorders. Models for MODY disorders can be generated using different *in vivo* and *in vitro* systems, such as rodents, human cadaveric islets, immortalized beta cell lines, hiPSCs and hESCs. Advantages (appear in green) and disadvantages (appear in red) for each type of model are presented.

the relations between TFs and their gene targets throughout pancreatic development and of the mechanisms underlying MODY symptoms. We will therefore focus on the analyses of MODY disorders that involve TFs.

Maturity Onset Diabetes of the Young 1

MODY1 is characterized by progressive β cell dysfunction, macrosomia, and neonatal hyperinsulinemic hypoglycemia in some of the cases. In 2017, Vethe et al. generated iPSCs from patients carrying I271fs mutation in *HNF4A* gene and differentiated them towards β-like cells (28). Comparing MODY1 iPSCs to iPSCs from a healthy member of the same family, they reported that the mutation did not alter the differentiation capacity of the cells. Mutated and control cells expressed similar levels of insulin, as well as other pancreatic proteins such as PDX1, NEUROD1 and MAFB. Comparing the proteomic landscape and function (by glucose stimulated insulin secretion (GSIS) test) of mutant and control β-like cells to adult human islet, they reported an immature state of the differentiated iPSCs.

A recent study from the same group by Ghila et al. used the same MODY1 iPSCs differentiated towards hormone producing islet-like cells, to investigate their miRNA expression profile (40). Their bioinformatic analysis revealed miRNAs that are differentially expressed between mutant and control cells. These miRNAs are composed of two subgroups, one that showed different expression during early differentiation stages (during the formation of posterior foregut) and the other was differentially expressed in later stages (after pancreatic endoderm formation). They observed alterations in miRNA networks related to *TP53* regulation in both stages. A combination of the miRNA data with transcriptomic data in MODY1 cells further highlighted an activation of *TP53* in the MODY1 cells, suggesting that mutations in *HNF4A* affect cell cycle arrest during late differentiation.

Ng et al. used iPSCs generated from a MODY1 patient carrying the same I271fs mutation. They showed that this

TABLE 1 | MODY models in hPSCs.

Type	Gene Name	Mutation Type	Type of cells	Reference
MODY1	HNF4A	p.I271fs	hiPSC	(27)
		p.I271fs	hiPSC	(28)
		p.I271fs	hiPSC	(29)
		p.Q268X	hiPSC	(30)
MODY2	GCK	V62A	hiPSC	(27)
MODY3	HNF1A	p.P291fsinsC	hiPSC	(27)
		p.R271W and p.P379fs	hiPSC	(31)
		p.S142F	hiPSC	(32)
		19/16bp δ-exon 1	hESC	(19)
		indel-exon 1	human β cell lines	(19)
		p.P291fsinsC	hiPSC	(33)
MODY4	PDX1	p.P33T	hiPSC	(34)
		p.C18R	hiPSC	(35)
		p.L36fs and p.A34fs	hESC	(36)
MODY5	HNF1B	g.1-1671del	hiPSC	(27)
		p.R177X	hiPSC	(37)
		p.S148L	hiPSC	(38)
MODY8	CEL	p.C563fsX673	hiPSC	(27)
MODY13	KCNJ11	p.E227K	hiPSC	(39)

mutation has haploinsufficiency, rather than a dominant negative mode of action, and that it leads to cytoplasmic mislocalization of the HNF4A TF (29). In order to assess the mutation effect on the developmental process, they focused on the hepatopancreatic foregut endoderm stage, an early stage of pancreatic differentiation. RNA sequencing (RNAseq) data of MODY1 cells showed an upregulation of hindgut HOX genes in parallel to downregulation of a set of pancreatic and liver genes including *GATA4*, *HNF1B*, and *PDX1*. Together, these results suggested that HNF4A has a role in hindgut repression. Downregulation of hepatic genes was also detected in further differentiated cells towards hepatocytes, while a significant reduction in *HNF1A* was detected in differentiated pancreatic β cells.

Braverman-Gross et al. generated MODY1 iPSCs from patients carrying a Q268X mutation in the *HNF4A* gene [previously shown to cause haploinsufficiency (41),]. The authors differentiated the cells towards pancreatic progenitors and exhibited an upregulation of some pancreatic markers, such as *PAX6*, *NEUROD1* and *NEOUROG3* in the MODY1 cells, indicating a possible partial compensatory mechanism for the mutation (30). RNAseq data of MODY1 cells from the primitive gut tube stage of differentiation revealed subgroups of putative HNF4A gene targets. Targets with less HNF4A binding sites and more sites for other TFs in their promotors were less affected by HNF4A haploinsufficiency, further suggesting a redundancy mechanism for the mutation.

Maturity Onset Diabetes of the Young 3

MODY3, caused by mutations in another TF, HNF1A, is characterized by gradual β cell dysfunction and progressive hyperglycemia. In 2015, Stepniewsky et al. generated two MODY3 iPSC lines derived from patients carrying either R271W or P379fs mutations in *HNF1A* (31). They then differentiated the cells towards hormone producing cells. Even though the differentiation capacity was rather poor and resulted in immature polyhormonal cells with low percentage of insulin-positive cells, they showed that the MODY3 cells had a similar capacity to differentiate as iPSCs generated from healthy individuals (31).

Yabe et al. generated MODY3 iPSCs carrying the most common mutation in *HNF1A* gene, P291fsinsC (33). They differentiated the iPSCs to pancreatic cells, and showed that the mutated RNA is abolished by nonsense mediate decay (NMD), consequently leading to minimal expression of the mutant protein, indicating haploinsufficiency mechanism for this MODY3 mutation as well.

Maturity Onset Diabetes of the Young 4

MODY4 is caused by mutated PDX1 TF, which usually results in defective insulin secretion, while homozygous mutations in this gene also lead to pancreatic agenesis. In 2016, Wang et al. generated two iPSC lines carrying heterozygous P33T and C18R mutations in *PDX1* gene (34, 35). By differentiating the cells towards pancreatic endoderm, they exhibited no difference in PDX1 expression and early pancreatic differentiation between mutant and control cells (42). At later stages, β-like cells derived from MODY4 iPSCs had low insulin expression and reduced GSIS. Using CRISPR/Cas9 gene editing, they also induced the same mutations in homozygous state, as well as heterozygous frame shift mutation in control iPSCs. The mutated cells showed impairment in differentiation towards pancreatic precursors by reduced C-peptide expression and reduced GSIS. In addition, they showed reduction of transcription levels in pancreatic genes including *NEUROD1*, *ISL1*, and *INS*, which were previously shown to be direct PDX1 targets (43).

Maturity Onset Diabetes of the Young 5

MODY5, caused by mutations in HNF1B TF, is characterized by reduced insulin secretion and renal cysts in some patients. Yabe et al. generated MODY5 iPSCs from a patient carrying R177X mutation in *HNF1B* (37). As in the case of their MODY3 study, they showed that NMD destruction of the mutant RNA is taking place, hinting for haploinsufficiency of HNF1B as the underlying cause of MODY5. Teo et al. generated iPSCs from MODY5 patients, carrying a different mutation, S148L (38). Differentiated MODY5 cells showed upregulation of definitive endoderm and early pancreatic genes, including *SOX17*, *FOXA2*, *GATA4*, *GATA6* and *PDX1*, as well as *HNF1B* itself, suggesting a compensatory gene expression circuit. Using luciferase assays they also showed that the increase in *PDX1* expression was directly related to the S148L mutant allele. The only pancreatic gene that was downregulated in the MODY5 cells was *PAX6*. It was hypothesized that this occurred by an indirect regulation, as *PAX6* promoter was not found to bind HNF1B. This downregulation could explain some of the diabetic symptoms of MODY5 patients.

EMBRYONIC STEM CELL MODELS FOR MATURITY ONSET DIABETES OF THE YOUNG

The scarcity of MODY patients and tissues donors, as well as inherent disadvantages of iPSC-based disease models, as discussed below, call for additional *in vitro* MODY models. One of the promising models currently being used are based on targeted gene editing, which became much simpler and prevalent with the introduction of the CRISPR/Cas9 methodology. Some groups recently combined gene editing of human embryonic stem cells together with pancreatic differentiation, to study the effects of MODY-related gene disruption (**Table 1**).

In 2016, Zhu et al. used the HUES8 ESC line to generate 62 ESC sublines carrying mutations in eight different pancreatic TFs, in order to study their role in poly hormonal β-like cell differentiation. One of these genes was *PDX1*, the gene causing MODY4 disease, for which they created two homozygous lines carrying biallelic frame shift mutation, L36fs and A34fs (36). ESCs carrying the same heterozygous monoallelic mutations showed a reduction in PDX1 expression compared to WT cells, indicating haploinsufficiency mechanism for these mutants, leading to decrease in endocrine hormone gene expression (*INS*, *SST*, *GCG*, *GHRL*) in differentiated cells.

Cardenas-Diaz et al. generated HNF1A homozygous and heterozygous deletion mutations in MEL1 and H1 ESC lines (19). They differentiated the cells and showed effect of the mutation in the β-like cells' stage, where they observed a reduction in the expression of pancreatic TFs including *PDX1*, *RFX6*, *HNF4A* and *PAX4*. Mutant cells showed an increase in *ARX* gene levels, accompanied by higher GCG levels and lower *INS* expression in the mutant cells. These results indicated a role for *HNF1*A in inhibiting α cell development. Mutations in *HNF1A* also impaired the β-like cells GSIS, and cell with heterozygous mutations showed decreased mitochondrial respiration. In addition, they revealed a downregulation in the heterozygous cells of *LINKA*, a primate-specific lncRNA, and suggested it has a role as a mediator of *HNF1A* regulation for subset of *HNF1A* targets, specifically related to mitochondrial respiration and pancreatic genes expression.

LESSONS LEARNED FROM MATURITY ONSET DIABETES OF THE YOUNG MODELS IN PSCs

The diverse studies discussed above shed light on roles of TFs during the different stages of pancreatic development. Some of these TFs may have roles in a specific time point of the differentiation. Focusing on that exact step may be crucial to understanding the effects of a gene mutation. A mutation in *PDX1*, for example, was shown to affect the cells only at the terminal stage of differentiation (42). In contrast, mutations in *HNF4A* were found to alter foregut formation (29) and affect posterior foregut genes (30). Although insulin expression was reduced in mutant *HNF4A* pancreatic β-like cells in one study (29), in another, similar cells did not functionally differ from control cells (28). It is possible that compensatory mechanisms overcome the mutation and enable the mutant cells to fully or partially differentiate towards β-like cells. Such compensatory mechanism was shown for cells carrying mutations in *HNF1B*, where the mutant protein caused a possible direct upregulation of *HNF1B* itself and of *HLXB9*, and indirect upregulation of *PDX1* and other early pancreatic genes (38). HNF genes, including *HNF4A*, *HNF1A* and *HNF1B* are known to cross-regulate each other during pancreas development (44), and may cause the upregulation of shared targets when one of them is mutated. It is also likely that the variability of differentiation protocols used in different studies influence the final outcome (45)—highlighting the need for a uniform, efficient differentiation protocol, from the pluripotent stage to β cells.

Within each of the 14 known genes causing MODY, various mutations have been identified. The vast majority of MODY mutations are heterozygous, however, in some of the MODY genes a biallelic mutation may occur, usually leading to a more severe phenotype. Homozygous mutations in GCG, PDX1, ABCC8, and KCNJ11 cause permanent or transient neonatal diabetes, as well as pancreatic agenesis (as is the case in PDX1) or neurological abnormalities (in NEUROD1) (6, 9). MODY models in iPSCs were only generated from heterozygous patients, but gene editing using CRISPR/Cas9 studies generated homozygous cells that showed some differences from their heterozygous parallels. For example, cells homozygous for the P33T mutation in *PDX1* gene showed impairment in pancreatic differentiation to a much higher extent than the heterozygous cells (42). HNF1A homozygous mutants showed lower insulin levels than the heterozygous upon differentiation while heterozygous mutation affected the cellular respiration more than the homozygous knock-out mutation (19).

Since MODY disorders are typically characterized by heterozygous mutations, and symptoms of patients with homozygous mutations are distinct, it is hypothesized that a dosage dependent mechanism underlies MODY genes' mode of action. One such dosage dependent model is haploinsufficiency (29, 33, 36, 37, 41). Thus, in a patient with heterozygous mutation, only one allele forms the gene product which is not enough for the normal function of the protein. The mechanisms underlying haploinsufficiency are varied and include threshold requirement of protein amount (*i.e.* in the case of TF that has to bind multiple sites of the DNA), or imbalanced stoichiometry of multi subunits complexes (46). Understanding the exact mechanism of action is important for future therapeutic purposes. While some haploinsufficiency genes are dosage sensitive and their upregulation may be toxic (46, 47), in other cases, activation of the normal allele transcription may rescue the haploinsufficiency disease symptoms (48).

MODY symptoms are affected not only from the mono or biallelic expression of a mutation, but also from the specific type of mutation, even within the same gene. Hence, specific mutations in a gene can cause MODY, while other mutations in the same gene can cause other types of disease. Permanent neonatal diabetes can be caused by mutations in *INS* and *KCNJ11*

genes, while other mutations in these genes will cause MODY10 and MODY13, respectively, with symptoms presenting only later in life; congenital hyperinsulinism can be caused by mutations in *ABCC8*, *KCNJ11*, *GCK*, *HNF1A*, and *HNF4A* genes, the same genes that, when harboring different mutations, cause MODY12, MODY13, MODY2, MODY3, and MODY1, respectively (9, 49, 50). Even within patients with the same type of MODY, symptoms and cellular gene profiling may be different due to different types of mutations. For example, cells carrying P33T mutation in *PDX1* show lower levels of PDX1, NKX6.1 and C-peptide than cells carrying C18R mutation in the same gene (42).

Although generating a specific heterozygous mutation may be technically challenging (51), these observations highlight the need to model the exact type and location of a mutation, especially when using gene editing.

CHALLENGES OF PSC BASED MATURITY ONSET DIABETES OF THE YOUNG MODELING

Using hPSCs for disease modeling, specifically monogenic developmental diseases such as MODY, have great benefits (52). In the case of MODY, one of the most important reasons to use hPSCs is the lack of appropriate animal models that recapitulate the human diabetic phenotypes (13). Furthermore, using hPSCs enables studying the complex pancreatic developmental process and assessing the effects of different mutations on its progress. This cannot be achieved using adult, differentiated tissues. However, when modeling a disease using hPSC, there are several issues one should consider (**Figure 1**).

Genomic Aberrations

Since hPSCs are cultured and passaged *in vitro*, and iPSCs are going through the clonal process of reprogramming; they are prone to gain genomic aberrations. Such aberrations include large chromosomal aberrations, sub-chromosomal aberrations, copy number variations (CNVs), and point mutations (53, 54). These changes in genome content may affect the differentiation performance and global gene expression (55).

Although a normal karyotype is validated in all MODY iPSC models, usually by Giemsa band staining, in order to avoid clones containing large chromosomal gains or deletions, smaller mutations are more difficult to identify. Of note, it was found that hPSCs bear CNV hot spots, mainly on chromosomes 1, 12, 17q, 20q, and X (56). This finding may be crucial when modeling genes located within these genomic loci. These genes include *HNF4A*, located on chromosome 20q13, *HNF1A* on chromosome 12q24, and *HNF1B* on chromosome 17q12.

Recent studies showed that some hPSC lines harbor point mutations mainly in *TP53*, coding for the tumor suppressor p53 (57). p53 is a master regulator of apoptosis, cell cycle, and proliferation and was recently suggested to be involved in cell cycle arrest of MODY4 cells (40). Since *TP53* mutations probably provide the cells with selective advantages and since these mutations remain and expand in the culture during pancreatic differentiation (57), they could affect the phenotypes observed in the models they are derived from and their interpretation. These mutations should thus be tightly monitored when using hPSC for disease modeling.

Epigenetic Aberrations

Another type of aberrations affecting hPSCs is epigenetic aberrations, including DNA methylation alterations, loss of parental imprinting, and variation in X chromosome inactivation (58). Specific genomic regions are prone to acquire hypermethylation leading to gene silencing during *in vitro* culturing and during reprogramming. These may affect the differentiation capacity of the cells, as was shown for *TSPYL5*, which was recently found to cause aberrant differentiation of cells upon its silencing (59). Various methylation aberrations were shown to originate from the reprogramming process of iPSCs, altering several genes in different cell lines (60). These aberrations, however, were suggested to diminish over time and passaging (61). Other methylation aberrations, usually corelated with cancerous mutations, provide growth advantage and are selected for during culturing (58).

Parental imprinted genes are genes that are expressed from a single allele, while the other allele is methylated and silenced depending on the parental origin. Wang et al. (43) found a decrease in PDX1 targets upon its haploinsufficiency in pancreatic progenitors differentiated cells (43). Two of these genes were *NNAT* and *MEG3*, paternally and maternally imprinted genes, respectively, which are related to insulin synthesis and secretion. This finding is based on comparing isogenic control iPSCs and mutant generated *via* CRISPR editing (43). However, many of the MODY studies are based on mutant and control iPSCs from different genetic and epigenetic backgrounds, such as diseased and healthy family members. In these cases, imprinted genes may be influenced by loss of imprinting aberrations, that consequently cause an expression upregulation through the biallelic expression (62).

Another epigenetic aspect relates to MODY, and a diabetes model is the epigenetic memory affecting differentiation capacity. It was previously shown that hESC and iPSC can be differentiated towards β-like cells in similar efficiencies (21) and that the majority of differences between lines in that context were related to the genetic background of the hPSC donor (63). iPSCs derived from specific somatic cells were thought to differentiate better towards the same cell-type of origin. Specifically, iPSCs generated from human pancreatic β cells were shown to differentiate better than ESC or iPSC derived from fibroblasts towards pancreatic progenitor cells (64). It was proposed that this observation originates from the open chromatin sites in the β cell-derived iPSCs, that were found in genomic regulatory regions related to endoderm and pancreatic islets' development, indicating an epigenetic memory of iPSCs (65, 66). This feature of iPSCs can be beneficial when modeling MODY, since β cells are usually formed and function in patients (6).

Gene Editing

The high rates of point mutations and genetic aberration occurrence in hPSCs and their clonal mode of enrichment, together with differences in differentiation capacity between cell lines, highlight the need for carefully supervised controls.

Many of the MODY studies discussed above used non-isogenic cells as control to the mutated cells, which may affect their conclusions. The introduction of gene editing and, specifically, the CRISPR/Cas9 methodology enables the formation of isogenic lines, with the same genetic background, enabling the study of the exact effect of a mutant gene. This can be done by either introducing a mutation to a healthy control background, or by correcting a mutation in iPSCs generated from patients. MODY models that utilized CRISPR editing used the former option, and moreover, created both monoallelic and biallelic mutations to study dosage dependence effect of the mutated gene (19, 36, 42).

Though the CRISPR system enables many promising applications (20), it has some limitations that should be considered. While the immune response and obstacles in vector delivery are the main pitfalls for its *in vivo* use in regenerative medicine, the off-target and low efficiency are the main disadvantages for *in vitro* disease modeling approaches (67). Off-targets are genomic regions that may be mutated, undesirably, when using Cas9, especially when it is constitutively expressed. It was also suggested that CRISPR editing in hPSCs is sub-optimal in the sense of efficiency, possibly due to TP53-mediated cell cycle arrest and apoptosis which are induced by the Cas9. These obstacles may be tackled by predicting the off-targets, restricting the Cas9 expression and using improved CRISPR protocols (67).

β Cell Differentiation

MODY modeling requires the differentiation of mutant ESCs or iPSCs towards pancreatic cells. As mentioned above, differentiation protocols are diverse and the differentiation capacity varies between cell lines because of genetic and epigenetic background. Since different studies are based on different differentiation protocols, and use different types of cells (and in the case of iPSCs—different reprogramming protocols), the final conclusions may be affected and even cause discrepancies between studies (28, 29). Current protocols generate β-like cells, which are not fully mature *in vitro*, thus limiting the study of MODY mutations' effect on the final stages of maturation and function of mature β cells. During recent years, several groups generated hESCs and iPSCs derived islet-like organoid. Such organoids containing MODY mutations are yet to be done. They could contribute to the study of these mutations' effect on pancreatic cells other than β cells, for example the impact of *HNF1A* mutation on α cells (19) or even on the development of the islet as a whole (13, 26, 45, 49).

CONCLUSIONS

In this review, we discussed the great power, as well as the limitations, of using hPSCs for modeling the monogenic diabetes MODY group of disorders. MODY are relatively rare diseases, underdiagnosed with multiple existing subtypes—modeling all MODY types is an ongoing quest. Indeed, nowadays, hPSCs models were generated only for a few MODY types (**Table 1**). Models generated so far contributed to our knowledge of essential pancreatic TF mode of action, the relation between them, and the mechanisms that cause the disease when those are mutated. These models are essential to studying human pancreatic development.

The field of pancreatic developmental disease modeling in hPSCs is still facing challenges, including the requirement for an improved robust and unified differentiation protocol for the generation of mature functional β-like cells *in vitro*. Modeling disease using hPSCs requires tight monitoring of genetic and epigenetic aberrations that may be acquired during reprogramming, culturing, and gene editing (**Figure 1**). However, the great improvement of gene editing technology as well as the increase in clinical identification of MODY patients will further promote MODY research thus, helping better understanding of human pancreatic development and offering new treatment options for patients.

AUTHOR CONTRIBUTIONS

CB-G wrote the manuscript with support and supervision from NB. All authors contributed to the article and approved the submitted version.

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Harnessing Proliferation for the Expansion of Stem Cell-Derived Pancreatic Cells: Advantages and Limitations

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Restoring the number of glucose-responsive β-cells in patients living with diabetes is critical for achieving normoglycemia since functional β-cells are lost during the progression of both type 1 and 2 diabetes. Stem cell-derived β-cell replacement therapies offer an unprecedented opportunity to replace the lost β-cell mass, yet differentiation efficiencies and the final yield of insulin-expressing β-like cells are low when using established protocols. Driving cellular proliferation at targeted points during stem cell-derived pancreatic progenitor to β-like cell differentiation can serve as unique means to expand the final cell therapeutic product needed to restore insulin levels. Numerous studies have examined the effects of β-cell replication upon functionality, using primary islets *in vitro* and mouse models *in vivo*, yet studies that focus on proliferation in stem cell-derived pancreatic models are only just emerging in the field. This mini review will discuss the current literature on cell proliferation in pancreatic cells, with a focus on the proliferative state of stem cell-derived pancreatic progenitors and β-like cells during their differentiation and maturation. The benefits of inducing proliferation to increase the final number of β-like cells will be compared against limitations associated with driving replication, such as the blunted capacity of proliferating β-like cells to maintain optimal β-cell function. Potential strategies that may bypass the challenges induced by the up-regulation of cell cycle-associated factors during β-cell differentiation will be proposed.

Keywords: human pluripotent stem cell, beta cell, proliferation, islet, diabetes, in vitro differentiation

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INTRODUCTION

Major advances in stem cell differentiation protocols for β-cell commitment have taken place throughout the past decade. Early reports that demonstrated the production of insulin$^+$ cells using *in vitro* differentiation protocols found that these cells were polyhormonal and did not form monohormonal β-cells *in vivo* (1–3). Subsequent studies have established that tightly-regulated sequential steps in differentiation that mimic human fetal islet development are required and that NKX6-1 expression is essential to generate pancreatic progenitors (PPs) that form monohormonal cells expressing both C-peptide and NKX6-1, which will be referred to as β-like cells in this review

(4–9). Stem cell-derived β-like cells demonstrate similar, yet not identical, characteristics to primary adult β-cells, including limited glucose-responsive insulin release, calcium dynamics, and mitochondria-driven oxidative phosphorylation (5, 8, 10–12). A defining feature of primary β-cells is their consistently low proliferation rate throughout adulthood, and the guided differentiation of human pluripotent stem cells (hPSCs) towards monohormonal β-like cells mimics this loss of replication, limiting the final number of cells available for further *in vitro* characterization or *in vivo* transplantation analyses. Promoting cell proliferation *in vitro* to generate higher yields of β-like cells can serve as a strategy to create a sufficient number of β-like cells for the reversal of hyperglycemia in diabetic patients. The following report will review current literature on the proliferation capacity of β-cells from primary human islets and in hPSCs differentiated to PPs and β-like cells. The balance between replication and maturation in stem cell-derived β-like cells and methods to increase total β-like cell yield for their potential application in therapeutics will be discussed.

CURRENT EVIDENCE OF HUMAN β-CELL PROLIFERATION

Replication During Fetal Development

β-cell proliferation rates during human islet development have been observed in multiple reports, but the limited availability of human fetal pancreatic tissue samples prevents the level of examination established with rodent models. What is evident from the current information available is that fetal pancreatic samples have comparatively high rates of replication in insulin-expressing cells when assessed against adult tissue samples. β-cell replication has been reported to be retained at ~3% from 10–23 weeks of gestational age in fetal pancreata, but is typically reduced to less than 1% in children less than 2 years old, although high variation in the percentage of proliferating β-cells has been noted during the first year of infancy (up to 5.28% KI67$^+$ β-cells) (13–18). Early stage (7.5 to 9.5 weeks of gestational age) human fetal pancreata transplanted under the kidney capsule of SCID mice demonstrated increased proliferation in progenitor PDX1 pancreatic cells and in early insulin$^+$ endocrine cells, but not in NGN3$^+$ endocrine-committed cells, which may suggest that the maturation status of endocrine cells is inversely related with cell replication (19). Further assessment of the correlation between proliferating insulin-expressing cells and their expression of maturation markers is required to conclude this.

Replication in Mature β-Cells

Low replication is found throughout all endocrine cell types of the adult islet, with approximately 0.38% of islet cells replicating in adults at one time (17). The human β-cell has been reported to have low (less than 0.5%) to no replication occurring under physiological conditions (17, 20). However, a recent report identified subsets of β-cells from donors that are highly proliferative (21). KI67$^+$ β-cells expressed distinct cell surface markers (CD9, CD44, CD49F, PDGFRA) and up-regulated signaling through ERK1/2, STAT3, and STAT5, potentially providing novel markers for identifying proliferating β-cells. Differences in β-cell replication rates within the developing and mature islet are likely due to the maturation status of cells. Fetal β-cells demonstrate a functionally immature phenotype when compared to adult cells and favour cell proliferation over glucose metabolism (10, 22, 23). The changes in fetal versus adult β-cell replication may also be driven by differing mechanisms of shared pathways. For example, activation of Glucagon-Like Peptide 1 Receptor (GLP-1R), which classically potentiates insulin release, also drives proliferation in adolescent, but not adult, β-cells through calcineurin -regulated transcription of cell cycle-associated genes (24). Further examination of developing β-cells can therefore reveal unique systems for driving replication.

β-Cell Proliferation in Diabetes and in Pregnancy

Reports on β-cell replication in patients with diabetes have contrasting findings. Islets from patients with type 1 diabetes do not display increased β-cell proliferation when compared to proliferation in control patient β-cells (16). However, patients with "recent onset" of type 1 diabetes, defined as less than 18 months since diagnosis, demonstrated a 10 times higher rate of β-cell replication compared to control patient β-cells, and this was not observed in patients with long-term type 1 diabetes or in patients with type 2 diabetes (25). This study found that islets from donors with recent onset type 1 diabetes, presenting with insulitis, had higher replication than islets without insulitis, suggesting that inflammation may drive proliferation during this short time frame. β-cells from patients with type 2 diabetes demonstrate unchanged proliferation when compared to control patients, yet proliferation is up-regulated in nondiabetic obese patients (26). This may mark a compensatory mechanism associated with balancing potential obesity stresses present in the period before gradual β-cell failure and apoptosis in type 2 islets. Recent reporting also suggests that human islets undergo adaptive mechanisms under pregnancy to increase the β-cell compartment, and that cadaveric sections from pregnant patients at later gestational ages (32–40 weeks) had increased β-cell proliferation when compared to β-cells from control patients (27). Insulin$^+$ staining near duct cells has been found in obese and pregnant patients and suggests a potential target for β-cell neogenesis, but examining proliferation or neogenesis in humans is limited due to their low detection and the limited availability of samples (18, 27–29). However, it does remain that inducing β-cell expansion can serve as a therapeutic for insulin restoration in patients with β-cell dysfunction.

Comparison of β-Cell Proliferation Between Rodents and Humans

Though rodent models of diabetes are often used to better understand the *in vivo* function of β-cells, evidence in the literature suggests that human β-cells do not demonstrate the same range of proliferation. The application of multiple techniques to induce proliferation in sorted primary β-cells *in vitro*—such as extracellular matrix supplementation and growth factor treatment—revealed that these techniques readily drove rodent β-cell replication but not human replication (30).

Screening primary human β-cells for mitogens that had been previously identified to initiate proliferation in rodent β-cells revealed that many factors were unable to initiate significant replication, with the dual-specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A)-inhibiting compound harmine being the only mitogen that induced significantly increased replication with treatment (31). Indeed, harmine is unique in that it has been repeatedly reported in the literature to induce β-cell and other pancreatic cell replication (32–37). Differing effects on β-cell replication through shared signaling mechanisms between rodent and human β-cells may be due to additional cellular factors that inhibit proliferation, such as the high accumulation of p16 in adult human β-cells (38–40). Thus, careful interpretation of factors that induce rodent β-cell expansion should be taken when examining human β-cells.

PROLIFERATION RATES DURING hPSC DIFFERENTIATION TO β-LIKE CELLS

Pancreatic and Endocrine Progenitor Replication

Sources of hPSCs, such as embryonic or induced pluripotent stem cells, maintain the unique characteristic of self-renewal prior to lineage-specific differentiation. In order to give rise to the monohormonal β-like cell type that resembles primary β-cells, hPSCs are differentiated to pancreatic progenitor cells that demonstrate co-expression of transcription factors PDX1 and NKX6-1. These cells contain differentiation capacity towards all major pancreatic cell lineages (exocrine, ductal, and endocrine) and bypass the polyhormonal cell fate observed in C-peptide-expressing cells that have no detectable NKX6-1 (41–44). A significant portion of end-stage pancreatic progenitor cells have been found to still contain the replication marker KI67 (~30%–50%) (5, 45, 46). Although KI67 expression is present, pancreatic progenitors demonstrate up-regulation of the cyclin dependent kinase (CDK) inhibitors p21 and p16, suggesting that these cells may be exiting the cell cycle (47). The specification and proliferative rates of pancreatic progenitors can be affected by factors controlling differentiation. YAP, a factor required for Hippo signaling, has been shown to decrease during the differentiation from posterior foregut-like cells (PDX1/NKX6-1$^-$) towards PDX1/NKX6-1$^+$ pancreatic progenitors (48). However, chemical inhibition of YAP signaling during this developmental stage decreased both the percentage of PDX1/NKX6-1$^+$ cells and of KI67$^+$ cells, demonstrating that proper commitment towards pancreatic progenitors is also accompanied by proliferation of this compartment (48).

As pancreatic progenitors proceed to differentiate towards more specified endocrine progenitors, characterized by Chromogranin A$^+$/NKX6-1$^+$ expression, the expression of proliferation markers continues to decrease (47). In the same study that examined the effects of YAP signaling modification on pancreatic progenitor commitment and proliferation, it was found that promoting YAP activity during endocrine progenitor commitment through to β-like cell differentiation led to increased proliferation in cultured cells (48). However, this reduced the final percentage of monohormonal β-like cells, while inhibiting YAP during these stages increased the percentage of monohormonal β-like cells. The authors of this study tested the CDK inhibitor roscovitine during endocrine progenitor differentiation and found that endocrine progenitor commitment was not affected by CDK inhibition (48). In contrast, disrupting the cell cycle using the compound aphidicolin throughout endocrine progenitor and β-like cell commitment, which arrested endocrine progenitor cells at G1 and inhibited the completion of S phase, improved the differentiation of endocrine progenitor cells to β-like cells, but this effect was not seen in the same degree with CDK inhibition alone (49). This may suggest that compounds that disrupt cell cycle progression in endocrine progenitors enhance differentiation to end-stage β-like cells.

β-Like Cell Replication

At the stage where monohormonal hPSC-derived β-like cells emerge, only a small subset of end-stage β-like cells are actively replicating (~1%), as demonstrated using common markers of proliferation such as KI67 or 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays (5, 32, 48, 50, 51). As mentioned in the preceding section, modifications to the proliferation of pancreatic or endocrine progenitors can impact the final population of β-like cells, which demonstrates a balance that must be maintained between successful β-like cell production and progenitor cell expansion. Although low proliferation rates in β-like cells follows the events seen during primary β-cell development and maturation, it limits the expansion of hPSC-derived β-like cells for further *in vitro* analyses and *in vivo* transplantation studies. For this reason, the search for methods to target β-like cell-specific replication *in vitro* is under continuous investigation. A notable report from the Melton group demonstrated that the leukemia inhibitory factor (LIF) drove proliferation in β-like cells expressing the corresponding receptor (LIFR). Activation of this pathway induced the expansion of β-like cells *in vitro* and was also able to enhance harmine-induced proliferation in treated β-like cells (32). To improve specificity and minimize the off-target effects from small molecule treatments, a recent study developed a zinc-binding prodrug for the deployment of compounds selectively in zinc-rich β-like cells. The study utilized β-like cells in an *in vitro* 3D platform to screen for molecules that targeted β-like cell replication. This novel platform allowed for the development of a harmine-carrying zinc-binding prodrug that was more effective than harmine alone at expanding β-like cells (37). Therefore, further development of compounds that induce targeted proliferation provide promising therapeutic avenues for both inducing and regulating β-like cell replication.

CAVEATS LINKED TO PROMOTING PROLIFERATION DURING hPSC DIFFERENTIATION TO β-LIKE CELLS

Inducible control over replication during the differentiation of hPSC-derived β-like cells is a potent strategy for restoring the number of β-cells for patients that have insufficient β-cell mass.

Before this can be pursued for basic research and potential clinical use, all challenges associated with cell cycle manipulation must be addressed.

A general issue that arises with the use of stem cell-derived cell sources is ensuring that proliferative capacity within end-stage β-like cells is tightly regulated to avoid uncontrolled cell growth. While cellular outgrowths have been occasionally detected following transplantation of cultures containing pancreatic progenitors (3, 7), endocrine progenitors (10), or end-stage cultures that failed to commit to the endocrine lineages (52), no outgrowths have been reported from transplantation of end-stage populations sorted for insulin-expressing cells (10). These findings from insulin-purified cell sorts are not surprising as human embryonic stem cell (hESC)-derived endocrine cells are typically post-mitotic, and there has been evidence that expanded pancreatic progenitors do not induce outgrowth when transplanted to mice (44). However, if proliferation is induced within β-like cells during *in vitro* differentiation, blocking proliferation prior to transplantation would become necessary to eliminate the risk of outgrowths.

The induction of proliferation during hPSC pancreatic differentiation to expand a transplantable population of β-like cells is challenged by reports that have documented inhibited commitment and maturation of pancreatic progenitors to end-stage β-like cells. Attempts made at expanding the pancreatic progenitor population have found that PDX1 cells generated do not necessarily express NKX6-1 and that pancreatic marker expression varied with passage (44, 46). The inverse relationship between proliferation and functional maturation of β-like cells was further supported in a recent study examining the cell surface CD9 marker (52). Depleting the stem cell-derived population of $CD9^+$ β-like cells removed the population of immature β-like cells, leaving cells that demonstrated higher expression of genes associated with β-cell maturity and insulin secretion. This study also established that human fetal pancreatic C-$peptide^+$/$KI67^+$ cells expressed higher frequencies of $CD9^+$ compared to the C-$peptide^+$/$KI67^-$ cells, confirming the higher proliferative status of the $CD9^+$ fraction *in vivo*. As mentioned previously, recent findings from the Melton lab identified YAP as one factor that could drive proliferation of β-like cells while reducing their maturation when up-regulated during their differentiation (32, 48). This group had also identified that WNT signaling was found in replicating epithelial progenitor cells and is down-regulated in mature endocrine-like cells (53). The TGF-β receptor inhibitor SB431542 has been shown to induce replication in adult human β-cells (54–56). However, SMAD2/3 inhibition through TGF-β can be detrimental in inducing monohormonal β-like cells when applied during pancreatic progenitor differentiation, instead leading towards early lineage polyhormonal endocrine cells that give rise to glucagon-expressing cells (1, 42). Prolonged TGF-β inhibition through ALK5 can also reduce β-like cell glucose-responsiveness when extended beyond endocrine progenitor commitment during β-like cell differentiation (12). Thus, exit from the cell cycle appears to enhance stem cell-derived cultures towards mature β-like cells *in vitro*. The inverse relationship between the maturation and proliferation of β-like cells can be further supported when looking at studies from primary β-cells in human and rodent islets. Signaling pathways such as NOTCH and mTOR have all demonstrated up-regulation in proliferating β-cells at the cost of their functionality (57–59). These findings present a challenge for successfully promoting proliferation in mature β-like cells since there are valid concerns with proliferation inducing characteristics of immature β-like cells and promoting unregulated expansion of transplanted grafts.

POTENTIAL STRATEGIES AND FUTURE DIRECTIONS

The end goal of stem cell-derived β-like cell research is to develop a transplantable system of cells that fully replicate β-cell function. Ideal tactics to achieve this would be to optimize current differentiation protocols, such as through manual purification of mature populations or treatments that increase final β-like cells (10, 12, 41, 52, 60, 61), and to improve *in vivo* site support for transplanted β-like cells (62–64). Despite the limitations listed above, expanding β-like cells or their progenitors *in vitro* still presents a promising strategy to further support these approaches. The final yield of end-stage β-like cells generated using current hPSC-based differentiation protocols is relatively poor and time-consuming, taking 23 days to 1 month to reach β-like cells (5, 10, 12, 53, 60, 65). Therefore, identifying strategies that promote controlled pancreas-specified cell proliferation *in vitro* could facilitate the manufacturing processes necessary to move forward with translational options.

Previous studies have identified methods to expand definitive endoderm, foregut endoderm, and pancreatic progenitor populations (**Figure 1**) (44, 46, 66, 67). While these approaches demonstrated the feasibility to induce self-renewal without impacting the developmental potential of hPSC-derived cells, these methods proved difficult to scale up. As a result of this, the generation of pancreatic cells for clinical purposes focuses on the expansion of undifferentiated hESCs, which can be grown and expanded in large bioreactors in the absence of special matrices or stromal cells (8, 68, 69). However, this approach is far from perfect, as a combination of proliferative and apoptotic events occurring during the differentiation process lead to a net outcome of 1 β-like cell for every 2 hESCs seeded (5). Therefore, being able to prevent these losses or expand cells at specific developmental stages will be key to increasing the β-cell yield. Strategies such as throughput screening, previously used to establish proliferative factors in primary β-cells, can be adapted for hESC-derived endocrine progenitor and β-like cell screening (31, 35, 70). With this tool, it is possible to screen for chemical or genetic regulators that provide control over proliferation in pancreatic cells. In addition to using compound-based treatments previously mentioned, such as harmine (34, 35, 37), another method for inducing replication could be through viral delivery of controllable systems (70–72). Once proliferative drivers have been identified, rigorous examination of the expanded population must confirm

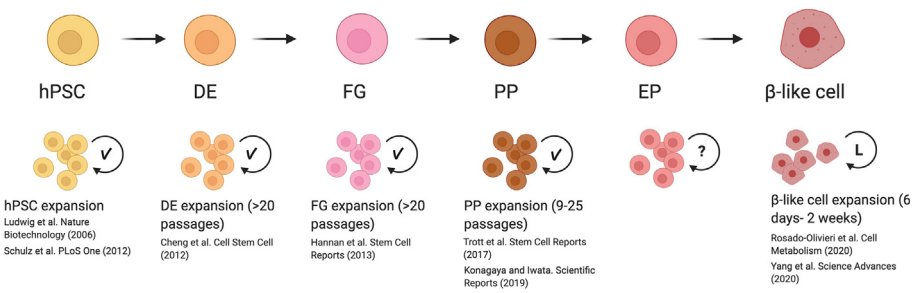

FIGURE 1 | Reported mechanisms and caveats for expanding cells during β-like cell differentiation. Previously reported methods for inducing replication of human pluripotent stem cells (hPSC) to end-stage β-like cells demonstrated that hPSC, definitive endoderm (DE), foregut (FG), and pancreatic progenitor (PP) stages are able to undergo significant expansion with the development of optimized culture conditions (indicated with check mark). These expanded cells are able to maintain their developmental potential with serial passaging. Although proliferation rates have been reported for endocrine progenitors (EP), methods to induce their replication *in vitro* are not well-studied (indicated with question mark). Inducing replication in β-like cells has been successfully initiated. However, β-like cells were only examined after a limited period of expansion *in vitro* (indicated by L), and the long-term effects of driving proliferation are not known. This figure was created using BioRender.com.

that no aberrant mutations have been acquired during the process and that expanded cells retain the ability to form β-like cells. Importantly, if proliferation is induced in β-like cells, one would need to show that cell cycle progression can be blocked and confirm that β-like cells retain maturation features, such as the ability to recapitulate first and second phases of insulin secretion in response to glucose challenge and other secretagogues, and that these cells express molecular markers indicative of bona fide β-cells (10, 12, 60).

Ultimately, the successful establishment of controlled proliferation of hESC-derived differentiating pancreatic cells will allow for the large-scale production of glucose-regulating cells and reduce the high costs associated with growing large batches of undifferentiated hPSCs from the initial steps of differentiation. Strict guidelines for the proposed protocols must ensure that the end product population generate islet-like cells with the ability to normalize glycemia without the risk of teratoma or outgrowth formation. The combination of transient expansion with other strategies to optimize β-like cell differentiation will inform the development of an accessible cellular bank for patients, and ideally contribute to their permanent halt of exogenous therapies for glucose management.

AUTHOR CONTRIBUTIONS

AO and MCN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Stem Cell-Based Clinical Trials for Diabetes Mellitus

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Since its introduction more than twenty years ago, intraportal allogeneic cadaveric islet transplantation has been shown to be a promising therapy for patients with Type I Diabetes (T1D). Despite its positive outcome, the impact of islet transplantation has been limited due to a number of confounding issues, including the limited availability of cadaveric islets, the typically lifelong dependence of immunosuppressive drugs, and the lack of coverage of transplant costs by health insurance companies in some countries. Despite improvements in the immunosuppressive regimen, the number of required islets remains high, with two or more donors per patient often needed. Insulin independence is typically achieved upon islet transplantation, but on average just 25% of patients do not require exogenous insulin injections five years after. For these reasons, implementation of islet transplantation has been restricted almost exclusively to patients with brittle T1D who cannot avoid hypoglycemic events despite optimized insulin therapy. To improve C-peptide levels in patients with both T1 and T2 Diabetes, numerous clinical trials have explored the efficacy of mesenchymal stem cells (MSCs), both as supporting cells to protect existing β cells, and as source for newly generated β cells. Transplantation of MSCs is found to be effective for T2D patients, but its efficacy in T1D is controversial, as the ability of MSCs to differentiate into functional β cells *in vitro* is poor, and transdifferentiation *in vivo* does not seem to occur. Instead, to address limitations related to supply, human embryonic stem cell (hESC)-derived β cells are being explored as surrogates for cadaveric islets. Transplantation of allogeneic hESC-derived insulin-producing organoids has recently entered Phase I and Phase II clinical trials. Stem cell replacement therapies overcome the barrier of finite availability, but they still face immune rejection. Immune protective strategies, including coupling hESC-derived insulin-producing organoids with macroencapsulation devices and microencapsulation technologies, are being tested to balance the necessity of immune protection with the need for vascularization. Here, we compare the diverse human stem cell approaches and outcomes of recently completed and ongoing clinical trials, and discuss innovative strategies developed to overcome the most significant challenges remaining for transplanting stem cell-derived β cells.

Keywords: stem cells, type 1 diabetes (T1D), type 2 diabetes (T2D), clinical trial (CT), transplantation, encapsulation, islets

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WHY THE NEED FOR STEM-CELL BASED THERAPY IN DIABETES?

Intraportal allogeneic cadaveric islet transplantation is considered the best available treatment for patients with Type 1 Diabetes who cannot control their blood glucose levels with exogenous insulin, despite optimal intensive medical management. It consists of the isolation of pancreatic islets from deceased donors, and their infusion into the liver through the portal vein, which results in engraftment in the hepatic parenchyma (1).

Over two decades have passed since the first seven Type 1 diabetic patients were treated with what is known as the Edmonton Protocol (2), a procedure developed by Dr. James Shapiro and his team. Before the introduction of this ground-breaking protocol, the success rate of islet transplantation (measured as percentage of patients able to remain insulin independent for more than one year) was only 8% (3). Modifications to the standard protocol led to an unprecedent 100% success rate in the first seven patients (2). These modifications included a steroid-free immunosuppressive regimen, the use of xeno-protein-free media during islet isolation, and the immediate transplantation of the purified islets from multiple donors (mean islet mass of 11,547 ± 1,604 islet equivalents per kilogram of body weight), to reach an adequate islet mass capable of restoring normoglycemia. Despite the short-term success in maintaining insulin independence during the first year after transplantation, only 11% of those patients remained insulin-independent after five years. Further improvements to the Edmonton protocol over the last twenty years have markedly increased the safety of islet transplantation, with regard to the rate of adverse events related to the infusion procedure, and to the immunosuppression regimen.

Adverse events categorized as "possibly or definitely related" to the infusion procedure include peritoneal hemorrhage, hepatic hematoma or hemorrhage, portal vein thrombosis, and abnormal liver function, while those related to immunosuppression include leukopenia, mucosal inflammation, graft vs host disease, pneumonia, increased blood creatinine, renal disorder, skin disorder, and hypertension. Data from annual reports released by the Collaborative Islet Transplant Registry (CITR, https://citregistry.org/), which includes clinical trial data from 37 islet transplantation centers (28 in North America, seven in Europe, three in Australia) collected between 1999–2016, indicate that the rate of adverse events in the first 30 days following transplantation dropped from 66 to 22%. Reports from the most recent clinical trial data being collected are not yet publicly available. Although islet transplantation has become one of the safest and least invasive transplant procedures, it currently still requires life-long immunosuppression. In addition, long-term insulin independence, which is often reached right after transplantation, declines over time. According to the latest CITR annual report (10th Annual Report, released in January 2017), the percentage of patients remaining insulin-independent after one year is approximately 50%, and a drop to 25% is observed after five years. A drop in insulin-independency was also reported by the more recent phase 3 CIT trial of human islet-after-kidney transplantation (4), where only 16.7% of the patients retained insulin independence three years after transplantation. These percentages are much lower than what was reported for patients undergoing total pancreas transplantation. Based on a report summarizing data from 2005 to 2016 (from one single transplantation center), 75% of the patients who obtained pancreas transplantation following total pancreatectomy remained insulin-independent [until their time of death, or until the present day (5)].

While these numbers indicate that certain aspects of cadaveric islet transplantation need to be further optimized to achieve long lasting relief from exogenous insulin injections, it is important to note that many clinical goals are still achieved with this procedure, especially with regards to the restoration of hypoglycemia awareness and protection from severe hypoglycemic events (6–8). Hypoglycemia unawareness, a state in which a person is unaware of inappropriately low blood glucose levels, is a severe, relatively frequent and potentially life threatening complication occurring in approximately 40% of patients with type 1 diabetes mellitus (T1DM) (9) (10).

Cell survival and graft rejection are the two key unresolved challenges for increasing insulin independence rates in islet transplantation. Currently, most standard islet transplantations are performed through infusion into the portal vein. Despite encouraging results, the liver might not be the optimal place for transplanted islets as entrapment in the hepatic vasculature results in hypoxia (11), and the revascularization process can take up to 14 days to be fully established (12). In addition to hypoxia that impairs β cell function and survival, acute graft loss is caused by instant blood-mediated inflammatory reaction (IBMIR), which results in activation of the complement cascade, clot formation, and lymphocyte recruitment (13). Together, hypoxia and IBMIR lead to destruction of more than 50% of transplanted islets in the first 48 h following infusion into the portal vein (14). Only a few alternative transplantation sites have been tested in clinical trials so far: the bone marrow (15), the muscle of the forearm (16, 17), and the omentum (18–20). Despite positive outcomes for autologous islet transplantation, clinical trials have shown that the bone marrow site is not a suitable alternative site for pancreatic islet allotransplantation in T1D patients, due to recurrence of autoimmunity (21). Survival of alloislets in the intramuscular site has also been shown to be limited so far (17). The omentum represents a promising site, but the protocols utilizing this site need further optimizing to ensure better vascularization and improved management of immunosuppression for long term success (19).

Risks associated with life-long immune suppressive drugs, together with the limited availability of cadaveric islets, are the two prominent current obstacles to a broader use of islet transplantation for the treatment of diabetes. Immune suppression in general remains critical to prevent rejection of the graft. A combination of induction (administered only at the time of transplant) and maintenance (administered for long-term regime) immune suppressive agents are necessary for graft survival, and despite the advancements in the immunosuppressive regimen (22), the majority of the patients still require additional islet infusions (10th Annual Report, CITR).

Islet transplantation is a government-funded, standard-of care therapy in Canada, Europe, China, and other parts of Asia (23), but only for a minority of T1D patients suffering from glycemic lability, hypoglycemia unawareness, severe hypoglycemic episodes, and/or diabetic ketoacidosis, despite optimal intensive medical management. These extreme scenarios are often referred to as "brittle" diabetes. Given the limited supply of cadaveric islets, most transplant centers also limit enrollment only to T1D patients who have complete loss of C-peptide production (24). Even when islet transplantation is government-funded, access to this procedure may still be problematic. In Canada, while islet transplantation is available in the province of Alberta, access to it is more difficult in all the other provinces, where the procedure is not always recognized as a non-research therapy (25). Access to islet transplantation is highly restricted in the United States where the procedure is not approved by health insurance companies as it is still considered an experimental treatment and requires filing of an investigational new drug application (NDA) with the Food and Drug Administration (FDA). This is one of the possible reasons why the total number of centers performing islet transplantations and the total number of transplantations performed per year have declined in the US since 2014, according to the 10th Annual CITR Report. Interestingly, this decline is evident not only for centers in North America, but also in the European and Australian centers that are part of the CITR. The exact reasons for this reduced activity for these centers need to be determined, but American Centers have observed a reduction in pancreas donors since the mid-2000s (https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/#). Unfortunately, among all organs isolated for transplantation the pancreas has become the organ with the lowest donation rate [approximately 11 donors per 100 eligible deaths were recorded in 2018 (26)].

A curated review of islet transplant trials registered on ClinicalTrials.gov (24) also revealed that although there are a number of newly registered trials focusing on testing alternative implant sites and innovative approaches to reduce graft rejection, including encapsulation devices and immune modulators, the overall number of clinical trials for cadaveric islet transplantation is not growing.

While it is highly likely that cell survival and graft rejection will continue to improve in the future, the low supply of cadaveric islets remains the critical limitation prohibiting widespread use of this therapy. In contrast, the prevalence of patients with T1D is increasing globally (27). Based on a recent diabetes forecasting model (28), by 2030 the total number of people with T1D and T2D in the United States alone will increase to 50 million, a 54% increase from 2015.

One possible pathway to a treatment, and perhaps a cure, for a broad number of diabetic patients, would be access to an alternate, unlimited source of insulin-producing cells that can reconstitute physiological glucose homeostasis, eliminating the reliance on organ donors.

Stem cell-derived β-cell therapy overcomes the barrier of limited donor availability, while also possibly representing a more cost-effective therapy compared to exogenous insulin. Although the future cost of stem cell-derived β-cell therapy is unknown, a speculative cost-effectiveness analysis from an early health technology assessment study (29) calculates that 8–9 years after transplantation both cadaveric islet transplantation and stem cell-derived β-cell transplantation would be more cost-effective than exogenous insulin therapy. This calculation assumes that the manufacturing costs of stem cell-derived β cells will be similar to the costs necessary to isolate cadaveric islets. In both cases, successful long-term engraftment is essential for the therapies to become profitable (30). The cost of stem cell-derived β-cell therapy will depend on a number of variables, including the requirement of immunosuppression, the duration of graft survival, and, most importantly, the optimization of the manufacturing process (31). While avoidance of autoimmune rejection will reduce the price, higher upfront costs are needed for the development of a scalable manufacturing process and the creation of stem cell banks for clinical use. Emerging technologies]such as stirred suspension bioreactor culture, wave bag bioreactor culture, multiplate culture, and roller bottle culture (32)] may eventually allow for mass production of stem cell-derived β cells and islet-like organoids. If a manufacturing expenses can be reduced, early health technology assessment studies demonstrated that stem cell-derived β-cell therapy will be a cost-effective (33, 34).

In addition to endogenous cell therapies, wearable computerized devices, such as insulin pumps and closed-loop systems, present alternative options for the delivery of exogenous insulin, and clinical trials are currently testing their efficacy. Insulin pumps (and sensor-augmented insulin pumps) can be programmed to continuously deliver a basal level of insulin as well as extra doses (bolus) during mealtimes. In contrast to these pumps that require manual adjustments and input from the patient, closed-loop systems (also known as artificial pancreas or automated insulin delivery systems) constitute a combined sensing-delivery system in which an external glucose sensor directs delivery of insulin from a sensor-responsive pump guided by real-time glucose sensor readings. A recent clinical trial on T1D patients reported that closed loop systems maintain control within the near normoglycemic range up to 71 ± 12% of the time. This represents a marked improvement over sensor-augmented insulin pumps, with which the normoglycemic range is maintained up to 59 ± 14% of the time (35). Moreover, closed loop systems are also able to improve glycated hemoglobin levels (HbA1c), an indicator of long-term systemic glucose levels. Similar improvements were also noticed when comparing sensor-augmented pumps against hybrid closed-loop system (where insulin is continuously administered, except during boosts at mealtime) (36), or bihormonal closed loop systems (37) that release both insulin and glucagon. The higher efficacy obtained with the bihormonal closed loop system represents an important achievement considering that glucagon's stability in solution is much lower than that of insulin, and that its remarkably dose-response relationship requires tight regulation of its release. A meta-analysis comparing 40 studies resulted in similar finding (38), asserting that closed loop systems are more efficient than any other insulin pump therapies, and represent an efficacious and safe approach for management of T1 diabetes. While closed loop systems clearly constitute a marked improvement in blood glucose control, there remains room for improvement of the algorithms

controlling insulin release, for instance during physical activity. Variables such as the duration and intensity of physical exercise, and the proper timing of hormone release relative to food intake remain hard to control with a fully automated closed-loop system (39, 40). Other concerns regard the wearability, and the cost-effectiveness, especially if considering the constant rise in the price of insulin (a 200% increase from 2002 to 2013 (41), and a 14% annual increase from 2012 to 2018 (https://healthcostinstitute.org/)).

The technical advances described above have dramatically improved the lives of patients with diabetes. However, the limitations of these systems, including need for attaching external devices that penetrate the skin and thus raise the chance of infection and scarring over time, the dependence on fully functional pumps and sensors whose dysfunction can result in rapid and life threatening changes in glucose levels, and the complexities of algorithms tasked with anticipating ever changing aspects of patients metabolism, indicate that they present a powerful temporal solution but not a cure for diabetes.

STEM CELL-BASED APPROACHES: PROTECTION OR RESTORATION OF β CELLS MASS

A curated list of completed, active, recruiting, and suspended stem cell-based clinical trials for both T1D and T2D, registered at ClinicalTrials.gov within the last ten years, is presented in **Table 1**. The majority of the recently completed and active trials use adult mesenchymal stem cells (MSC) derived from different origins, hematopoietic stem cells, or a combination of both. Although initial studies might have suggested the possibility of generating insulin-producing cells from MSCs, clear evidence supporting this hypothesis is currently lacking. Thus, the purpose of these current trials is to understand the mechanisms of protection provided by MSCs and evaluate their efficacy, especially in modulating the immune response. In contrast, a number of publications have demonstrated the potential for human embryonic stem cells (hESCs) (42–46) and induced pluripotent stem cells (iPSCs) (43, 47) to form functional mature insulin-producing β cells, and trials with such cells address the issue of directly restoring β cell mass. Despite these efforts being very promising, only three trials have so far utilized hESCs to derive pancreatic progenitors for β cell replacement therapy. However, successful preclinical studies in non-human primates, led by Vertex Pharmaceutical and Sigilon Therapeutics, are paving the road towards more pluripotent stem cell-based clinical trials.

MESENCHYMAL STEM CELL-BASED THERAPY

Mesenchymal stem cells (MSCs), or stromal stem cells, are currently the most widely used stem cells in clinical trials (www.clinicaltrials.gov). MSCs are multipotent adult stem cells that can be derived from both adult and neonatal tissues. Although adult bone marrow is the most prevalent source, MSCs can be obtained from almost all tissues that include a perivascular area (48, 49). MSCs are derived from the mesodermal germ layer and have a trilineage differentiation potential, that is the ability to differentiate *in vitro* into osteoblasts (bone tissue), chondroblasts (cartilage), and adipocytes (fat tissue) 29. A number of studies have also shown neuronal crest-derived MSCs (50–52) and given the high heterogeneity of MSCs it remains to be determined if additional sources besides the paraxial mesoderm and the neural crest exist. From a regulatory perspective, MSCs have been classified as an advanced therapy medicinal product (https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapy-medicinal-products-overview).

Although MSCs are emerging as the most promising source for allogeneic cell therapy (53), the therapeutic use of MSCs in T1D clinical trials is highly controversial. Three different hypotheses have been explored in clinical settings: (a) the use of MSC-derived pancreatic progenitors that develop into functional β cells capable of restoring normoglycemia, (b) the use of undifferentiated MSCs to generate β cells through direct transdifferentiation *in vivo* upon transplantation, and (c) the use of undifferentiated MSCs to support islet health and survival without differentiating into pancreatic progenitors (**Figure 1**). As of yet, strong evidence to support the hypothesis that MSCs can differentiate into functional mature β cells or islet-like organoids, both *in vitro* and *in vivo*, is lacking.

MSCs' MECHANISMS OF ACTION

Early studies have investigated the hypothesis that MSCs differentiate into insulin-producing cells (54–61). This was in part based on the observation that expression of insulin and other pancreatic transcription factors increase in differentiating MSCs. However, the mere presence of such markers, including PDX1, NGN3, NEUROD1, NKX6.1, and ISL, is not proof of fully matured β cells, as some of these factors are found to be expressed also upon expansion of MSCs *in vitro* (58), and during development of other cell types, such as neurons (62). Furthermore, the presence of these proteins alone does not guarantee mature β cell activities, as expression of non-β cell factors could interfere with critical processes, whereas expression of other markers essential for mature function (including, but not limited to, K^+-channels, Ca^{2+}-channels, secretory vesicles) might still be missing. Functionality of MSC-derived insulin-producing cells has been tested by glucose stimulated insulin secretion *in vitro* and by glucose tolerance in mice *in vivo*. The claimed ability to respond to glucose by secretion of insulin may have been overestimated as insulin levels, rather than the more appropriate C-peptide levels (57, 60, 61), were measured. Insulin secretion may not indicate true insulin production as the hormone is often present in culture media (63). This is supported by the observation that when both insulin and C-peptide levels were determined, a significant increase in secretion was observed only for insulin (56) but not for C-peptide, a normal by-product generated during the maturation of the hormone. Nevertheless, one study showed a four-fold increase

TABLE 1 | Completed and active stem-cell based clinical trials for T1D and T2D.

Trial ID	Study start date	Sponsor and Collaborators	Cell type	Diabetes subtype	Status	Official title	Purpose of the study	Treatment method
NCT01068951	2010-06-01	Uppsala University Hospital	MSCs	T1D	Completed	Open Study to Evaluate the Safety and Efficacy of Autologous Mesenchymal Stem Cells in Treatment of Recently Diagnosed Patients With Type 1 Diabetes Mellitus	Test if development of autoimmune diabetes may be halted by the immune modulatory properties of mesenchymal stem cells	Intravenous injection of autologous mesenchymal stem cells
NCT02239354	2014-09-01	ViaCyte. California Institute for Regenerative Medicine (CIRM)	hESCs	T1D	Suspended	A Prospective, Multicenter, Open-Label, First-in-Human Phase 1/2 Study With Two Cohorts to Evaluate the Safety, Tolerability, and Efficacy of Various Doses of VC-01™ Combination Product in Subjects With Type 1 Diabetes Mellitus	Test if VC-01™ combination product can be implanted subcutaneously and maintained safely for two years. It will also test if VC-01 is an effective treatment	Subcutenous transplantation of combination product VC-01 (PEC-01 cells loaded into PEC-Encap)
NCT03920397	2015-03-01	Universidade Federal do Rio de Janeiro	MSCs	T1D	Active / Recruiting	Allogenic Adipose Derived Mesenchymal Stem Cells and Vitamin D Supplementation in Patients With Recent-onset Type 1 Diabetes Mellitus	Unspecified	Intravenous injection of adipose tissue-derived stem/stromal cells and oral Cholecalciferol supplementation
NCT04078308	2015-07-06	Royan Institute. Tehran University of Medical Sciences, Iranian Stem Cell Council	MSCs	T1D	Active / Recruiting	Phase I/II Clinical Trial to Examine the Safety and Efficacy of Transplantation of Mesenchymal Stem Cells in New-onset Type 1 Diabetes Patients	Modulate immune response and improve regeneration	Intravenous injection of autologous mesenchymal stem cells
NCT02940418	2017-02-19	Sophia Al-Adwan	MSCs	T1D	Active / Recruiting	The Use of Mesenchymal Stromal Cells (MSC) in Type 1 Diabetes Mellitus in Adult Humans: Phase I Clinical Trial	Unspecified	Intravenous injection of allogenic adipose-derived mesenchymal cells with autologous bone marrow mononuclear cells
NCT03162926	2017-07-05	ViaCyte	hESCs	T1D	Completed	An Open-Label Study Evaluating the Safety and Tolerability of VC-02™ Combination Product in Subjects With Type 1 Diabetes Mellitus	Test if VC-02™ combination product can be implanted subcutaneously and maintained safely for up to four months	Subcutenous transplantation of combination product VC-02 (PEC-01 cells loaded into PEC-Direct). Up to six VC-02-20 implants
NCT03163511	2017-07-06	ViaCyte. California Institute for Regenerative Medicine (CIRM), Horizon 2020 - European Commission	hESCs	T1D	Active / Recruiting	An Open-Label, First-In-Human Study Evaluating the Safety, Tolerability, and Efficacy of VC-02™ Combination Product in Subjects With Type 1 Diabetes Mellitus and Hypoglycemia Unawareness	Test if VC-02™ combination product can be implanted subcutaneously and maintained safely for up to two years. It will also test if VC-02 is an effective treatment	Subcutenous transplantation of combination product VC-02 (PEC-01 cells loaded into PEC-Direct). Cohort 1: up to six VC-02-20 implants and up to two VC-02-300 implants. Cohort 2: up to ten VC-02-300 and up to two VC-02-20.
NCT03406585	2017-11-28	NextCell Pharma Ab	MSCs	T1D	Active / Recruiting	A Double-blinded, Randomized, Placebo-controlled Trial With Wharton's Jelly Derived Allogeneic Mesenchymal Stromal Cells (WJMSCs) for Preserving Endogenous Insulin Production in Adult Patients Diagnosed for Type 1 Diabetes	Unspecified	Transplantation of cell suspension with expanded allogenic MSC's procured from donated Wharton's Jelly from umbilical cord tissue
NCT03912480	2019-01-05	CAR-T (Shanghai) Biotechnology Co., Ltd.	MSCs	T1D	Active / Recruiting	Study on the Efficacy and Safety of Stem Cells From Human Exfoliated Teeth in Treating Diabetic Patients With Significantly Reduced Islet	Unspecified	Intravenous drip of dental pulp mesenchymal stem cells

(Continued)

TABLE 1 | Continued

Trial ID	Study start date	Sponsor and Collaborators	Cell type	Diabetes subtype	Status	Official title	Purpose of the study	Treatment method
NCT03973827	2019-05-17	NextCell Pharma Ab	MSCs	T1D	Active / Recruiting	An Open Label, Parallel Single Center Trial of Wharton's Jelly Derived Allogeneic Mesenchymal Stromal Cells Repeatedly Treated to Preserve Endogenous Insulin Production in Adult Patients Diagnosed With Type 1 Diabetes	Investigate safety and tolerance after a repeated allogeneic infusion of WJMSCs intravenously after one year following the repeated treatment.	Transplantation of cell suspension with expanded allogenic MSC's procured from donated Wharton's Jelly from umbilical cord tissue
NCT04061746	2020-02-13	Medical University of South Carolina. National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)	MSCs	T1D	Active / Recruiting	Cellular Therapy for Type 1 Diabetes Using Mesenchymal Stem Cells	Determine the safety and efficacy of allogeneic umbilical cord-derived mesenchymal stromal cells for the treatment of new-onset T1D and to understand the mechanisms of protection	Intravenous injection of autologous mesenchymal stem cells
NCT01719640	2011-01-01	Fuzhou General Hospital	MSCs	T2D	Completed	Autologous Bone Marrow Mesenchymal Stem Cell and Bone Marrow Mononuclear Cell Infusion in Type 2 Diabetes Mellitus	Provide signals for regeneration and improve recovery from inflammation-induced lesion	Intra-arterial pancreatic infusion of autologous bone marrow mononuclear cells in combination with autologous bone marrow mesenchymal stem cells
NCT01576328	2012-04-01	Mesoblast, Ltd.	MSCs	T2D	Completed	A Randomized, Placebo-Controlled Dose-Escalation Study to Assess the Safety and Tolerability of a Single Intravenous Infusion of Allogeneic Mesenchymal Precursor Cells (MPCs) in Patients With Type 2 Diabetes Sub-optimally Controlled on Metformin	Assess safety and tolerability of a single intravenous infusion of three doses of Mesenchymal Precursor Cells	Single intravenous infusion of MPCs
NCT01759823	2012-12-01	Postgraduate Institute of Medical Education and Research	MSCs	T2D	Completed	Efficacy and Safety of Autologous Bone Marrow Derived Stem Cell Transplantation in Patients With Type 2 Diabetes Mellitus	Unspecified	mesenchymal stem cell will be injected into superior pancreatic duodenal artery
NCT03343782	2017-11-01	Vinmec Research Institute of Stem Cell and Gene Technology	MSCs	T2D	Completed	Outcomes of Expanded Autologous Bone Marrow-derived Mesenchymal Stem Cells Therapy in Type 2 Diabetes	Evaluate safety and effectiveness of autologous bone marrow-derived mesenchymal stem cells transplantation	Transplantation of autologous bone marrow-derived mesenchymal stem cells
NCT03943940	2019-04-24	Van Hanh General Hospital	MSCs	T2D	Active / Recruiting	A Preliminary Safety and Efficacy Evaluation of Bone Marrow Mononuclear Cells (BM-MNCs) and Umbilical Cord Tissue-derived Mesenchymal Stem Cells (UC-MSC) Infusion for Type 2 Diabetes Mellitus (T2DM) Patients	Unspecified	Intravenous injection of autologous bone marrow mononuclear cells and allogeneic umbilical cord tissue-derived mesenchymal stem cells

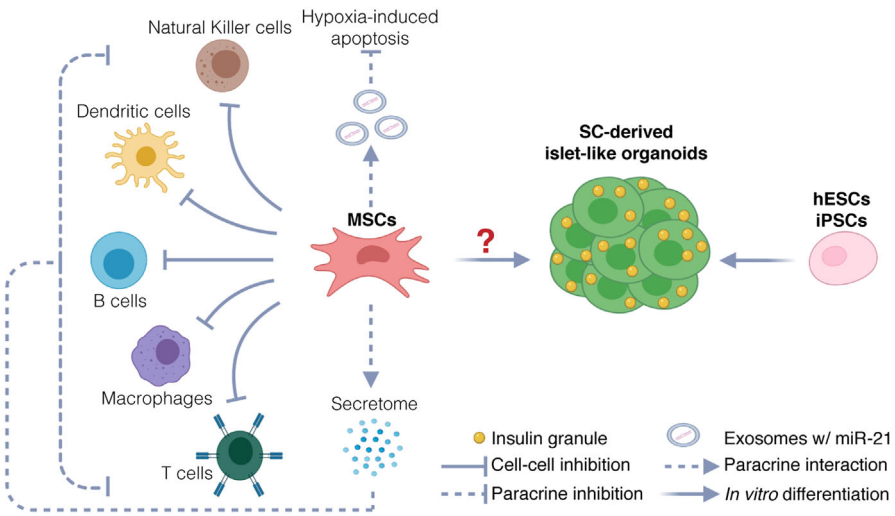

FIGURE 1 | Potential therapeutic mechanisms. Potential mechanisms include protection of endogenous islets and restoration of β cells mass. MSCs could protect endogenous β cells *via* immunomodulation and inhibition of hypoxia-induced apoptosis. Immunomodulation is exerted *via* two mechanisms: inhibition through direct cell-cell interaction with immune cells, and inhibition through paracrine activity, by secretion of chemokines, cytokines, and growth factors (secretome). Inhibition of hypoxia-induced apoptosis could be exerted through release of exosomes carrying miR21, targeting messenger RNAs involved in the hypoxia-mediated ER stress preceding apoptosis. The therapeutic use of MSCs as source for generating stem-cell derived β cells and islet-like organoids is uncertain. hESCs and iPSCs instead are used to generate functional islet-like organoids to restore β cell mass.

in C-peptide secretion upon glucose stimulation *in vitro* (58), suggesting that some MSCs may differentiate into glucose-responsive insulin secreting cells. Glucose tolerance tests have shown some ability in decreasing blood glucose levels upon transplantation into pancreactomized or Streptozotocin (STZ)-induced diabetic mice, although at suboptimal levels. Guo et al. (60) showed an initial maintenance of low blood glucose levels, followed by a drastic increase in blood sugar just 14 days after transplantation. Thus, in the absence of human C-peptide measurements it is difficult to conclude whether the initial low blood glucose levels in mice transplanted with MSC-derived insulin-producing cells were due to the presence of human C-peptide or because of persisting mouse C-peptide, especially without a proper control (STZ-treated mice without transplanted cells). The time-window was extended in the study by Dong-Qui et al. (57), but with a less favorable outcome. Although mice transplanted with MSC-derived insulin-producing cells showed an initial reduction in blood glucose levels by almost 50%, these mice developed tumors and became diabetic within 45 days. Tumor mass is most likely derived from undifferentiated MSCs as bone marrow-derived MSCs have shown to spontaneously transform into neoplastic cells during long-term culture *in vitro*. The neoplastic propensity is not observed in every study (58), possibly due to differences in the source of MSCs, the differentiation protocols employed, or the differentiation stage of the transplanted cells (64). Normoglycemia was also not determined in the study conducted by Kamalaveni et al. (58). Low levels of human C-peptide were detectable in response to glucose only 60 days after transplantation, and although still measurable 150 days post-transplantation, the range was highly variable and below therapeutic purposes (range 0.0–7.97 pmol/ml). Absence of pro-insulin transcript, suggesting the inefficiency in generating mature insulin-producing cells, was also observed in the study from Phadnis et al. (55). Furthermore, none of these studies has extensively investigated the transcriptome profile of the MSC-derived insulin-producing cells, their heterogeneity, and the percentages of poly-hormonal cells versus mono-hormonal cells. Neither were critical functional aspects of mature β cells analyzed, such as biphasic dynamic insulin secretion, proper calcium signaling, mitochondrial respiratory function, and induction of mitochondrial oxidative phosphorylation for glucose oxidation. Summarily, the evidence for differentiation of MSCs into β cells remains unconvincing. In addition, cell tracing studies in mice do not support direct transdifferentiation of multi-potent MSCs into pancreatic progenitors after transplantation *in vivo* (65–67).

Conversely, a number of pre-clinical studies performed over the last 15 years support the hypothesis that MSCs protect islet grafts (68–73) *via* at least two different mechanisms, improvement of cell survival, and immune-modulation. Back in 2012, Ezquer at al. (67) showed that intravenous administration of murine bone marrow MSCs in STZ-treated mice improves blood glucose levels, decreases glycated hemoglobin to levels similar to non-diabetic mice, and increases insulin total insulin levels. Fluorescence-tracing confirmed that MSCs do not differentiate into insulin producing cells, but engraft in lymphoid organs where they restore both the systemic and the local balance of regulatory T cells, increase anti-inflammatory markers such as IL13, and decrease proinflammatory markers such as IL1 beta, IL18, tumor necrosis factor alpha (TNF alpha), and MCP1. In addition, STZ-treated mice transplanted with MSCs also showed an increase in EGF, a trophic factor involved in cell survival. A recent study (74) has

shown that highly proliferative bone marrow MSCs can promote autochthonous β cell regeneration *in vivo* in mice with partial pancreatomy. Higher levels of proliferation were reported based on an increase in number of bromodeoxyuridine positive cells, and a plausible mechanism points to the downregulation of the FoxO1 pathway. In addition to enhanced proliferation, MSC-treated mice also displayed an increase in EGF and total insulin content together with a decrease in interferon gamma and TNF alpha. These data suggest that at least some of the beneficial effects of MSC treatment are mediated *via* a reduction of inflammation.

MSCs-BASED CLINICAL TRIALS

Despite the lack of convincing evidence from pre-clinical studies, clinical trials have been performed testing the hypothesis that MSC-derived pancreatic progenitors generated *in vitro* maturate into β cells *in vivo*, either alone (75) or upon co-transplantation with bone marrow-derived stem cells (76). Although both trials reported positive outcomes in terms of improvement in HbA1c, in addition to an increase in serum C-peptide, a decrease in glutamic acid decarboxylase antibodies (GAD), and a decrease in exogenous insulin requirement, clear evidence for the presence of mature functioning MSC-derived β cells is still lacking. What is more likely is that the benefits of MSC transplantation derive from the immune-modulation and/or the protective role of these cells towards endogenous islets. In fact, co-infusion of MSCs-derived islet pancreatic progenitors with bone marrow-derived hematopoietic stem cells resulted in better long-term control of hyperglycemia as compared with MSC-pancreatic progenitors only (75, 76).

For the reasons listed above, the most recent trials (**Table 1**) focus on the third hypothesis: MSCs support islet health and survival *via* indirect means. Potential mechanisms of action include a paracrine effect through secretion of growth factors (77), modulation of extracellular matrix, ability to scavenge reactive oxygen species (ROS), ability to protect against hypoxia-induced apoptosis through micro RNAs (miRNAs) derived from exosomes (78), and the ability to modulate the immune system (79) (80, 81) through inhibition of T-cell proliferation and promotion of regulatory T-cells, or through interactions with other immune cell types, such as macrophages, B-cells, dendritic cells, and natural killer cells (53).

Meta-analyses of data from completed clinical trials suggest that MSCs can protect islets in T2D but the effect in T1D patients remains questionable. A clear interpretation of early MSC-based clinical trials for T1D has been challenged by the limited number of enrolled patients, and/or by the trial's design that does not allow a proper statistical analysis. A trial conducted by Mesples and his team in 2013 (80) to test the efficacy of autologous bone marrow stem cell transplant reported improvements especially in the reduction of anti-pancreatic islet antibodies. The follow up study at 12 months showed negative value in islet cell antibodies (ICA), GAD, and insulin antibody levels, followed by an increased level of C-peptide and decreased levels of blood glucose and HbA1c. However, a decrease in blood glucose and HbA1c was also seen in the only available control patient, and the levels of C-peptide were not fully maintained in one of the two enrolled patients after 12 months. The small number of treated patients and controls rendered the interpretation of the data inconclusive. In 2015, Carlsson and his team conducted a similar trial (81) on twenty adult patients with newly diagnosed T1D. Treated patients showed preservation or even increase in C-peptide levels in response to a mixed-meal tolerance test 12 months after transplantation.

Although each of these trials suggested that MSC-based therapy promotes β cell health and function in T1D patients, systematic reviews and meta-analysis studies of controlled clinical trials are still debating their positive outcomes. A meta-analysis performed in 2018 (82), comprising 9 randomized-controlled trials and 14 self-controlled trials, concluded that the pooled effect of hematopoietic stem cells therapy, MSC-based therapy, and co-infusion of hematopoietic and multipotent MSCs, resulted in an increased C-peptide level, compared with conventional insulin therapy, whereas trials based on umbilical cord blood-derived MSCs did not reach a significance. A separate meta-analysis of 6 controlled T1D trials published in 2019 (83) showed that there was no difference in the levels of stimulated C-peptide and fasting C-peptide. The reduction in HbA1c was the only difference observed between treated and control patients. Results from ongoing randomized-controlled trials, with larger number of enrolled patients and controls, are needed to elucidate the efficacy of MSC therapy for T1D.

Contrary to the questionable benefits of MSC-based therapies for T1D, MSC-based clinical trials for T2D have shown a constant and robust efficacy. T2D MSC-based trials make use of multipotent MSCs derived from different sources (**Table 1**). From the first trial in 2009 (84) to the most recent trials (85–87) improvements have been observed in C-pep levels, HbA1c values, and reductions in the required insulin dosage. A systematic review of 10 T2D MSC-based trials confirmed a significant increase in the levels of stimulated C-peptide and fasting C-peptide (83). However, despite the large number of *in vitro* studies and *in vivo* pre-clinical studies already conducted, the exact mechanism by which MSCs improve outcomes still remains to be elucidated. Whether the discrepancy between T1D and T2D trials outcomes is caused by technical limitations relative to how the trials for T1D patients have been designed, or by differences in the etiology, needs to be also elucidated.

PLURIPOTENT STEM CELL-BASED CLINICAL TRIALS

Human embryonic stem cells (hESCs) are pluripotent cells isolated from the inner cell mass (ICM) of the blastocyst (88). They possess self-renewal capacity, genomic stability, and can give rise to all three lineages (endoderm, mesoderm, and ectoderm). Induced pluripotent stem cells (iPSCs) (89) are generated from somatic cells by ectopic overexpression of specific transcription factors. iPSCs also have the capacity of self-renewal and differentiation potential, though their genomic stability is still questionable. hESCs and iPSCs maintain their pluripotency after expansion (90), thus fulfilling that need of unlimited supply required for therapeutic purposes. Although iPSCs are emerging as a potential alternative to hESCs, their ability to differentiate into mature pancreatic endocrine cells has not yet reached the same quality observed with hESC protocols (91).

In 2015, the first T1D patient was treated with a hESC-based pancreatic progenitor transplant in Edmonton. The study was driven by the regenerative medicine company ViaCyte, under the supervision of J. Shapiro's team. The purpose of the trial (NCT02239354, submitted in 2014) was to test a combination of hESC-derived pancreatic progenitor cells (PEC-01) (92, 93) expected to mature into functional insulin-producing cells upon transplantation based on prior studies with surrogate animals, within an encapsulation device called PEC-Encap (VC-01) (94). The first transplantation consisted of 40 million pancreatic progenitor cells, divided into two encapsulation devices implanted subcutaneously in the abdomen, along with six smaller encapsulation devises implanted subcutaneously in the arm, serving as sentinels to be removed at different time points to follow cell survival and maturation. This first encapsulation device was designed to protect the pancreatic progenitor cells from the immune system, preventing both allogeneic (foreign organ) reaction and autoimmune rejection, eliminating the necessity of immunosuppressive drugs. The device had a semipermeable membrane that allowed exchange of molecules but not cells. VC-01 (consisting of the combination of PEC-01 cells and PEC-Encap device) was meant to be evaluated in an open-label, dose-escalating Phase 1/2 study in T1D patients with minimal insulin-producing β-cell function. The trial was suspended due to inconsistencies in cell survival and poor cell engraftment, primarily caused by a foreign body response, similar to a wound healing which clogged the membrane and prevented vascularization. This first trial indicated the necessity for optimization of the encapsulation device.

In 2017 ViaCyte launched a second 12-months trial (NCT03162926) which introduced an alternative encapsulation device (PEC-Direct, VC-02), with a modified membrane that does not provide immune protection, but allows vascularization, and therefore requires the re-introduction of immunosuppressants. No changes were performed in the type of hESC-derived pancreatic progenitors used. Successful outcomes led to the currently ongoing 2-year trial (NCT03163511), aimed at testing safety and tolerability of VC-02 implanted subcutaneously in T1D subjects with hypoglycemia unawareness. The purpose of this trial is also to test whether VC-02 is an effective treatment. PEC-01 cells were able to engraft, survive, and produce measurable C-peptide levels (95). Preliminary results from a small subset of patients (six out of 18) showed substantial engraftment of sentinel devices containing insulin positive cells (9 months after transplantation), and production of C-peptide in all patients up to 12-months (with some patients already reaching 15, 18, or 21 months). Moreover, the immunosuppression regimen prevented allogeneic and autoimmune destruction of the cells, without causing a foreign body response. The intended islet mass transplanted was intentionally insufficient to normalize HbA1c levels, therefore no data regarding the efficacy is available. Although these positive outcomes are restricted to 30% of the transplanted patients, further optimization of microencapsulation device materials might improve future outcomes. In support of this notion, a press release from Viacyte from August 2020 (https://viacyte.com/news-events/) announced a clinical phase agreement with Gore, a materials science company, for the development of a modified version of the original PEC-Encap, which has the potential to eliminate the need for immunosuppression while still allowing vascularization.

In addition to Viacyte, two other companies, Vertex Pharmaceutical and Sigilon Therapeutics, are moving forward towards clinical trials with stem cell derived beta cells. These companies are taking a different approach in terms of cell type and immune protection. Viacyte's cells (PEC-01) (92, 93) consist of a mixture of hESC-derived multipotent pancreatic progenitors (which can differentiate into endocrine, exocrine, or ductal cells) and immature hormone-producing cells. This choice derives from the observation that immature progenitors can better overcome the inflammation initiated by the transplantation procedure (96). Vertex Pharmaceutical and Sigilon Therapeutics produce stem cell-derived islet-like organoids which lack the progenitor population. Islet-like organoids generated by Vertex have been tested in a pre-clinical study performed on non-human primates, mimicking cadaveric islet transplantation. Organoids were delivered through the portal vein, in combination with immunosuppressants. The transplanted organoids successfully engrafted in the liver and were functional over a period of 6 weeks. Although the transplanted amount did not lead to insulin independence, the study showed a 60% reduction in the required insulin dosage (International Society for Stem Cell Research (ISSCR) Annual Meeting 2019). Vertex is also developing its own macroencapsulation device, consisting of a porous membrane which allows immune protection. The first pre-clinical study in pigs showed that the device was able to confer immune protection, while still balancing cell survival and foreign body reaction (ISSCR Annual Meeting 2019). Alice Tomei and her team recently reported an alternative encapsulation strategy, termed conformal coating, consisting in a uniformly thin hydrogel layer that conforms to the islet shape. Preliminary data in mice revealed that the conformal-coated stem cell-derived islets could reverse diabetes and maintain euglycemia for more than 80 days (97). Positive results were also obtained from pre-clinical studies performed on macaques by Sigilon (98), using a different encapsulation strategy. Rather than generating islet-like organoids that aggregate solely by cell-cell interaction, the company utilize a microencapsulation technology consisting of gel-based spheres that can hold up to 30,000 cells (99). Chemical modifications on the surface of the spheres allows for immune protection. Endocrine cells within these clusters were shown to remain functional after transplantation for up to four months (98).

The timing for clinical trials has not been released, but both companies aim to bring their technology into clinical use in the near future.

It is worth noting that numerous companies are developing and clinically testing encapsulation devices that, although initially aimed at preserving human pancreatic islets, could be quickly applied to stem cell-derived therapies. An example of such a company is BetaO2 Technologies, which developed a bioartificial pancreas, called Beta-Air, designed to contain macro-encapsulated human islets together with an oxygen tank. Human islets encapsulated within an alginate-based hydrogel are protected from the immune system by a permselective membrane, and are continuously supplied with oxygen. The bioartificial pancreas was tested in patients with T1D in 2014 (NCT02064309), and the

company is currently developing a second-generation device specifically adapted for stem cell-derived pancreatic clusters.

FUTURE DIRECTIONS

After decades of MSC-based clinical trials for both T1 and T2 diabetic patients, hESCs and iPSCs-based β cell replacement therapies are finally becoming a tangible reality with the first hESC-derived islet-like organoids transplanted in T1D patients in 2014. A tremendous amount of clinical testing is now necessary to investigate the many aspects involved in stem cell transplantation, including the long-term safety of each encapsulation device, the optimal implant size to reach a therapeutic effect, and the long-term viability of the transplanted cells. Improvements of cell survival in subcutaneous and/or intramuscular space would alleviate safety concerns and allow for easier transplant monitoring. A preclinical study on non-human primates has shown that long-term survival (over 800 days) of human islets transplanted subcutaneously can be achieved when islets are mixed with a matrix, termed islet viability matrix, consisting of human collagen 1, l-glutamine, fetal bovine serum, sodium bicarbonate and medium199 (100). Similarly, numerous groups are developing drug-eluting scaffolds to modulate the immune reaction. These biomaterials have been shown to not only reduce local inflammation following transplantation (101) but also maintain long-term graft survival (102). Islet viability matrixes and drug eluting matrices/scaffolds should be tested in clinical trials, in combination with encapsulation devices. Co-transplantation of autologous non-endocrine tissues which may help cell survival and engraftment may also be considered, such as co-transplantation with parathyroid gland tissue, a method currently tested with cadaveric islets in the intramuscular space (NCT03977662).

Efforts to compare the quality of the cell mixtures required to achieve optimal metabolic control are crucial. Currently, three different cell mixtures can be generated: pancreatic progenitors, islet-like organoids, and enriched β cell clusters; of those, only the first two are explored in clinical trials. Numerous studies have shown that cell-cell communication between different endocrine cells are critical for correct glucose responsiveness and electrical coupling of stimulus with insulin secretion (103). Current ongoing clinical trials are utilizing a mixture of pancreatic progenitor cells which can differentiate and mature into endocrine, exocrine, or ductal cells *in vivo*. Although the architecture of these organoids more likely resembles that of endogenous human islets, it is still not known whether immature polyhormonal cells remain in small quantities after transplantation, and whether they may disrupt proper function over time. The use of mature enriched β cell clusters has not been tested in clinical settings, but considering that enriched β cell clusters lack other islet cell types, such aggregates may not provide optimal metabolic control either. Furthermore, human islets contain specialized β cells, termed hub cells, which have reduced β cell identity but regulate efficient islet response to changes in glucose levels (104). Enrichment strategies that aim at targeting highly insulin expressing cells may therefore exclude β cell hubs. Previous studies have shown that enriched β cell clusters that have already reached maturity *in vitro* can still continue to mature *in vivo*, and generate mono-hormonal glucagon and somatostatin positive cells (105). Whether these *in vivo* matured β cell clusters contain β cell hubs remains to be determined. Transplantation of islet-like organoids, consisting of mature β, alpha, and delta cells, which have been individually differentiated, and subsequently clustered based on endogenous percentages, has also not yet been tested in pre-clinical nor clinical settings, although protocols for the *in vitro* generation of both β and alpha cells have been optimized (32, 106). The generation of somatostatin-producing cells is currently achieved in small percentages as a bio-product of the β cell differentiation protocols (105).

Another aspect to consider is the use of iPSCs over hESCs. iPSCs have some advantages in terms of safety, at least in the sphere of alloimmunity, but from a technical perspective the time-consuming generation of iPSC lines from each single patient may pose an insurmountable economic burden. This is one of the reasons why numerous studies are currently testing alternative methods to eliminate overall immune rejection. These methods can be classified into two categories: induction of immune tolerance, and gene editing to generate 'cloaked' cells invisible to the immune system. Immune tolerance can be induced with tolerogenic cytokines and immunomodulatory proteins such as CTLA-4, and PD-L1 (107), whereas the generation of 'cloaked' cells is attempted by removal of HLA proteins, mainly through genome editing (108–110). ViaCyte, in partnership with CRISPR Therapeutics, is currently developing immune-evasive stem cell lines that combine both strategies. Approaches aimed at inducing immune protection have the potential concern of creating cells that cannot be recognized and thus eliminated by the immune system if they should become infected by a virus or should form a teratoma, a major concern in stem cell therapy. A possible solution may be provided by the introduction of inducible suicide genes, such as the inducible Caspase-9 (iC9) (111), combined with its in-frame insertion into a locus transcriptionally active in undifferentiated stem cells, such as SOX2 (112). Overall, while there are challenges that still need to be addressed, generating immune "cloaked" cells would remove the need for immune-suppressive regimen, thus broadening the applicability of stem cell therapies to treat patients afflicted by both type 1 and type 2 diabetes.

AUTHOR CONTRIBUTIONS

EdK wrote the manuscript with support from MH. All authors contributed to the article and approved the submitted version.

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Lessons from Human Islet Transplantation Inform Stem Cell-Based Approaches in the Treatment of Diabetes

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Diabetes mellitus is characterized by the body's inability to control blood glucose levels within a physiological range due to loss and/or dysfunction of insulin producing beta cells. Progressive beta cell loss leads to hyperglycemia and if untreated can lead to severe complications and/or death. Treatments at this time are limited to pharmacologic therapies, including exogenous insulin or oral/injectable agents that improve insulin sensitivity or augment endogenous insulin secretion. Cell transplantation can restore physiologic endogenous insulin production and minimize hyper- and hypoglycemic excursions. Islet isolation procedures and management of transplant recipients have advanced over the last several decades; both tight glycemic control and insulin independence are achievable. Research has been conducted in isolating islets, monitoring islet function, and mitigating the immune response. However, this procedure is still only performed in a small minority of patients. One major barrier is the scarcity of human pancreatic islet donors, variation in donor pancreas quality, and variability in islet isolation success. Advances have been made in generation of glucose responsive human stem cell derived beta cells (sBCs) and islets from human pluripotent stem cells using directed differentiation. This is an emerging promising treatment for patients with diabetes because they could potentially serve as an unlimited source of functional, glucose-responsive beta cells. Challenges exist in their generation including long term survival of grafts, safety of transplantation, and protection from the immune response. This review focuses on the progress made in islet allo- and auto transplantation and how these advances may be extrapolated to the sBC context.

Keywords: islet transplantation, type 1 diabetes, diabetes treatment, total pancreatectomy with islet autotransplant (TPIAT), allotransplantation, type 2 diabetes, beta cell transplantation, stem cell differentiation

INTRODUCTION

Patients With Insulin Deficient Forms of Diabetes May Be Considered for Islet Transplantation

Diabetes is characterized by the body's inability to control blood glucose within a tight physiological range, due to insulin deficiency from beta cell loss or dysfunction and/or insulin resistance. Prolonged or recurrent hyperglycemia can lead to macro and microvascular complications, associated with substantial morbidity and early mortality (1, 2).

Type 1 diabetes is an autoimmune attack on a patient's own insulin producing beta cells. If left untreated, severe insulin deficiency leads to hyperglycemia, diabetic ketoacidosis and potentially death. Treatment options at this time are limited to exogenous insulin; although progress has been made in the precision of delivery of insulin and blood glucose monitoring (2), patients are at risk for life-threatening hypoglycemia (3). While therapies targeted specifically at risk relatives have shown some promise in delaying onset of disease (4) there is still no cure for type 1 diabetes. Allogeneic islet transplantation may be considered in highly selected type 1 diabetes patients with either repeated severe hypoglycemic, significant glycemic variability, or microvascular complications (usually renal failure necessitating kidney transplant) (5). In the United States islet allotransplant is considered investigational and only performed in the context of a research study but is offered as standard clinical care in areas of Canada, Europe, and elsewhere. However, only a limited supply of suitable cadaveric donor pancreases are available for islet isolation and transplant.

In contrast, type 2 diabetes is a condition of insulin resistance and progressive beta cell decline (6). Patients are often treated with oral or injectable agents that improve endogenous insulin function. In some cases, patients with type 2 diabetes are also treated with insulin. Because the large islet mass needed to overcome insulin resistance is unlikely to be obtained with isolated islets, patients with type 2 diabetes are generally not considered candidates for islet transplantation. However, a renewable cell source could overcome this barrier of insufficient islet mass.

While type 1 and 2 diabetes are the more common causes of glucose dysfunction, beta cell loss and dysfunction can also occur in the setting of persistent inflammation and stress within the pancreas due to chronic pancreatitis (7). This is a painful and disabling condition that can be treated with analgesics, procedural interventions, and sometimes removal of the pancreas, rendering the patient without endocrine or exocrine pancreatic function. Total pancreatectomy and intraportal islet cell autotransplantation (TPIAT) can provide pain relief and sustained islet graft function in these patients. In this procedure, patients receive their own islets and therefore donor pancreases are not required. However, because of the damage and fibrosis from pancreatitis, these individuals usually have a sub-optimal islet mass and only about 1 out of every 3 achieves insulin independence.

In these etiologies of dysglycemia, replacement of beta cell function is a potential treatment to alleviate glycemic variability and reduce risk for the complications associated with long term hyperglycemia. A common challenge is obtaining a sufficient number of islets to successfully treat individual patients and offer cell therapy to a larger number of patients with diabetes. Here we review the uses of allo- and auto- islet transplantation and how stem cell derived beta cells (sBCs) or islets may overcome barriers and limitations currently inherent in islet transplantation (**Table 1**).

Islet Allotransplantation

Allogenic transplantation of cadaveric islets as a functional source of beta cells has become a treatment for patients with type 1 diabetes, particularly those with either life-threatening hypoglycemia or diabetes-related kidney failure requiring kidney transplantation. Although whole organ pancreas transplants can also be performed for these individuals (8–10), the appeal of islet transplantation is the lack of major surgery and very low risk for procedural complications. There have been improvements in isolation of islets and the procedure is considered minimally invasive. Initial transplants of pancreatic islets were trialed as early as the 1970s, with initially low rates of success (11). A turning point came with the introduction of the Edmonton Protocol in 2000 (12)—by introducing glucocorticoid-free immunosuppression and using multiple donors to increase islet mass, all 7 patients transplanted in the initial Edmonton trial achieved insulin independence. Subsequent refinements in immunosuppression protocols have improved the longevity of insulin independence (5, 13).

Even when insulin independence is not achieved, islet transplantation is highly successful in preventing severe hypoglycemia, if islet graft function is maintained (5, 14, 15). While success of islet transplants have generally improved over the last 20 years (5), variability in achieving insulin independence and concern for immunosuppression impact on kidney function (16) remain a concern. In addition, donor tissue availability

TABLE 1 | Considerations in islet transplantation.

	Allotransplantation	Autotransplantation	Stem cell transplantation
Patient Population	Patients with type 1 diabetes and severe hypoglycemia, glycemic variability or microvascular complications	Patients with chronic pancreatitis undergoing pancreatectomy	Investigational; type 1 diabetes with potential application to other forms of diabetes
Source of Islet Material	Cadaveric islets	Autologous transplant	Human pluripotent stem cells (induced pluripotent stem cells or embryonic stem cells)
Limitations	- Immunosuppression required - Limited supply of donors	- Limited cell mass, from one's own diseased pancreas	-Remains investigational - Need to scale up to sufficient functional mass to reverse diabetes in humans

continues to be the limiting factor of allogenic islet transplantation as a treatment for diabetes as 2–3 donors are typically required to obtain the necessary beta cell mass required for transplantation (17).

Despite improving long-term outcomes after islet allotransplantation, challenges remain around the longevity of insulin independence. Transplanted islets are subjected to non-immune attrition, and at risk for alloimmune rejection and recurrent autoimmunity (18–20). Immunosuppressive drugs necessary for islet allotransplant also carry risk for beta cell toxicity (21). Although intraportally transplanted islets are rarely accessible for study, limited histopathology of intraportal islet allografts have shown amyloid deposition, postulated due to over-stimulation of insulin production from a marginal islet mass or immunosuppressive drug toxicity (22); a recent report documenting absence of islet amyloid in an islet autograft patient with marginal islet mass suggests drug toxicity as a more likely culprit (23). More recently de-differentiation of the mature beta cell phenotype was observed in two islet allotransplant recipients, possibly consequences of hypoxia and metabolic stress (23). Innate immune destruction of islets stimulated upon intraportal infusion of islets has led to study of alternate sites for transplant, including omentum, bone marrow, intramuscular, and subcutaneous sites, though none has yet established the same efficacy as the liver (24–27).

As with any organ transplant, alloimmune rejection can occur in islet transplant and is more common in patients exhibiting high levels of HLA-sensitization pre-transplant (28–31). Unfortunately, immune rejection is difficult to treat due to limitations in early detection and lack of effective treatment strategies (10, 18, 32–34). Genetically engineered human beta cell lines can be used *in vivo* to augment the immune response to evaluate immune interactions and perhaps protect transplanted beta cells from immune destruction (35). Recurrent autoimmunity has been associated with positive autoantibodies, but the presence of autoreactive T-cell studies is more strongly associated with islet graft failure (36–39). Potential strategies to address these immunologic losses include encapsulation and use of bioengineered scaffold devices with enhanced vascularization and/or local drug release.

Islet Autotransplantation

TPIAT is a treatment option for patients suffering from intractable abdominal pain from chronic pancreatitis. Total pancreatectomy provides pain relief by removing the primary source of chronic pain but results in complete exocrine insufficiency and insulin deficient diabetes. By combining total pancreatectomy with islet transplantation, patients can maintain some beta cell mass with insulin secretory capacity, in order to mitigate the severity of post-operative diabetes (40). Unlike allotransplantation, TPIAT does not require immunosuppression and patients serve as their own islet donors (41). Rather the challenge with islet transplantation is obtaining a sufficient number or mass of islets from a diseased pancreas.

TPIAT was first performed in the 1970s at the University of Minnesota (42) and since then has been adapted to many centers worldwide as a treatment for chronic pancreatitis (43–46). Because of the limitations in obtaining sufficient islet mass, only around one out of every three individuals is insulin independent after the procedure, but the majority preserve some endogenous insulin secretion benefiting glycemic control (41). Younger age and higher islet mass transplantation are predictors for functional graft survival (47) and normal preoperative glucose status can also improve post-operative graft success (48). Improvements have been made in the isolation of the islets and minimization of ischemia to the pancreatic islets (41). Work has been done to minimize risk of ischemia by avoiding prolonged cold ischemia to the isolated tissue but length of time has not been shown to have a detrimental effect on islet isolation and location of isolation (remote or onsite) does not affect insulin independence (49). Although some patients have maintained insulin independence for >10 years after TPIAT, as seen with Considerations in islet transplantation, insulin independence, and islet graft function wane over time (50). It is possible that metabolic strain due to glucotoxicity, exposure to toxins and medications (51) or the inability for islet neogenesis to occur in the liver (52) may contribute to the observed decline in islet graft function. An autologous renewable cell source could address the diabetes challenges after TPIAT by increasing islet mass and providing potential to "redose" islets later to address the apparent slow loss of islet mass over time after the procedure. For obvious reasons, autologous transplantation can only occur once in a patient's lifetime and cadaveric islets require a deceased donor, therefore a source of renewable islet sources could benefit patients with islet dysfunction, either due to diabetes or chronic pancreatitis.

Moving Beyond Allo and Autotransplantation

There is clearly a need for access to a renewable cell source for allotransplantation, and for re-transplant after autotransplantation or in chronic pancreatitis. There are some key lessons to be learned from allo- and autotransplantation for the future of cell therapy. From islet autotransplantation, we have functional data to establish a dose-response in the absence of targeted immunity– Islet graft function (C-peptide positivity) is nearly universal when a minimum threshold of 5,000 IEQ/kg is transplanted in the autograft setting, suggesting this may be an appropriate minimum "dose" target for a stem cell-derived therapy [particularly if immune barriers are fully addressed (41)]. Islet attrition occurs due to immune and non-immune stressors, and thus engineering the proper microenviroment for renewable cell sources may enhance the potential for long-term benefit. Encapsulation, engineered scaffolds, and alternate transplant sites are particularly relevant to stem cell therapy, where encapsulation may also both immunoprotect and to "contain" the cell product and sites outside the liver may be desired for safety. Addressing auto and alloimmunity, such as through encapsulation approaches, will continue to be a need for stem cell derived therapy.

A Future for Stem Cell Derived Islets

Given the limitations of donor availability, the generation of glucose responsive human sBCs and islets from human pluripotent stem cells (hPSCs) are a potential future treatment for those with diabetes. Both human embryonic stem cells

(hESCs) and induced pluripotent stem cells (iPSCs) are potential sources of hPSCs from which sBCs can be generated. hESCs are have been derived from blastocysts (53, 54). iPSCs are somatic cells that can be taken from a patient blood sample or fibroblast and reprogrammed with defined factors to the pluripotent state (55). Both hESCs and iPSCs are able to undergo differentiation and self-renewal to generate an unlimited source of potentially therapeutic cells. Much work has been done to direct the differentiation from hPSCs to the pancreatic lineage through stepwise differentiation protocols (56–59) (**Figure 1**). These cells are functionally mature (60) and display insulin secretory properties similar to human islets (61–63). Transplantation of stem cell derived pancreatic endoderm can mature to functional islets *in vivo* in rodents (58). Further work has been done to make this process functional and scalable (64). While in their infancy, current and future studies are underway in humans to investigate safety and efficacy of hPSC derived islets (NCT02239354, NCT03163511, and NCT02939118). These advances are the initial steps to providing renewable, functional islets to patients with beta cell dysfunction (65).

While great progress has been made in the development of these sBCs, there are several challenges and factors to consider. One factor to consider is the presence of off-target or undifferentiated cells that could interfere with the functional sBCs or be tumorigenic. Current and future clinical approaches using partially mature (pancreatic endoderm) or fully mature islets for implantation may reduce this risk, but close clinical follow up will be needed.

A second consideration is the role of immunogenicity of the sBCs or stem-cell derived islets. hESC derived cells, in the absence of genome editing, are subject to risk for alloimmune rejection which may require immunosuppression or encapsulation. Using iPSCs subverts this risk of alloimmunity and would be ideal for a cell source in TPIAT. Autologous sBCs have been successfully derived from patients with type 1 diabetes (62), but in this setting would remain at risk for autoimmune attack by autoreactive T-cells against pancreatic beta cells characteristic of type 1 diabetes. Therapeutic strategies for delaying this autoimmune attack have shown promise in at-risk relatives (4) but there is not yet an accepted therapy for halting this immune response. Even in the setting of allogeneic islet or simultaneous pancreas-kidney transplantation, where multi-drug immunosuppression is administered, islet autoimmunity can recur (66). Transplanted islet exosome profiling can be used as a way to monitor for evidence of recurrent autoimmunity (67). This can be tracked from a peripheral blood sample from a patient and may be a marker of beta cell injury patients who have undergone islet transplantation. Plasma detection of glutamate decarboxylase (GAD-65) can serve as a marker of beta cell loss after transplantation (68). Although hPSCs can evade allogenic response (69) once fully differentiated, these cells lose their immunologic privilege (70). Additionally, strategies being explored include the use of genetically engineered immune silent cells. Advances in genome engineering using CRISPR/Cas9 allows for modification of hPSCs (71) and can knock out HLA surface molecules implicated in autoimmunity (72, 73).

Pluripotent stem cells may be better poised to overcome the immunologic challenges of allotransplantation when combined with genetic engineering, encapsulation, or scaffolding technology. Bioengineered scaffolds offer novel opportunity to improve islet vascularization and optimize the islet microenvironment to protect grafts (27, 74, 75). Macroencapsulation of sBCs have been explored as a way to protect sBCs *in vivo* (76, 77) which could block transplanted sBCs from an immune attack but provide an environment to allow the survival of transplanted tissue. Trials are underway of to encapsulate sBCs for transplantation (78). These considerations will be important aspects to consider prior to considering transplantation of sBCs.

DISCUSSION

While much progress has been made in transplantation functional islet tissue as a treatment for diabetes, there are still many aspects that must be faced. While sBCs and islets can be generated on a large scale, there are still challenges to ensure

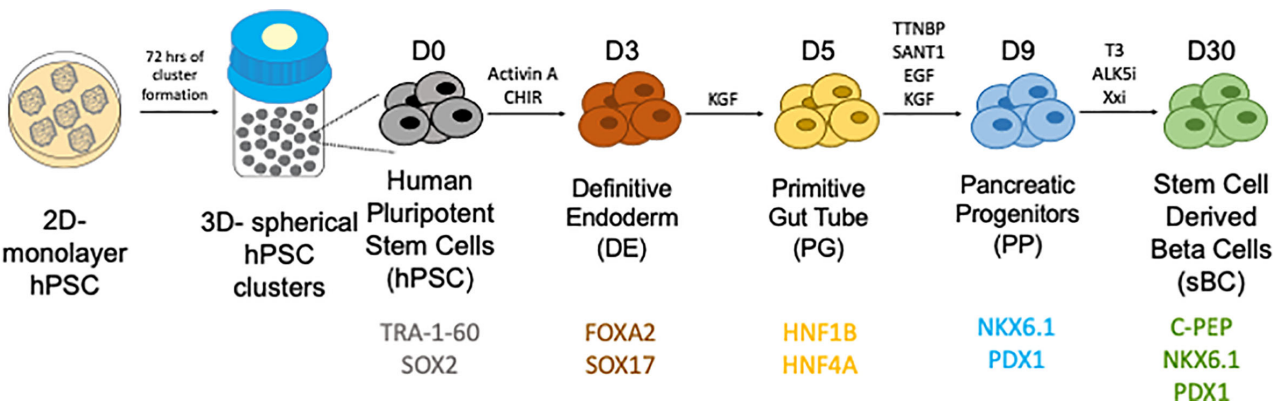

FIGURE 1 | Generation of stem cell derived beta cells from human pluripotent stem cells through a stepwise differentiation protocol. Courtesy of Dr. Holger Russ.

protection from rejection, continued functionality and assurance of safety. Lessons learned from allo and auto islet transplantation will be helpful to apply in the sBC context.

AUTHOR CONTRIBUTIONS

TT is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis. TT researched data and wrote the manuscript. MB researched data and reviewed/edited manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Human Pluripotent Stem Cells to Model Islet Defects in Diabetes

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Diabetes mellitus is characterized by elevated levels of blood glucose and is ultimately caused by insufficient insulin production from pancreatic beta cells. Different research models have been utilized to unravel the molecular mechanisms leading to the onset of diabetes. The generation of pancreatic endocrine cells from human pluripotent stem cells constitutes an approach to study genetic defects leading to impaired beta cell development and function. Here, we review the recent progress in generating and characterizing functional stem cell-derived beta cells. We summarize the diabetes disease modeling possibilities that stem cells offer and the challenges that lie ahead to further improve these models.

Keywords: diabetes, insulin, modeling, stem cells, genetic defects, insulin secretion

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INTRODUCTION

More than 450 million people worldwide are diagnosed with diabetes, a number unfortunately expected to increase dramatically in the next decades (1). Diabetes unfolds when the pancreatic beta cells fail to secrete enough insulin to meet physiological demand, resulting in abnormally high blood glucose levels. Our understanding of the distinct molecular mechanisms that lead to beta cell failure in the different types of diabetes has remarkably improved thanks to progress in the genetic characterization of people with diabetes and the development of animal and cellular models (2). Among these models, the generation of islet cells from human pluripotent stem cells is gaining traction as a useful approach to dissect diabetes molecular mechanisms (3). In this review, we aim to summarize recent progress in diabetes disease modeling using human pluripotent stem cells, discussing current limitations and potential improvements. We particularly focus on advances in functional islet cell generation, and how these cells may be utilized to study beta cell insulin secretory defects.

Beta cell failure leading to diabetes occurs in different ways. While in type 1 diabetes, beta cells are destroyed by cytotoxic T lymphocytes (4), in type 2 diabetes, which represents 90% of all diabetes cases, the beta cells are dysfunctional as a result of maladaptation to elevated demand for insulin secretion, usually in the context of systemic insulin resistance (5, 6). Both type 1 and type 2 diabetes (T2D) result from interactions between a polygenic background and environmental factors like viral infections or obesity (7). Other forms of diabetes that are less frequent result from highly penetrant monogenic mutations that impair beta cell development and/or function. They can manifest at birth, transiently or permanently, in what is known as neonatal diabetes, or in the young adult (10–25 years of age), termed maturity onset diabetes of the young (MODY) (2, 8). While genetic variants in ~30 loci are associated with neonatal diabetes and MODY, over 50% of clinically

diagnosed cases remain genetically unexplained despite continuous efforts to find causative genetic variants by using genome sequencing (9, 10).

The characterization of the genetic defects associated with these different types of diabetes has improved the understanding of the molecular mechanisms that trigger or increase the risk for this disease. Genome-wide association studies have so far identified over 400 association signals across ~200 loci associated with T2D. These genetic variants are particularly enriched in coding and non-coding genomic regions characteristic of pancreatic islet cells, highlighting their central role in the development of diabetes (11–13). Interestingly, several genetic variants associated with T2D are in loci of genes that are also mutated in cases of neonatal diabetes and MODY [e.g. *KCNJ11* (14, 15), *HNF1A* (16, 17), *GCK* (18, 19)]. These genes are critical for beta cell function and the severity of the disease is determined by the precise molecular mechanism disrupted by the particular genetic variant and its functional impact. There is a spectrum within diabetes in which the pathogenetic mechanisms might range from protein-truncating mutations causing neonatal diabetes due to pancreas developmental failure (20, 21), to increased T2D risk due to regulatory variants modulating adult islet cell function (22, 23). While genetic studies have identified numerous candidate genetic variants associated with different types of diabetes, functional validation of their impact on glucose homeostasis requires models that recapitulate as faithfully as possible human islet physiology.

Rodent animal models have provided abundant knowledge of pancreatic development and beta cell physiology. The generation of genetically modified mouse models have contributed to understanding the role of genes involved in these processes (21, 24). However, animal models have inherent limitations due to key differences with humans at the genetic and physiological level (25, 26). Primary human islets obtained from the pancreas of cadaveric donors are a valuable research material to study diabetes. They have been used to study particular aspects of human islet physiology (27) and to understand how genetic variation affects islet function (28). However, human islet preparations are scarce and exhibit considerable variability in terms of purity, function, and cell type composition after isolation (29–32). Furthermore, isolated human islets are challenging to keep in culture for extended periods of time, and the ability to use them to study the effect of particular genetic variants is limited by the current capabilities to genetically manipulate them. As an alternative, there have been many attempts to generate immortalized human beta cells resulting in the derivation of several cell lines that are now widely used in research. They constitute a renewable source of beta-like cells that can be used to perform diverse *in vitro* experiments. In particular, EndoC-βH lines have proven to be a particularly useful model since they present glucose-stimulated insulin secretion *in vitro* and are transcriptomically similar to primary beta cells (33, 34). Such lines can be utilized to study the impact of particular genetic variants and perform drug screenings since they are amenable to genetic modification and other perturbations (23, 34). A drawback of these cells is that they are aneuploid, which can be a confounding factor for genetic studies (35). They also proliferate, which compromises the functional characteristics of adult beta cells (36, 37). This has been resolved in conditionally immortalized versions of this cell line where the SV40LT oncogene used to transform them can be removed by inducible genetic recombination (37, 38); these cells continue to be a useful resource for the field.

Differentiated human pluripotent stem cells (hPSCs) represent another source of human beta cells. hPSCs can be derived from human embryos (human embryonic stem cells, hESCs) (39) or from somatic cells *via* nuclear reprogramming (human induced pluripotent stem cells, hiPSCs) (40). Notably, hiPSCs can be obtained from somatic cells of people that carry diabetes-associated genetic variants. By doing so, pluripotent cell lines preserving the donor genetic background can then be differentiated *in vitro* into particular cell types to model the molecular consequences of the genetic variant under study (41). Importantly, hPSCs are amenable to different genome editing approaches, facilitating the correction or introduction of desired genetic variants. This is a useful approach to generate optimal isogenic controls or to create new models when donor sources are not available (42).

Here we discuss the possibilities of using hPSCs to model the impact of diabetes-associated genetic variants on the physiology of the beta cell, focusing on the molecular mechanisms impairing insulin secretion.

BETA CELL INSULIN SECRETION DEFECTS

All forms of diabetes have in common the ultimate dysfunction of the pancreatic beta cells and the consequent inadequate circulating insulin levels. Beta cells constitute about 60% of the cells in the human islets. They are highly intermingled with the other endocrine cells, in particular with glucagon producing alpha cells, the second most abundant type, a configuration that is crucial for the optimal function of the beta cells (43, 44), and somatostatin secreting delta cells that dampen the release of both insulin and glucagon (45). The particular organization of human islet cells is remarkably heterogeneous, with variable islet size and cell type composition across parts of the pancreas, but also showing important variation across individuals and from birth to adulthood (29–31).

In conjunction with glucagon secreting alpha cells, beta cells keep human fasting blood glucose concentrations around 5 mM, normoglycemia, by adjusting their insulin secretion output (44). Beta cells are fine-tuned glucose sensors with an intricate machinery that enables them to respond with exquisite precision to deviations from normoglycemia, such as during meals, to minimize glucose excursions (46, 47). Genetic variants that result in the disruption of these molecular mechanisms impact the capacity of beta cells to secrete insulin in a regulated manner. These can cause reduced insulin secretion, leading to the development of different forms of diabetes, or increased insulin secretion (hyperinsulinism) (20, 48). We discuss some of these in detail below (summarized in **Table 1** and **Figure 1**).

TABLE 1 | Genetic defects leading to dysregulated beta cell insulin secretion.

Mechanism affected	Genes	Impact of genetic defect	Type of disease	References
Glucose import and metabolism	*GCK*	Reduced or increased glucokinase activity results in abnormal glycolytic flux, ATP generation, and insulin secretion	ND, MODY, T2D, CHI	(18, 19, 49)
	G6PC2	Loss of function mutations are associated with reduced fasting glycemia	T2D	(50)
	SLC2A2 (GLUT2)	Loss of function mutations result in impaired glucose uptake	ND, T2D	(11, 51)
	HK1	Abnormal silencing of HK1 in beta-cells results in increased glycolytic flux, ATP generation and insulin secretion	CHI	(52)
	SLC16A1 (MCT1)	Promoter mutations impair SLC16A1 silencing in beta-cells, resulting in abnormal pyruvate uptake, increased ATP generation, and insulin secretion	CHI	(53)
	GLUD1	Gain of function mutations result in increased entrance of glutamate in TCA cycle, increased ATP generation, and insulin secretion	CHI	(54)
	HADH	Loss of function mutations result in abnormal activation of GLUD1, increased glutamate into TCA, ATP generation, and insulin secretion	CHI	(55, 56)
	UCP2	Gain or loss of function mutations alter the mitochondrial uncoupling activity of UCP2, resulting in abnormal ATP generation and insulin secretion	T2D, CHI	(57, 58)
	mtDNA	Mitochondrial DNA mutations impair oxidative phosphorylation, ATP generation, and insulin secretion	–	(59)
Membrane depolarization	*KCNJ11*	Gain or loss of function mutations result in abnormal closure or opening of the channel, altered membrane depolarization, and insulin secretion	ND, MODY, T2D, CHI	(55, 60–62)
	ABCC8	Gain or loss of function mutations result in abnormal closure or opening of the channel, altered membrane depolarization, and insulin secretion	ND, MODY, T2D, CHI	(14, 15, 55, 63, 64)
	KCNQ1	Genetic variants in this locus are associated with T2D risk.	T2D	(11, 65)
Membrane receptors	*GLP1R*	Genetic variants in this locus are associated with lower fasting glucose levels. Altered GLP-1 signaling affects amplification of insulin secretion.	T2D	(66)
	GIPR	Genetic variants associated with reduced GIP signaling, impair incretin-mediated amplification of insulin secretion.	T2D	(67)
	MTNR1B	A genetic variant increasing melatonin signaling lowers cAMP levels, inhibiting insulin secretion.	T2D	(68)
Insulin synthesis and secretion	*INS*	Loss of function mutations disrupt INS protein synthesis, folding, transport or bioactivity.	ND, MODY, T2D	(11, 69)
	SLC30A8 (ZNT8)	Different coding genetic variants increase risk or protect against T2D.	T2D	(70–72)
	ADCY5	Non-coding genetic variant reduces ADCY5 expression, which couples glucose to cAMP generation, increasing T2D risk.	T2D	(73, 74)
ER homeostasis	*WFS1*	Loss of function mutations lead to elevated ER stress and beta cell dysfunction.	ND, T2D	(75)
	CDKAL1	Loss of function mutations induce beta cell ER stress and hypersensitivity to glucotoxicity and lipotoxicity.	T2D	(76)
	THADA	Coding genetic variants associated with increased T2D risk.	T2D	(77)
	MANF	Loss of function mutations cause childhood diabetes and a neurodevelopmental disorder.	ND, T2D	(78, 79)
	YIPF5	Loss of function mutations impaired ER-to-Golgi trafficking leading to increased beta cell ER-stress.	ND	(80)
Transcriptional regulation	*PDX1*	Loss of function mutations impair transcriptional regulation of pancreatic development and adult islet cell function.	ND, MODY, T2D	(81–84)
	RFX6	Loss of function mutations impair transcriptional regulation of pancreatic development and adult islet cell function.	ND, MODY	(85–87)
	NEUROD1	Loss of function mutations impair transcriptional regulation of pancreatic development and adult islet cell function.	ND, MODY	(88–90)
	GLIS3	Coding and non-coding genetic variants impair transcriptional pancreatic development and adult islet cell function	ND, MODY, T2D	(91–93)
	HNF1A	Coding and non-coding genetic variants impair transcriptional pancreatic development and adult islet cell function.	MODY, T2D, CHI	(16, 17, 94)
	HNF1B	Coding and non-coding genetic variants impair transcriptional pancreatic development and adult islet cell function.	ND, MODY, T2D	(95, 96)
	HNF4A	Coding and non-coding genetic variants impair transcriptional pancreatic development and adult islet cell function.	MODY, T2D, CHI	(54, 97)
	TCF7L2	Coding and non-coding genetic variants impair transcriptional pancreatic development and adult islet cell function.	T2D	(98, 99)

ND, Neonatal Diabetes; MODY, Maturity Onset Diabetes of the Young; T2D, Type 2 Diabetes; CHI, Congenital Hyperinsulinism.

Summary of genetic variants that impact on molecular mechanisms involved in insulin secretion by the beta cell, classified by the mechanism affected and detailing the impact of the genetic defect.

Islet cells are profusely vascularized, and this facilitates the sensing of circulating blood glucose levels. Glucose is imported into human beta cells primarily *via* glucose transporters 1 (GLUT1) and 3 (GLUT3). Glucose transporter 2 (GLUT2), the main transporter in rodent beta cells, is expressed at lower levels in human beta cells (100). Interestingly, genetic variants associated with T2D are found in *GLUT2* (51) suggesting an important role in human beta cells.

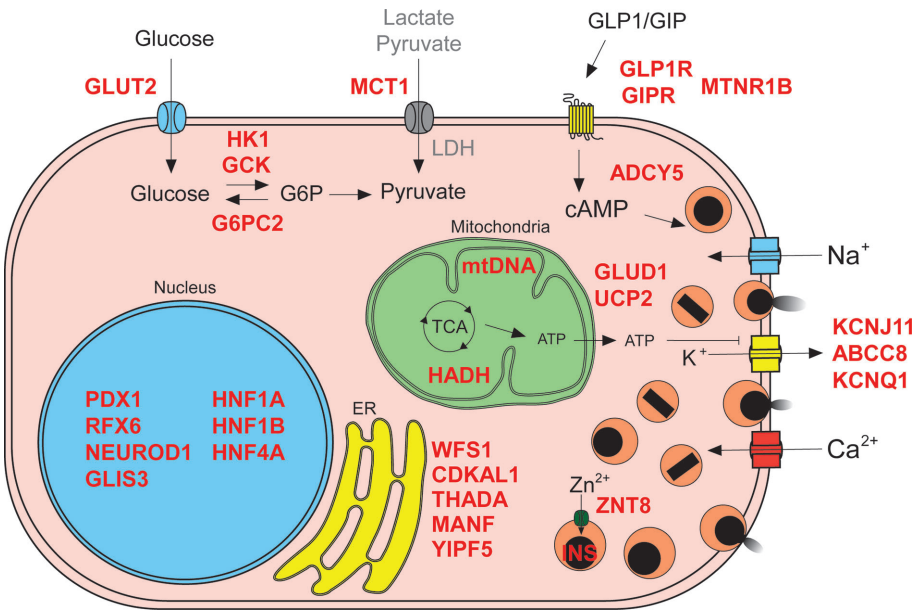

FIGURE 1 | Insulin secretion molecular mechanisms affected in diabetes. Genetic defects can impair different processes involved in regulated insulin secretion (known genes affected in red text): glucose import and metabolism (G6P, glucose 6-phosphate; LDH, lactate dehydrogenase; TCA, tricarboxylic acid cycle; abnormal beta cell metabolism of non-glucose carbon sources due to failure in silencing of disallowed genes depicted in gray text), membrane depolarization, membrane receptors, insulin synthesis and secretion, endoplasmic reticulum (ER) homeostasis, and transcriptional regulation.

Imported glucose is phosphorylated by glucokinase (GCK), a low affinity hexokinase. Diverse genetic defects in *GCK* lead to different kinds of insulin secretion phenotypes, resulting in a range of disease severity, from neonatal diabetes and MODY, to increased T2D risk and congenital hyperinsulinism (18, 19, 49). Other regulators of glucose phosphorylation have also been implicated in insulin secretion dysregulation. For example, glucose-6-phosphatase 2 (*G6PC2*) harbors genetic variants associated with reduced fasting glycemia (50), while the abnormal beta cell expression of "disallowed gene" (101) hexokinase I (*HK1*) has been linked to congenital hyperinsulinism (52).

Once phosphorylated, glucose is retained within the beta cell and it enters the glycolytic pathway to generate pyruvate. Pyruvate is then further oxidized in the mitochondrial tricarboxylic acid cycle, generating abundant chemical energy in the form of ATP, and thus increasing the ATP to ADP ratio. This results in depolarization of the beta cell membrane *via* closure of the ATP-sensitive potassium channels (K^+_{ATP}), triggering insulin secretion. Thus, oxidative metabolism of pyruvate constitutes a crucial coupling process enabling regulated insulin secretion (102). As an alternative to this canonical, one-state model of insulin secretion, recent work by Lewandowski et al. proposes a dynamic model in which beta cells in high glucose conditions oscillate between two states: a biosynthetic state in which conversion of ADP and phosphoenolpyruvate into ATP and pyruvate by pyruvate kinase results in closure of the K^+_{ATP} channels, triggering exocytosis, followed by a state of active oxidative phosphorylation that supports the elevated ATP to ADP ratio sustaining membrane depolarization until exocytosis-associated processes reduce the ATP levels (103, 104). Activators of pyruvate kinase resulted in potentiated GSIS in both rodent and human islets, suggesting that pyruvate kinase may be a potential therapeutic target for T2D (53). Different defects related to the abnormal incorporation of metabolites into the tricarboxylic acid cycle are associated with congenital hyperinsulinism and T2D. Impaired silencing of the pyruvate and lactate transporter *SLC16A1* (*MCT1*), a beta cell disallowed gene, results in congenital hyperinsulinism (53). Gain of function missense mutations in *GLUD1*, glutamate dehydrogenase, or loss of function mutations in hydroxyacyl-coenzyme A dehydrogenase (*HADH*) increase incorporation of glutamate into the TCA cycle leading to congenital hyperinsulinism (54–56). Also, genetic variation in the mitochondrial uncoupler *UCP2* has been associated with T2D and congenital hyperinsulinism (57, 58). In addition, mitochondrial DNA mutations that impair ATP generation cause syndromes that present with diabetes of variable severity (59).

The increase of ATP to ADP ratio triggers the closure of membrane K^+_{ATP} channels, formed by the proteins KCNJ11 and ABCC8 (105). Channel closure leads to depolarization of cell membrane and opening of additional Na^+ and Ca^{2+} channels. Ca^{2+} influx crucially couples membrane depolarization with insulin exocytosis, in a process mediated by the Ca^{2+}-sensing proteins synaptotagmins which trigger the fusion of insulin granules with the plasma membrane (105). Gain of function mutations in *KCNJ11* and *ABCC8* resulting in constant channel opening are the most common cause of neonatal diabetes due to islet physiology defects (15, 60, 63). Genetic variation in these genes can also cause MODY and increased T2D risk (61, 62).

Loss of function mutations that result in constant K_{ATP} closure, or impair its trafficking to the membrane, lead to congenital hyperinsulinism (14, 55). Furthermore, genetic variants in the voltage-gated K^+ channel KCNQ1 are associated with increased T2D risk (65).

Influx of Ca^{2+} ions into depolarized beta cells induces insulin exocytosis by activating the synaptotagmins and SNARE proteins that regulate the fusion of insulin granules with the plasma membrane (106). This exocytosis machinery is not only regulated by the intrinsic pathway triggered by membrane depolarization but is also critically modulated by the intracellular levels of cyclic AMP (cAMP), in what is known as the amplifying pathway (107). Incretin hormones glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP), released by intestinal enteroendocrine cells, potentiate insulin secretion upon binding to their cognate G-protein coupled receptors in the membrane of beta cells (108, 109). This binding results in the generation of cAMP and activation of protein kinase A pathway resulting in augmented K^+ channel inhibition, Ca^{2+} influx, and insulin exocytosis (110). GLP-1 also regulates the alpha cells in a glucose-dependent manner, inhibiting glucagon at high glucose levels, and thereby further contributing to glucose homeostasis (111, 112). GIP stimulates glucagon secretion in a glucose-dependent manner in healthy individuals, with enhanced activity at lower glycemia (113). However, GIP stimulates glucagon secretion even in the presence of hyperglycemia in subjects with T2D, and thereby could contribute to the pathogenesis of T2D (113). Genetic variants found in both the incretin receptors genes *GLP1R* and *GIPR* have been associated with increased and decreased risk of T2D (66, 67). Melatonin receptor 1 B (*MTNR1B)*, another G-protein coupled receptor present in the membrane of beta cells, has also been linked to T2D. A genetic variant increasing the expression of *MTNR1B* has been shown to lower cAMP levels in beta cells, leading to reduced insulin secretion (68). Furthermore, a genetic variant that results in reduced expression of the adenyl cyclase five (*ADCY5*), which regulates beta cell cAMP levels, has been associated with increased risk of T2D (73, 74).

Insulin protein is synthesized in remarkable amounts, representing up to 50% of beta cell total protein synthesis (114), and imposes a high demand on the protein folding machinery of the endoplasmic reticulum (ER). These processes are controlled by the unfolded protein response (UPR) pathway, which is highly efficient in beta cells in order to cope with the insulin biosynthesis-induced ER-stress (115, 116). After potassium channel defects, coding mutations in the insulin gene are the second most common cause of neonatal diabetes due to beta cell dysfunction. These missense mutations cause defects in proinsulin translation, folding, or processing, and may induce high levels of ER-stress that leads to dysfunction of the beta cell. Some *INS* coding mutations can also cause MODY (69) and genetic variants in the *INS/IGF2* locus have been associated with T2D increased risk (11). The fine-tuning of ER-stress levels in beta cells is crucial for the proper functioning of these busy insulin factories. Coding mutations in components of the UPR pathway can cause neonatal diabetes or increased risk for T2D (e.g. *WFS1*, *CDKAL*, *THADA*, *MANF*, *YIPF5*) (11, 75–80). Processed proinsulin molecules are tightly packaged as Zn^{2+} complexed crystals in dense core exocytotic granules. Genetic variants in the Zn^{2+} transporter *SLC30A8* (*ZNT8*), present in the membrane of insulin granules, have been associated with T2D susceptibility (70, 71). A rare loss of function mutation in ZNT8 protects against T2D, making this Zn^{2+} transporter a potentially interesting therapeutic target (72).

While coding and non-coding genetic variants linked to diabetes often impact mechanisms regulating insulin secretion from beta cells, some of them perturb the development of the pancreas, islets, and beta cells themselves (23, 48). The expression levels of insulin secretion machinery components is controlled by transcription factors that conform gene regulatory networks governing the beta cell transcriptional program (117). However, many of these transcription factors are also involved in regulating beta cell development (e.g. FOXA2, PDX1, MNX1, NEUROD1, PTF1A, HNF1A, RFX6) and genetic defects in their loci might lead to a wide variety of diabetes phenotypes (20). While highly damaging transcription factor mutations can cause developmental defects leading to pancreatic agenesis and neonatal diabetes, other genetic variants with milder effects might lead to MODY with different clinical features and penetrance (17, 81, 118), increased T2D risk (91, 98), or even congenital hyperinsulinism (54, 94). Epigenetic profiling of human islets has enabled the characterization of their regulatory landscape, showing that dense enhancer areas are enriched in genetic variants associated with T2D risk (13). Furthermore, a recent study characterizing human islet chromatin architecture resulted in the identification of 3D higher-order hubs of enhancers and promoters (23). These regions are enriched for genetic variants that impact on the heritability of islet-cell traits. We summarize in **Table 1** a list of genes that harbor genetic variants specifically linked to dysregulated insulin secretion. The impact of a given genetic variant will depend on how deleterious it is for a particular mechanism controlling insulin secretion, thus determining the diabetes phenotype and the possible therapeutic interventions. Given the wide spectrum in the functional consequences of coding and non-coding genetic variants, we need suitable research models that enable precise dissection of the detailed mechanisms by which these genetic variants impair human islet physiology.

MODELING INSULIN SECRETION DEFECTS USING STEM CELL DERIVED ISLET CELLS

The generation of hPSC-derived beta cells typically relies on differentiation protocols recapitulating the inductive signaling cues that instruct pancreatic development *in vivo*. These protocols have been devised based on knowledge gained from developmental biology, mostly using mouse models, that deciphered the dynamic signaling environment required for pancreas specification, endocrinogenesis, and beta cell formation (119, 120). With this information, different research teams have empirically determined the recipe of recombinant

proteins and small molecules that reproduce developmental signals in a stepwise manner. These efforts have crystallized in differentiation protocols that make possible the efficient derivation of beta cells from hPSCs (**Figure 2**).

The first report demonstrating the feasibility of generating insulin-producing cells from human embryonic stem cells *in vitro* relied on an spontaneous differentiation approach (122). The first directed differentiation protocol was reported by D'Amour and colleagues from the company CyThera (now Viacyte Inc.). They devised a multistage, adherent culture differentiation protocol, that relied on a first step of efficient definitive endoderm induction (123), followed by four additional steps to induce primitive gut tube, posterior foregut, pancreatic progenitors, and hormone expressing cells (124). While this pioneer protocol generated relatively few insulin producing cells (about 7%), these cells became functionally mature after implantation into mice. Furthermore, the implanted cells were able to protect against streptozotocin induced diabetes (125).

These results sparked intense effort to develop improved protocols leading to more efficient and robust ways to obtain hPSC-derived beta cells over the next decade. Modulation of additional signaling pathways (e.g. FGF, TGF-beta, EGF, PKC) in a time-wise manner enhanced the differentiation efficiency of pancreatic progenitors and endocrine cells (126–132). However, detailed characterization of the hPSC-derived beta cells showed that these insulin expressing cells were frequently co-expressing other hormones, like glucagon or somatostatin, (usually termed as "polyhormonal" cells) (133–135). Polyhormonal cells had impaired glucose stimulated insulin secretion (134), aberrant epigenetic profiles (136), inappropriate glucose transporter GLUT1 expression, imbalanced $K+_{ATP}$ channel subunit expression (137), and resembled human fetal beta cells at the transcriptomic level (138).

A critical realization was the importance of beta cell programming transcription NKX6-1 for beta cell development and functionality (139). NKX6-1 expression at the pancreatic progenitor stage of the differentiation was shown to be crucial for the generation of "monohormonal" beta cells, expressing insulin together with NKX6-1 (INS+NKX6-1+ beta cells) (140). Delaying NEUROG3 induction to later stages, when PDX1+NKX6-1+ progenitors are more abundant, increased the fraction of insulin$^+$ glucagon$^-$ beta cells (140, 141). Protocols generating functional beta cells *in vitro* from hPSCs were reported in 2014 (121, 142). Both differentiation protocols have similarities in the length, stages, and signaling cues used, resulting in abundant INS+NKX6-1+ beta cells. Endocrine cell differentiation was induced by a combination of ALK5 (a TGF-beta receptor) and NOTCH signal inhibitors. Thyroid hormone triiodothyronine (T3) was used to induce the expression of MAFA, a beta cell maturation marker (143, 144). The stem cell-derived beta cells secreted insulin in response to high glucose under static conditions, however, a more detailed analysis of dynamic insulin secretion and calcium influx showed the response was minimal compared to human islets (121). In both studies, the implantation of these functional hPSC-derived beta cells rescued diabetes in mice and had increasing levels of human insulin produced by the grafts over time.

These landmark reports demonstrated a viable path towards the generation of glucose-responsive hPSC-derived beta cells *in vitro*, despite the cells not matching the fine-tuned responses of human islets. It is important to recognize that human islets isolated from cadaveric donors, while presently used as the gold-standard control, have the limitation of considerable variability across islet preparations from different donors in terms of purity, cell-type composition, functionality, and expression of important beta cell markers (30, 121, 145).

Subsequent studies have built on these protocols and further refined them to achieve a higher percentage of hPSC-derived beta cells with better functional responses. For example, different studies have demonstrated how NKX6-1 expression can be increased by aggregating the pancreatic progenitors (146, 147) or by adding EGF and Nicotinamide (148). Also, Rho-associated kinase (ROCK) inhibitors were shown to boost the expression levels of NKX6-1 (149) and the numbers and maturation of hPSC-derived beta cells (150). ROCK inhibitor Y-27632 together with TGF-beta ligand Activin A was reported to induce the formation of endocrine cell enriched protrusions in a 3D-aggregate differentiation setup (151). Performing the differentiation in 3D suspension conditions, in an attempt to recapitulate the developing pancreas cytoarchitecture, has improved the generation of pancreatic progenitors and endocrine cells, as well as the reproducibility and scalability of the differentiation (141, 142, 146, 152).

Induction of endocrine cell formation in these differentiation protocols has relied on the modulation of NOTCH (using gamma

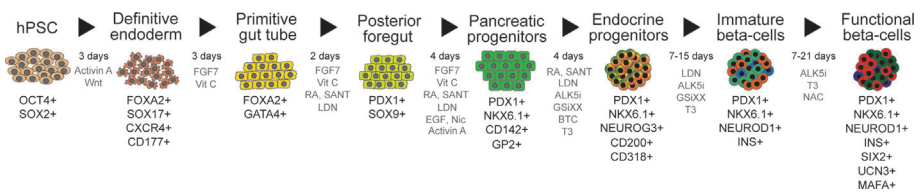

FIGURE 2 | Multistage differentiation protocol to generate functional islet cells from human pluripotent stem cells. Current islet cell differentiation protocols mimic pancreatic developmental stages. Here we represent the commonly used stages [based on (121)], with their usual duration in days, together with cell markers used for the characterization of the differentiated cells (black text) and the cocktails of signaling molecules utilized to induce differentiation (gray text; FGF7, fibroblast growth factor 7; VitC, vitamin C, ascorbic acid; RA, retinoic acid; SANT, SANT-1, a sonic hedgehog signaling inhibitor; LDN, LDN-193189, a BMP inhibitor; EGF, epidermal growth factor; Nic, nicotinamide; ALK5i, a TGF-beta inhibitor; GSiXX, gamma secretase inhibitor used to inhibit Notch signaling; BTC, betacellulin; T3, triiodothyronine; NAC, N-Acetylcysteine).

secretase inhibitors), TGF-beta (ALK5 receptor inhibitors), and EGF (EGF and betacellulin ligands) signaling to trigger NEUROG3 expression. Interestingly, a newly developed 2D planar differentiation protocol generated cells with improved function using latrunculin A to depolymerize the cytoskeleton during endocrine induction, demonstrating that the cytoskeletal state of cells during differentiation can also modulate NEUROG3 expression. These cells were also able to reverse diabetes in STZ treated mice faster than cells generated with a 3D suspension protocol (153). Appropriate timing of NEUROG3 expression is important for beta cell lineage commitment. Its induction in pancreatic progenitor cells expressing PDX1+NKX6-1+ favors the generation of beta cells, while inducing at earlier stages will result in polyhormonal cells that appear to largely resolve into alpha cells (129, 140, 141, 148). Regulatory genomics analyses of embryonic and stem cell derived pancreatic progenitors identified TEAD and YAP as important regulators critical for pancreatic progenitors outgrowth (154). These effectors of the Hippo signaling pathway form part of the gene regulatory network that recruits pancreatic progenitor enhancers and controls their proliferation. Disruption of the TEAD-YAP complex with verteporfin results in reduced proliferation of mouse, zebrafish, and hPSC-derived pancreatic progenitors (154). Additional studies on the role of TEAD-YAP in pancreatic progenitors have shown that cell confinement prevents YAP nuclear accumulation and is a prerequisite for NEUROG3 upregulation (155). In this model, endocrinogenesis is triggered by the disruption of extracellular matrix signaling *via* integrin alpha 5, which maintains the expression of NEUROG3 repressor complex YAP1-TEAD4-HES1. Consistent with this, the use of verteporfin in stem cell-derived pancreatic progenitors resulted in reduced progenitor proliferation, increased NEUROG3 expression, and more C-peptide+ cells (155, 156).

Other approaches to improve the function of stem cell derived beta cells have relied on enrichment steps at various stages and controlling 3D cluster size. For example, enrichment of GP2+ pancreatic progenitors led to the generation of increased numbers of beta cells (157, 158), while enrichment of later differentiation stages using an INS-GFP reporter cell line or magnetic-based enrichment for ITGA1 improved the functionality of the stem-cell derived islet-like aggregates (159, 160). Optimal cluster diameter is important in order to avoid necrosis in the cell cluster core due to hypoxia, maintain a good surface to volume relationship, and is critical for glucose sensing and insulin release dynamics. Across mammalian species with different pancreas sizes, the diameter of islets averages 100–200 μm. The fact that mammalian islets do not scale with the weight of the animal suggests there is an optimal size for the function of these endocrine miniorgans (161, 162). Recapitulating the size of human islets by spontaneous reaggregation (159, 163) or controlled forced aggregation using micropatterned culture plates improves the functionality of stem cell-derived beta cells (160).

The signaling cues required in the later stages of the differentiation protocols to induce maturation are not completely identified. Recent studies have shown that better functioning hPSC-derived beta cells are generated when serum-free media with no added factors is used in the later stages (159, 163). Velazco-Cruz and colleagues reported remarkable acquisition of dynamic glucose stimulated insulin secretion, including robust first and second phases, following the omission of TGF-beta inhibition together with cluster resizing and use of serum-free media during the last stage of the differentiation process. Letting the stem cell-derived islet-like cells establish their own niche and paracrine/autocrine signaling might be a better alternative to achieve more functional cell types (44, 164).

A common problem in the field of hPSC differentiation is the robustness of a given protocol applied to different hPSC lines. In most instances, protocols are optimized specifically for one or few cell lines, and they tend to yield variable differentiation efficiencies when other cell lines are used. In the case of pancreatic differentiation, reports have shown how a particular differentiation protocol results in different percentages of pancreatic progenitors and insulin expressing cell numbers depending on the hPSC line used (148, 165). This is an important obstacle to the wide application of published differentiation protocols, affecting reproducibility. It also complicates the generation of beta cells from diverse patient-derived hiPSCs for disease modeling purposes.

The variability in the efficiency of a particular differentiation protocol has been attributed to the hPSC line genetic background, which can condition its response to inductive cues (166, 167). Recent studies suggest that specific genetic variants in hiPSC lines may alter the differentiation efficiency towards definitive endoderm (168). By using pools of 125 different iPSCs and single-cell RNA sequencing, the authors mapped the population variation during definitive endoderm differentiation stages. They identified several molecular markers predictive of differentiation efficiency, demonstrating that it can be altered by germline genetic variants. Despite intense efforts to improve *in vitro* differentiation protocols, currently they only partially recapitulate the optimal *in vivo* signaling environment. Missing signaling cues are probably better tolerated in some cell lines than in others, explaining this apparent genetic background-determined fitness to respond efficiently to a given protocol. A partial solution to the problem of variability in differentiation efficiency across cell lines is the generation of genome edited cell lines. Genome editing technologies have made possible the introduction and correction of point mutations in hPSCs (169–171). In particular, CRISPR-Cas9 technology has proven particularly useful to efficiently generate isogenic cell line pairs. These can be obtained either by correcting the genetic variant of interest in patient-derived iPSC, or by introducing mutations in a hPSC line that differentiates robustly to the cell type of interest (172, 173). CRISPR can also be used to elucidate which signaling pathways and mechanisms are important to achieve a particular differentiation stage. A recent report illustrates this approach by using a genome-wide CRISPR screening to identify JNK-JUN signaling as a barrier for pluripotency exit and endoderm differentiation (174).

Generation of patient-derived hiPSCs and their differentiations towards the pancreatic lineage has facilitated the generation of cellular models to study diabetes. In combination with genome

editing technologies, these approaches make it now feasible to study how a particular genetic variant impacts pancreas development and beta cell physiology. The first diabetes disease modeling studies assessed the ability of patient-derived hiPSCs and healthy donor controls to efficiently differentiate into beta cells (175, 176). CRISPR-Cas9 genome editing has been exploited to correct point mutations associated with diabetes in patient-derived hiPSCs or to generate knockouts (KOs) of critical pancreatic and beta cell genes (177–181). Maxwell and colleagues showed that they were able to generate functional beta cells using a CRISPR-Cas9 edited iPSC line obtained from a patient with *WFS1* mutation. The corrected cells exhibited robust first- and second-phase insulin secretion in response to glucose challenge and restored euglycemia when implanted into diabetic mice, while the unedited controls did not (182).

Diabetes disease modeling studies based on hPSCs have been mostly focused on genes that cause neonatal diabetes, since the expected severe phenotype due to the developmental defect is assumed to be easier to detect. Together with patient-derived hiPSCs, several KO hPSC lines have been genome engineered to study neonatal diabetes disease phenotypes. Several reports have studied the outcomes of disrupting critical pancreatic developmental genes like *NEUROG3* (177), *PDX1* (183), *GLIS3* (184), *RFX6*, *PTF1A*, *MNX1*, *HES1*, *ARX* (178, 185), *GATA4*, *GATA6* (180, 181), or *SIX2* (186). Similar approaches have been exploited to dissect the disease mechanisms behind mutations in *HNF1B* (176) and *HNF4A* (187) that cause MODY or a rare mutation in *STAT3* gene causing neonatal diabetes (179). Genes that harbor genetic variants associated with increased risk of T2D have also been knocked out with CRISPR in hPSCs to study their role in beta cell development and function (e.g. *CDKAL1*, *KCNQ1*, *KCNJ11*) (188, 189).

Beyond genetic defects impairing pancreatic and beta cell development, those directly affecting beta cell insulin secretion are more challenging to study due to the current limitations of the hPSC-based models, in particular the functional immaturity of the derived beta cells. Genetic defects in K^+_{ATP} channel genes (64, 188, 190), the insulin gene (191, 192), or the ER-stress related genes *WFS1, YIPF5* and *MANF* (79, 80, 175) that cause neonatal diabetes and congenital hyperinsulinism have been modeled with hPSCs using diverse strategies. These include detailed characterization of the *in vitro* obtained pancreatic cell populations, their expression of relevant beta cell markers, their tolerance to different stresses, and their functionality in response to glucose and other secretagogues. Additional characterization with *in vivo* studies allows the assessment of how defective cells respond to systemic environment cues in terms of further differentiation, maturation, and acquisition of regulated insulin secretion. Phenotyping of the implanted cell populations may be particularly useful when the disease mechanisms do not clearly manifest *in vitro*. This is of particular importance when considering the modeling of strict insulin secretion defects, where the functionality of the beta cells generated *in vitro* may be too immature to correctly ascertain a particular phenotype.

In order to model insulin secretion defects reliably, we will need completely functional hPSC-derived beta cells that are as comparable as possible to the ones found in native human islets. Major obstacles in this quest are: i) the lack of differentiation protocols that robustly generate functional beta cells and are widely applicable to any hPSC line; ii) the high variability of human islet preparations, which makes them a problematic gold-standard control to rely upon; iii) the absence of standardized phenotyping methods for hPSC-derived beta cells and human islets which hinders faithful comparison of results across laboratories.

CHARACTERIZATION OF STEM CELL DERIVED BETA CELLS: HOW DO THEY COMPARE TO HUMAN ADULT BETA CELLS?

Beta cell differentiation protocols are technically complex: they have multiple stages, last over a month, and utilize combinations of recombinant proteins and small molecules at different dosages. During the course of any differentiation, many aspects can deviate from the optimal parameters, leading to poor reproducibility and consistency across experiments. In order to minimize experimental variation and minimize costs, laboratories differentiating hPSC usually implement standard operating procedures to prepare culture reagents and execute the differentiation experiments. Current approaches to characterize the outcomes of the hPSC differentiations towards beta cells rely on a battery of methods applied at select stages of the differentiation process. These methods commonly include, but are not limited to, flow cytometry, immunohistochemistry, and RT-qPCR. It is not uncommon for differentiation experiments to fail due to poor definitive endoderm induction, limited expression of pancreatic progenitor markers, or reduced number of INS+ cells. Since the differentiation of one cell type into the next is not 100% efficient, it is critical to address the identity of the cells in the population at given time points. The percentage of cells reaching definitive endoderm stage, the abundance of PDX1+NKX6-1+ pancreatic progenitors, and the fraction of INS+NKX6-1+ cells are examples of common flow cytometry quantifications. They are proxies for the efficiency and quality of the differentiation in terms of achieving bona-fide beta cells. The ultrastructure of hPSC-derived beta cells and human islets has been compared using electron microscopy, using insulin granule morphology as another indicator of beta cell maturity (121, 142, 153, 160).

Current differentiation protocols yield 40–75% INS+ cells in their later stages, although only 20–52% usually represent bona-fide beta cells expressing INS+NKX6-1+ (see an example of differentiation protocol presented in **Figure 2**). Furthermore, the proportions of cell populations can widely vary between experiments and different cell lines (148, 165). One of the important aspects of human pancreatic development that is still poorly understood is the fate allocation of the different endocrine cell types. The timing of NEUROG3 upregulation seems to influence the fate selection of the endocrine precursors (193), which have been shown to be unipotent (194). Endocrine

cell fate selection is likely determined by heterogeneous spatiotemporal signals present in the niche of the trunk domain endocrine progenitors. For example, different ligands of the EGF family can modulate the cell-fate selection: betacellulin was reported to promote differentiation into the beta cell lineage when added to mouse embryonic explant cultures (195). This effect was later shown be mediated *via* EGFR-PI3K/AKT-RAC1 signaling resulting in apical polarity inhibition, NOTCH signaling reduction, and induction of NEUROG3 expression (196).

In comparison with the stem cell differentiation outcomes, *in vivo* pancreatic development is also a highly heterogeneous process. Human islet endocrine cell composition varies depending on the islet size and location (161). It is also highly variable across individuals (30) and ages (197). This heterogeneity probably reflects the complexity of endocrine cell fate allocation during development and the plasticity of the pancreas to adapt during the life of the individual to the different metabolic needs.

At the transcriptomic level, gene expression profiling of stem cell-derived islet cells is determined by bulk RT-qPCR or RNAseq at different stages. Sorting of antibody-stained or INS-GFP reporter lines have been used to study beta cell transcriptomes. Several reports have compared the transcriptome of stem cell-derived islet and beta cells with human islets (121, 141, 142, 153, 160, 163, 198). Hrvatin and colleagues compared the transcriptome of stem cell-derived beta cells to both fetal and adult human beta cells (138). RNAseq analyses showed that the INS+ cells generated with that differentiation protocol were transcriptionally closer to fetal beta cells than to adult ones. They had reduced expression levels of genes associated with the functionality and maturation of the beta cell like *PDX1, NKX6-1*, *MAFA*, *GLI3*, and *MNX1.* Recent reports describing the generation of hPSC-derived beta cells with dynamic glucose stimulated insulin secretion (GSIS) have curiously shown that some important mature beta cell markers associated with functionality are expressed at much lower levels compared to adult beta cells (i.e. MAFA, UCN3, SIX3) (159, 163, 186, 198, 199). It is therefore unclear what should be considered a reliable marker of functional maturity for hPSC-derived beta cells, especially when the expression levels of some of these markers are age-dependent, being low in functional juvenile islets and taking years to increase (200).

Arising single cell technologies are generating a new important source of knowledge that can be utilized in the quest of generating better beta cells. Single cell transcriptomics, mass spectrometry, and epigenomics are providing new insights on the development and physiology of pancreas, islets, and beta cells (30, 201–208). Single cell transcriptomics has proven particularly useful to investigate the differentiation of hPSCs into beta cells by providing novel information about the heterogeneity of the stage-specific populations, their differentiation trajectories, the role of putative regulators of fate decisions, as well as a mean to assess the impact of diabetes-associated genetic variants. Using single-cell RT-qPCR, Petersen and colleagues explored the trajectories of stem cell derived pancreatic progenitors differentiating towards beta cells (209). They identified two pancreatic progenitor populations that give rise to "monohormonal" beta cells. This suggests the existence of alternative differentiation routes toward beta cells, *via* a progenitor stage that expresses NKX6-1 before or after NEUROG3 upregulation. Single-cell RNA sequencing approaches enable the transcriptional profiling of thousands of cells simultaneously. Krentz and colleagues used this approach to characterize mouse and hPSC-derived endocrine progenitors (210). Exploiting fluorescent reporter mouse strains and hPSC lines labeling Neurog3 lineages, they described and compared the heterogeneity of the mouse and human endocrine progenitor populations and the gene markers they express. scRNA-seq can also aid in the interrogation of the molecular mechanisms behind mutations causing neonatal diabetes (182, 192). Veres et al. used scRNA-seq to chart the differentiation trajectories of stem cell-derived populations, showing the presence of different endocrine and non-endocrine cell populations (159). Single cell transcriptomic technologies have been exploited to identify surface markers like ITGA1, which can be used to enrich for beta cells (159), or CD9 which can be used as a negative marker of functional beta cells (211). Similarly, single cell RNA sequencing analysis led to the observation that WNT signaling is reduced in endocrine cells compared to pancreatic progenitors. Chemical inhibition of WNT signaling in hPSC-derived progenitors induced differentiation to endocrine cells (212). By performing scRNA-seq on *in vitro* and grafted stem cell derived islets, Augsornworawat and colleagues were able to show that 6-month grafted cells undergo important transcriptomic changes, acquiring a gene expression profile more similar to human adult islets (199). scRNA-seq technologies thus offer a new window into the understanding of how transcriptomic regulation determines cell state. Part of its potential for the development of stem cell differentiation approaches relies on the direct comparison of the *in vitro* cells with their *in vivo* "real" counterpart. Enterprises like the human cell atlas are yielding body-wide datasets of single cell transcriptomic that are being used to benchmark *in vitro* stem cell differentiated cells (213).

The assessment of hPSC-derived beta cell functionality relies on methods established to characterize human islets. The most conventional method is the evaluation of insulin secretion in response to high glucose, either in a static setup or in a dynamic fashion using a perifusion setup. Additionally, different secretagogues can be used to probe the different insulin secretion mechanisms in place: K^{+}_{ATP} channel blockers (Tolbutamide), cAMP level modulators (Forskolin, IBMX), GLP1R ligands (Exendin-4, liraglutide), voltage dependent calcium channel agonists (Bay K 8644), non-glucose metabolic substrates (pyruvate, glutamine, leucine), and forced membrane depolarization (arginine, KCl).

The stimulation index (fold increase in insulin secretion from low to high glucose) of hPSC-derived islet cells in static GSIS reported by most studies ranges from 2 to 3, while human islet indexes have a median of about 7 (32). While static glucose stimulated insulin secretion is seemingly a straight-forward assay, there is a wide range of protocols used in the field for both hPSC-derived cells and human islets. They diverge in important critical points: the concentrations of low and high glucose used, the length of the stimulation, the number of cells/

aggregates/islets used for the test, the composition of the stimulation buffer, the washing steps, the length of the equilibration period, and the glucose concentration used during that time. Also, the selection of the samples for this assay is not always clearly reported: it is a common practice to hand-pick human islets of homogeneous aspect and size to perform GSIS, which might yield better results than randomly sampled islets. Many of these parameters and details may seem trivial but can introduce important systematic biases that make comparison of results across labs difficult. These comparisons could greatly benefit from the adoption of standardized practices in the functional assessment and phenotyping of both hPSC-derived cells and human islets. Furthermore, a stable positive control could be used in each GSIS test to have a reference point between experiments, but this is usually not possible due to the scarcity and high variability of human islets.

An important characteristic of human islets is their fine-tuned secretion of insulin in response to glucose. This can be clearly observed in dynamic GSIS assays using perifusion systems, where a robust first insulin secretion phase is followed by sustained second phase of insulin secretion with lower output (145). The acquisition of this biphasic insulin secretion pattern does not occur in human islets until birth (214). The levels of basal insulin secretion are also a good indicator of beta cell function. Immature beta cells have reduced glucose threshold for insulin secretion which leads to higher basal insulin secretion levels and a relatively low stimulation index (138, 215). Robust dynamic glucose stimulated insulin secretion of hPSC-derived beta cells has been only recently reported (160, 163). It seems to depend on a combination of abundant insulin positive cells in the aggregates, achieved either by high differentiation efficiencies involving late stage reaggregation in media containing no additional signaling cues and the expression of beta cell maturation marker SIX2 (163, 186), enrichment using fluorescent reporter lines (160) or surface marker antigens (159), followed by forced or spontaneous reaggregation, respectively. Interesting, Hogrebe et al. also showed that dynamic glucose stimulated insulin was achieved in beta cells generated using their planar differentiation protocol (153). A remaining challenge faced with hPSC-derived beta cell GSIS is the lower magnitude of insulin secretion in comparison to human islets. Davis and colleagues demonstrated that the disparity may be due to a metabolic bottleneck in the glycolytic pathway that can be ameliorated when glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK1) activities are bypassed (216).

Additional functional characterization has relied on surrogate indicators of insulin secretion, like the measurements of Ca^{2+} influx into the cytoplasm in response to different stimuli. Calcium imaging can be performed on dispersed individual cells or on whole aggregates/islets. It has been used to assess the function of hPSC-derived beta cells in some studies, showing that although calcium dynamics might be similar in a small fraction of cells, they are not as robust as in primary human islet cells (121, 142, 160). Electrophysiological studies of human beta cells using patch-clamp technique have demonstrated the electrical properties of their membranes in response to different stimuli (105, 217). In a recent study, Camunas-Soler and colleagues exploited Patch-seq technology to generate healthy and diseased human islet single cell transcriptomic profiles linked with their electrophysiological characteristics. This valuable dataset enabled them to generate predictive sets of genes that reliably linked gene expression to beta cell function and identify transcriptional programs that contribute to beta cell dysfunction in type 1 and type 2 diabetes (218). Basford and colleagues examined the electrophysiological properties of beta cells derived from an INS-GFP+ reporter stem cell. Compared to human adult beta cells, stem cell-derived beta cells presented heterogeneous K_{ATP} (45% of the cells) and Ca^{2+} (42% of the cells) channel currents and no Na^{+} channel currents (134). To the best of our knowledge, there are presently no reports of stem cell derived beta cells demonstrating electrophysiological properties identical to those of primary human adult beta cells.

Glycolysis coupled with efficient mitochondrial respiration is required for normal insulin secretion (219). During beta cell maturation, active DNA methylation silences the expression of disallowed genes (e.g. *HK1*, *LDHA*) that interfere with the glucose-secretion coupling (220). Neonatal acquisition of aerobic oxidative metabolism is a crucial step for the maturing beta cell, a process shown to be induced by non-canonical WNT4 signaling and estrogen related receptor gamma (ESRRG) (221, 222). Rates of O_2 consumption and CO_2 production can be used to evaluate the respiratory capacity of islets and hPSC-derived cells (223, 224), and serve as both a functionality and maturation surrogate marker in the efforts of making better beta cells (160, 221). Enrichment and reaggregation of hPSC-derived beta cells induced mitochondrial metabolic maturation, and the ultrastructure of mitochondria showed increased folding and stacking of cristae (160).

All hPSC-derived beta cell characterization approaches are ultimately benchmarked against human islets. Unfortunately, human islets typically demonstrate wide phenotypic variability across batches (30, 121). This particular point is frequently not suitably acknowledged, and it is particularly problematic when batches of poorly performing islets are used for the comparison to hPSC-derived beta cell preparations. Systematic evaluation of human islets batches at different levels (cell composition, functionality, transcriptomics, etc.) is a step in the right direction to highlight this variability and define a canonical human islet response (**Table 2**). This is illustrated by the Alberta Diabetes Institute IsletCore database collaborative initiative spearheaded by the MacDonald laboratory (32), where traceable phenotypes of over 300 human islets batches demonstrate the remarkable variation in functionality.

MODELING DIABETES WITH IMPLANTED STEM CELL DERIVED ISLET CELLS

An alternative approach to derive functionally mature hPSC-derived beta cells is to implant their precursors into immunocompromised host rodents. The first report describing this approach showed that a few months after implantation the grafts secreted human insulin in response to systemic glucose

TABLE 2 | Key characteristics of human islets.

Morphology: spheroid
Number of endocrine cells/islet: ~1500
Mean islet size: ~150 µm
Endocrine architecture and composition:
* Beta cells ~50–60% interspersed throughout the islet
* Alpha cells ~40% interspersed throughout the islet
* Delta cells ~10%
* Gamma and PP cells <1%

Stimulated insulin secretion
* High glucose-stimulated insulin challenge (static GSIS) stimulation index: ~7-fold
* High glucose-stimulated insulin challenge (dynamic GSIS): rapid biphasic response
* Potentiated secretion in response to cAMP modulators (e.g. Forskolin, IBMX)
* Potentiated secretion in response to incretin hormones (e.g. GIP, GLP-1)
* Increased secretion in response to membrane depolarization (e.g. KCl)
* Increased secretion in response to K_{ATP} channel activators (e.g. tolbutamide)
* Increased secretion in response to calcium channel agonists (e.g. Bay K 8644)
* Increased secretion in response to non-glucose nutrients (e.g. palmitate, leucine)

Key transcription factors and maturation markers: SIX2, SIX3, UCN3, MAFA, NKX6.1, INS, PDX1, GLIS3, MNX1
Morphology of mature insulin granules: dense core vesicles
Dithizone staining: brick red color
Respiratory capacity: primarily mitochondrial
Calcium signaling: increased pulsatile signaling in response to high glucose

List of various features of primary isolated human islets that can be taken into consideration for comparison to stem cell derived islet cells.

administration (125). Since then, multiple implantation sites (subcutaneous, intramuscular, renal subcapsular space, epididymal fat pad, pancreas) (121, 125, 225, 226) and several animal models (SCID-beige, NSG, NOG, NRG-Akita mice; nude rats) (125, 142, 227) have been employed with variable success.

Implantation in the renal subcapsular space is one of the preferred approaches since it is relatively easy to implant and retrieve the cells months later *via* survival nephrectomy. Upon implantation, cells become vascularized and interestingly their cytoarchitecture can undergo rearrangement (131), concomitantly with an increase in the functional maturation (121, 227). Endogenous pancreatic beta cells in recipient animals can be largely eliminated by administration of a beta cell toxin (e.g. alloxan or streptozotocin) either before or after implantation of hPSC-derived cells. The doses used are typically relatively harmless to the hPSC-derived cells owing to species differences in toxin sensitivity (228, 229). Graft function is monitored by measuring circulating human C-peptide levels (using assays that can distinguish the graft derived human *versus* recipient's endogenous C-peptide owing to sequence differences), and the response to glucose can be determined with intraperitoneal, intravenous, or oral glucose tolerance tests. Also, hPSC-derived pancreatic progenitors or endocrine cells can be transplanted within macro- or micro-encapsulation devices (132, 230–232).

An interesting phenomenon is the functional maturation of implanted beta cells with time. Rezania and colleagues described the progressive increase in circulating C-peptide levels for several weeks after implanting pancreatic progenitor cells or more matured cells (121, 131), a phenomenon which has also been observed by others (160, 192, 199). Furthermore, Rezania et al. observed faster diabetes recovery and achieved higher circulating C-peptide levels sooner when the implanted cells were further along in their differentiation prior to implant (121). It remains unclear what factors promote the apparently successful maturation of differentiated hPSCs post implant. One possibility is that immature hPSC-derived beta cells require a critical niche and systemic factors including vascularization and proper oxygenation to acquire full functionality (233). Interestingly, maturation of hPSC-derived pancreatic progenitors is accelerated in rats compared with mice (228), something that the authors correlated with increased levels of thyroid hormone in the rats, in line with the fact that thyroid hormone promotes beta cell maturation in rats (143) and in differentiating hPSCs (121). Sex hormones may also influence *in vivo* maturation of pancreatic progenitors since following implant, glucose-stimulated insulin secretion was observed in female mice before males (234). The systemic environment also provides periodic signals, which might entrain the circadian clock of the implanted stem cell-derived islet cells, leading to their functional maturation (235–238).

Several studies reporting the generation of hPSC-based diabetes disease models have relied on implanting mice with hPSC-derived beta cells. This constitutes a practical solution to study the function of the beta cells in a systemic environment, especially when the disease phenotype is not apparent *in vitro* (80, 175, 188, 191, 192). It is also useful to investigate the impairment of development caused by mutations perturbing critical regulators of islet cell development (177, 179, 180). Once grafts have matured, these models offer the possibility to dissect the effect of particular mutations on insulin secretion by carefully examining their responses to different stimuli.

An important aspect after implantation of hPSC-derived beta cells, is their capacity to survive the hypoxic environment of the implantation site until they become vascularized. This is a critical stress period that may result in the apoptosis of the most differentiated endocrine cells (239). Faleo and colleagues reported that this might be partially overcome by acclimatizing the cells to hypoxic conditions before implantation. In this regard, the format of the implanted cells likely also plays a critical role in successful engraftment, with smaller aggregates probably benefiting from faster vascularization kinetics as shown for engineered pseudoislets (240).

Another concern with stem cell derived islet cell implants, especially when using them on diabetic rodents, is the "pellet" effect, in which the non-regulated basal insulin secretion coming from immature beta cells might be enough to rescue hyperglycemia. This brings up the question of how many cells should be implanted to achieve an optimal working graft, which obviously will depend on the stage of differentiation and quality of the cells in terms of functionality (121). The composition and format (e.g. aggregate size) of the implanted cell population is also likely critical for a successful outcome. In fact, the formation of pancreatic progenitor cell aggregates prior to implant was shown to be essential for the formation endocrine cells (132). In order to investigate the *in vivo* maturation process of hPSC-derived beta cells, novel *in vivo* imaging technologies could be exploited. Radio tracer-based imaging of beta cell mass and function could prove to be particularly useful in this regard (241, 242).

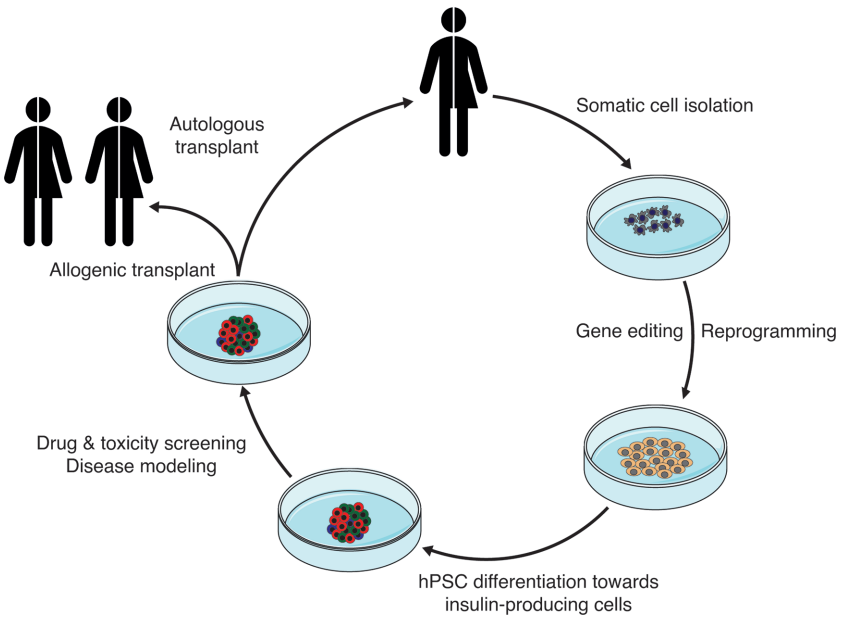

FIGURE 3 | Schematic showing the potential application of stem cell-derived insulin-producing cells for the treatment of diabetes.

Although intra islet paracrine signaling between the different endocrine cell types is crucial for fine-tuned insulin secretion (44, 45, 243), different reports have shown that diabetes can be rescued with nearly pure populations of islet beta cells with different efficiency (244, 245). Nair and colleagues described the implantation of 90% enriched hPSC-derived beta cells using an INS-GFP reporter cell line. While the grafts presented a few polyhormonal (INS+GCG+) cells 3 days after implantation, 8 weeks later, there was clear presence of GCG+ and SST+ monohormonal cells together with the beta cells, suggesting that the polyhormonal cells gave rise to monohormonal alpha and delta cells that likely mediate paracrine signaling contributing to optimal insulin secretion (160). The proportion of endocrine cell types in the implant to achieve the best glycemic control possible is an interesting question that requires further investigation.

Further understanding of the factors playing an important role in the functional maturation of implanted hPSC-derived beta cells will pave the way to the generation of better humanized mouse models to study insulin secretion. Ultimately, optimal control of implantation parameters will reduce the associated variability of these experiments enabling the careful assessment of the impact of genetic variants on insulin secretion.

CONCLUSION

Derivation of endocrine islet cells from hPSCs has become an attractive possibility to model diabetes disease and screen for new treatments (**Figure 3**). The progress in the last decade has made it feasible to obtain cells *in vitro* that closely resemble the native adult counterpart. Arising technologies like CRISPR-Cas9 genome editing and single cell transcriptomics are aiding in the generation of more reliable stem cell models, the refinement of the differentiation protocols and the characterization of the resulting differentiated islet cells. There are still important remaining questions in the quest for more functionally mature beta cells: how can we determine and increase beta cell specification? What are reliable mature beta cell markers and the key triggers of functional maturation? Together with detailed single cell transcriptomic characterization, improved characterization of the metabolism, proteomics and functional genomics of the hPSC-derived islets cells, and their comparison with human islets, will certainly pave the way forward. In this common effort, consensus in standardized characterization of the resulting hPSC progeny, the development of robust and reproducible differentiation protocols, and open dissemination of results, will enable prompt replication and speed up the implementation of successful strategies for beta cell generation and diabetes disease modeling.

AUTHOR CONTRIBUTIONS

Writing—original draft preparation, DB. Writing—review and editing, DB, DI, and TK. Funding acquisition, TK. All authors contributed to the article and approved the submitted version.

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REVIEW
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Modeling Type 1 Diabetes Using Pluripotent Stem Cell Technology

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Induced pluripotent stem cell (iPSC) technology is increasingly being used to create *in vitro* models of monogenic human disorders. This is possible because, by and large, the phenotypic consequences of such genetic variants are often confined to a specific and known cell type, and the genetic variants themselves can be clearly identified and controlled for using a standardized genetic background. In contrast, complex conditions such as autoimmune Type 1 diabetes (T1D) have a polygenic inheritance and are subject to diverse environmental influences. Moreover, the potential cell types thought to contribute to disease progression are many and varied. Furthermore, as HLA matching is critical for cell-cell interactions in disease pathogenesis, any model that seeks to test the involvement of particular cell types must take this restriction into account. As such, creation of an *in vitro* model of T1D will require a system that is cognizant of genetic background and enables the interaction of cells representing multiple lineages to be examined in the context of the relevant environmental disease triggers. In addition, as many of the lineages critical to the development of T1D cannot be easily generated from iPSCs, such models will likely require combinations of cell types derived from *in vitro* and *in vivo* sources. In this review we imagine what an ideal *in vitro* model of T1D might look like and discuss how the required elements could be feasibly assembled using existing technologies. We also examine recent advances towards this goal and discuss potential uses of this technology in contributing to our understanding of the mechanisms underlying this autoimmune condition.

Keywords: induced pluripotent stem cells, type 1 diabetes, macrophages, antigen presenting cells, T cells, T cell receptor

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an autoimmune disorder disease involving the specific destruction of insulin-producing pancreatic beta cells (1). Beta cell loss leads to primary insulin deficiency and subsequent hyperglycemia, which presents as clinical diabetes. A complex interplay of genetic and environmental factors is thought to trigger beta cell specific autoimmunity. The disease predominantly affects children and young adults and current estimates suggest that more than a million children around the world are affected by T1D, with the prevalence rising by almost 3% each year (2) Our knowledge of how the disorder develops remains imperfect and therefore attempts at preventing or curing the disease have largely not met with success (3).

A major deficit in understanding human T1D has been the lack of appropriate models. While multiple therapeutic interventions have been found effective in the Non obese diabetic (NOD) mouse T1D disease model, none have been translatable to humans (4). This has led investigators to question rodent models as a platform for testing disease therapeutics and has also resulted in a quest for human T1D models (5).

An idealized *in vitro* model of T1D would necessarily enable the incorporation of the large number of variables and cell types that have been implicated in disease development. Indeed, understanding how different cell types and environmental factors interact to contribute to the pathogenesis of T1D will be critical for development of new models.

DISEASE PATHOGENESIS

Current understanding is that T1D is precipitated in genetically susceptible individuals by environmental triggers such as infections, diet, toxins or stress, which initiate the autoimmune response against beta cells. Failure of immune tolerance results in the expansion of autoreactive CD4+ and CD8+ T cells, autoantibody-producing B cells, and activation of the innate immune system, which then collude to lead to beta cell destruction (6, 7).

Most of our understanding of disease pathogenesis has been deduced from rodent models such as the NOD mouse (4). However, emerging data from human biobanks such as the Diabetes Virus Detection (DiViD) study (8) and the JDRF Network for Pancreatic Organ Donors (nPOD) (9) have yielded important details of the human disease pathology and highlighted the differences in rodent and human disease patterns.

The histological hallmark of the disease is the presence of insulitis, i.e., an infiltration of inflammatory cells consisting of T and B lymphocytes and macrophages around and within islets (10, 11). Although variable between subjects, CD8+ T cells have been found to be the predominant immune cell type in the insulitic lesion, followed by CD68+ macrophages, CD4+ T cells and CD20+ B cells (11, 12).

Abbreviations: iPSCs, Induced pluripotent stem cells; T1D, Type 1 Diabetes Mellitus; APC, Antigen presenting cell; TCR, T cell receptor.

OVERVIEW OF PATHOGENESIS

There are numerous hypotheses regarding the events initiating the processes that eventually lead to T1D. For example, a triggering event, such as a viral infection, may lead to an initial phase of beta cell death causing release of beta cell autoantigens (13). MHC Class I hyper-expression has also been noted on beta cells from T1D tissue samples, potentially making these cells prone to attack from self-reactive cytotoxic CD8+ T cells and further antigen release (14). Islet autoantigens are phagocytosed by antigen presenting cells in the islets, and carried to the draining pancreatic lymph nodes where they are presented to CD4+ and CD8+ T cells (15). Due to loss of central and peripheral tolerance, these self-antigens are recognized by autoreactive CD4+ T cells leading to their activation and proliferation. B cell activation leads to formation of plasma cells and the appearance of autoantibodies against islet proteins (16). These immune cells then infiltrate the islets leading to insulitis and progressive beta cell death (17).

With the initiation of the autoimmune attack, inflammatory cytokines are released which amplify the immune response. These include IL1 and 6, IFNγ, and TNFα. It has been suggested that some of these cytokines directly precipitate beta cell destruction, diminishing insulin secretion from the beta cells and activating cytotoxic T cells (18). They also enhance the expression of HLA class 1 molecules on the beta cells (19). Production of superoxide radicals and high concentrations of nitric oxide increase the damage to the beta cells (18). Inevitably, this damage leads to the further release of beta cell antigens, which may serve to create a feedback loop that reinforces ongoing beta cell destruction. This process is summarized in **Figure 1**.

THE CASE FOR ESTABLISHING HUMAN STEM CELL-BASED MODELS OF T1D

Studying the disease pathogenesis of human T1D has been challenging for many reasons. The retroperitoneal location of the organ and the inherent risk of pancreatitis make pancreatic biopsies a risky procedure (20, 21), leading to an understandable scarcity of pancreatic tissue samples from affected individuals. The scattered and sparse nature of the insulitic lesions means that multiple tissue samples from one organ are needed for a comprehensive analysis. The tissue itself is difficult to handle due to the high content of pancreatic enzymes, predisposing it to autolysis. In addition to this, T1D has a long pre-symptomatic phase which means that affected individuals only present with established disease where most of the beta cell mass has been lost, making the study of the early disease pathogenesis difficult (5). Therefore, mouse models have been widely used as surrogates of the human disease. However, recent studies on human pancreata have brought to light important differences between human and rodent disease patterns (8, 9, 22).

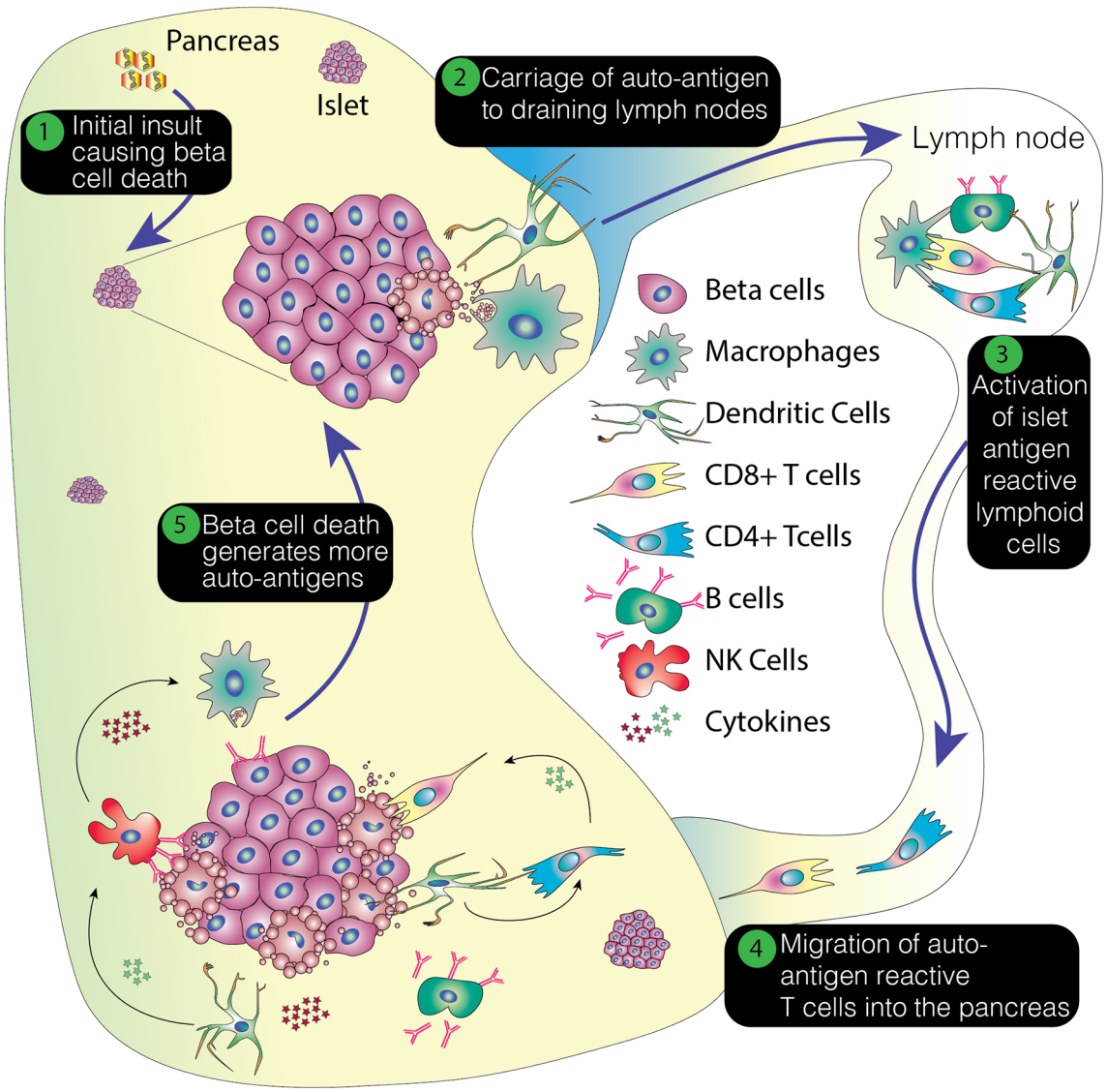

FIGURE 1 | Overview of the pathogenesis of T1D. This process envisages an initial insult that creates beta cell stress or death. The former potentially leading to the production of neoantigens and the latter resulting in the release of beta cell proteins. This damage results in the attraction of immune cells, with emigrant antigen presenting cells picking up and processing the proteins and conveying them to the pancreatic lymph nodes. Here, autoreactive or neoantigen specific T cells are recruited and these then migrate back to the pancreas, potentially promoting further inflammation, stress and cell death. This positive feedback loop ultimately results in the loss of beta cells.

ANIMAL MODELS—THE NON-OBESE DIABETIC (NOD) MOUSE

NOD mice are prone to spontaneously developing autoimmune diabetes, which mimics many features of human disease such as, islet infiltration by immune cells and the development of autoantibodies (23). However, in distinction to humans, affected mice display an intense insulitis including a peri-insulitic pattern of heavy infiltration, with clusters of lymphocytes often resembling tertiary lymphoid organs. A marked decrease in islet insulin content is seen after week 12 and, after a median period of 18 weeks, diabetes develops in most female mice (22).

HUMAN DATA AND HUMAN BIOBANKS

In recent years, biobanks have been established to collect pancreatic tissue specimens from donors with type 1 diabetes, autoantibody positive individuals, type 2 diabetics and healthy controls. The Diabetes Virus Detection study (DiViD) is one such biobank. DiViD was setup in Norway with the unique premise of collecting pancreatic biopsies from live adult patients newly diagnosed with type 1 diabetes (20). While this study provided valuable information, it also demonstrated the dangers associated with this investigative approach, as serious procedural complications in three out of the six enrolled patients

(postoperative leak and bleeding) led to early termination of the study (24). In contrast to the small scale focused DiViD study described above, the Juvenile Diabetes Research Foundation established a multicenter collaborative effort, the Network for Pancreatic Organ Donors with Diabetes (nPOD) in 2007. This tissue biobank supports collection of tissue samples from donors with T1D, autoantibody positive individuals, people with type 2 diabetes (T2DM), and pancreas transplant recipients (with T1D) (25, 26).

What have human data shown and what are the differences when compared to animal models?

Analysis of human data suggests that the presence of insulitis in human T1D is much lower than in the NOD mouse with only 10-30% of islets affected on average, however, the variation between patients is large with the DiViD study showing that insulitis varied between 5 and 58% in patients (8, 27). Insulitis was mainly seen to affect the insulin positive islets (33%) with low levels of inflammation found in insulin negative islets (2%) indicating that inflammatory cell influx is predominantly seen in the early phase of the disease with efflux of cells following beta cell destruction and depletion of autoantigen targets (9).

Another important finding from human studies has challenged the classical dogma that >90% of beta cell mass is lost at the time of disease presentation. In fact, it was found that individuals who develop diabetes beyond their teenage years may retain as many as 40% of their insulin positive islets (28), confirming previous knowledge that the disease process is more fulminant in younger cases.

The distribution of insulitis and the degree of immune cell infiltration in human samples is also found to be very different when compared to the NOD mouse (29). While the NOD mouse has been a useful model to study autoimmune diabetes, available human data is bringing to light important differences in pathology between human and rodent disease patterns. It is possible that these differences go some way to explaining why interventions that have been successful in either preventing or reversing the disease process in the NOD mouse have not yielded similar outcomes in human clinical trials (4, 30).

Although studies from human biobanks have gone some way towards filling the gaps in our knowledge, our understanding of the human disease is still incomplete. Issues of limited tissue availability still remain valid, particularly in the current era of enhanced modern diabetes management where death from diabetes related complications is rare. Therefore, there is an urgent need for alternative human models of the disease, which can address species specific aspects of human physiology and allow the study of interventions for disease prevention. This need has therefore paved the way for stem cell derived *in vitro* human disease models.

NEWER WAYS OF MODELING DISEASE: PLURIPOTENT STEM CELLS

Disease models for T1D are necessary for understanding disease pathogenesis, as a platform for testing potential immune-modulatory therapies and for designing beta cell preservation strategies. *In vivo* studies on immune modulatory therapies have mainly been carried out in NOD mice, and whilst some of these therapies showed success in mice, this success has generally not translated to provide equal efficacy in human disease. This highlights the importance of having species specific disease models, which reflect the complexity and heterogeneity of the human disease process. A human *in vitro* disease model could provide a complementary experimental resource for studying the pathophysiology of the human disease and also for designing potential treatment strategies.

IPSCS GENERATION FROM HUMAN T1D SUBJECTS AND THEIR USE IN STUDYING DISEASE PATHOLOGY

iPSC technology provides an opportunity to generate patient specific cell lines that can be differentiated into tissues of interest and then be used for modeling disease pathology or potentially for cell replacement therapy. iPSCs have been generated from individuals with many different forms of diabetes including T2DM (31), cystic fibrosis related diabetes (32), neonatal diabetes, forms of monogenic diabetes (33, 34), maturity onset diabetes of the young (35–37) and T1D (38).

iPSCs have been successfully used to create human models of diabetes caused by monogenic disorders that effect beta cell development and function such as Wolfram syndrome (33) and insulin gene mutations (34). These experiments not only demonstrated the success of iPSC technology for modeling disease phenotypes but also provided proof of principle data for correcting the disease phenotype.

However, the potential of this system to investigate acquired forms of diabetes has only recently been investigated. T1D is a complex disease to model *in vitro* as the disease has a polygenic inheritance pattern with a heterogenous presentation and a strong influence of environmental factors as potential triggers of autoimmunity. Therefore, simply generating beta cells *in vitro* will likely be insufficient to reproduce the conditions that reflect the *in vivo* disease. Immune cells that have been identified in insulitis lesions from human pancreas, such as CD8+ T cells, CD4+ T cells, macrophages, dendritic cells and B cells (11) would also need to be generated *in vitro* and then co-cultured together with beta cells so as to mimic the pathologic process in the pancreas (39, 40). However, because of the complexity of T cell development, it is unlikely that methods for the generation of autoreactive T cells from iPSCs will be straightforward. As such, it is likely that, in the first instance, autoreactive T cells will need to be obtained from T1D tissue/cell donors. In such a scenario, immune interactions in the disease process could potentially be modeled by recreating key components such as antigen presenting cells and beta cells *in vitro* whilst obtaining autoreactive T-cells from *in vivo* sources.

A number of groups have examined iPSC derived beta cells in the context of T1D tissue donors (38, 41, 42) (**Table 1**). Maehr et al. were one of the first to report the generation of iPSC from

TABLE 1 | Generation of iPSCs and relevant cell types from T1D individuals.

Cell type	Starting cell	Results	Reference
Beta like cells	iPSCs generation from fibroblasts of 2 T1D individuals	Insulin & c-peptide positive beta like cells which were glucose responsive	Maehr et al. (38)
Islet like cells	iPSC from skin fibroblasts from 3 T1D individuals and 1-ND	Significant intra-individual variability found with only 1 of 3 iPSC clones from each donor being able to generate INS-positive cells	Thatava et al. (41)
Beta like cells	iPSC from skin fibroblasts from 3 T1D individuals and 3-ND	Generation of C-peptide+/NKX6-1 + glucose responsive beta cells with the ability to ameliorate alloxan induced diabetes in mice. No differences in morphology, marker profile, gene profile, functionality and propensity to cytokine induced stress seen in T1D versus ND iPSC-beta cells.	Millman et al. (42)
Beta like cells	iPSC from peripheral blood from 3 T1D individuals and 1-ND	iPSC beta cells from both, on undergoing ER stress elicit an immune activation response from autologous T cells from both T1D and non-diabetic individuals. T cell activation is specific to beta cells and exposure to iPSC- alpha cells elicits minimal immune activation.	Leite et al. (43)
Macrophages	iPSC from PBMC of 1 T1D individual & 1-ND	iPSC Macs displayed mature morphology and surface marker profile with ability of phagocytosis and capacity to process complex protein mixtures and present relevant epitopes derived from proinsulin C-peptide to TCRs derived from autologous islet infiltrating T cells leading to their activation.	Joshi et al. (44)

T1D, Type 1 Diabetes; ND, Non diabetic; PBMC, Peripheral blood mononuclear cells; ER, endoplasmic reticulum.

individuals with T1D and to differentiate these into beta-like insulin producing cells that were glucose responsive (38). More recently, Millman et al. reported on the generation of iPSCs from three T1D donors and compared their differentiation potential to iPSCs from non-diabetic individuals. Their study showed that beta cells derived from T1D iPSCs were similar to those from non-diabetic individuals in terms of their surface marker expression profile, morphology and *in vitro/in vivo* insulin secretion capacity. They were able to demonstrate that post transplantation into mice, T1D derived iPSC beta cells were equally efficient at rescuing the phenotype of alloxan induced diabetes (42). These experiments reinforce the view that T1D arises from factors that are not intrinsic to the beta cells, whether they are immune cells or environmental triggers. Consistent with this, Millman et al. also examined how the cytokine environment may contribute to beta cell death in the context of type 1 diabetes, hypothesizing that T1D beta cells may be more prone to cytokine induced damage. However, they found that beta cells derived from both diabetic and non-diabetic individuals were equally sensitive to cytokine induced stress, with both showing loss in expression of beta cell markers post exposure to inflammatory cytokines (42). This study therefore further underlines the fact that for studying T1D pathogenesis analyzing islet biology in isolation will not be sufficient and interactions with the immune system will be the key to understanding the complex mechanisms of autoimmune beta cell destruction.

A recent study by Hosokawa et al. described the generation of iPSCs derived from patients with fulminant diabetes, a subgroup of the Type 1b non-autoimmune or idiopathic T1D (45). The pathogenesis of this kind of diabetes, which has almost exclusively been reported from Japan, is not well understood. It differs from classical T1D by the rapidity of onset of symptoms, the degree of hyperglycemia and severity of ketoacidosis, almost complete loss of beta cells along with variable alpha cell loss, absence of islet autoantibodies and elevated levels of pancreatic enzymes (46). In distinction to work above related to classical T1D, Hosokawa and colleagues found beta cells generated from fulminant diabetes individuals had an increased sensitivity to proinflammatory cytokine induced damage (45). However since the pathogenesis of this kind of diabetes is believed to be different to classical Type 1A autoimmune diabetes, these results may not be extrapolatable to the classic human T1D disease pathology (46).

In a recent study, Leite et al. simulated T1D relevant immune interactions in an *in vitro* system. They generated iPSC derived beta cells and exposed these cells to ER stress, attempting to replicate the pro-inflammatory islet environment in T1D (**Table 1**). Interestingly, their experiments suggested that stressed iPSC derived beta cells elicit an immune activation response from autologous T cells from both T1D and non-diabetic individuals. They thus concluded that beta cells from T1D individuals are healthy to begin with and the process of islet inflammation makes them stressed and vulnerable to T cell mediated autoimmune destruction (43). This sequence of events is consistent with the hypothesis that an initial assault on beta cells, such as viral infections or an environmental toxin, trigger beta cell damage or stress, which subsequently leads to immune attack and widespread beta cell destruction.

The above experiments have focused on beta cells and their interaction with effector cells of the immune system, particularly CD8+ cytotoxic T cells. However, most current hypotheses regarding the genesis of T1D implicate CD4+ helper cells as underlying drivers of disease because of the very strong genetic association with HLA-class II (47). *In vivo*, the involvement of CD4+ requires the presence of antigen presenting cells: cells that could potentially take up and process antigens from damaged beta cells. For this reason, we investigated the generation of iPSC derived macrophages from individuals with T1D. We were able to demonstrate that these antigen presenting cells had mature functionality and were able to process and present islet lysate and purified synthetic C-peptide to autologous islet infiltrating CD4+ T-cells (44) (**Table 1**). Clearly, the major benefit of the iPSC system is the capacity to exactly match the HLA alleles of antigen presenting cells (APCs) to donor derived T cells. This important feature will be the key advantage of future iPSC-based models of T1D.

AN IDEALIZED MODEL OF T1D

The complexity of T1D pathogenesis presents a number of challenges for efforts to create an *in vitro* model that can be used to interrogate the many factors contributing to ongoing islet cell destruction. Key variables that would need to be addressed by a potential stem cell model of T1D include the different cellular insults used to trigger beta cell death or stress, the presence or absence of particular cell types, and, because T cells are an important player, HLA class matching. In order to visualize how such a model could be constructed, it is first necessary to briefly outline what is known about the role that putative environmental triggers and incriminated cell types play in disease onset and progress (**Figure 2**).

Model Inputs

Initiating Insults

Genetic Predisposition

T1D has a complex inheritance pattern with genome-wide association studies identifying more than 60 disease susceptibility loci. The HLA complex has been shown to have the strongest association with T1D and more than half the genetic susceptibility is attributed to this region. Other important loci are the cytotoxic T lymphocyte antigen 4 (CTLA4, IDDM12 locus), PTPN22 (gene encoding lymphoid tyrosine phosphatase) and the IL2RA (interleukin 2 receptor A) locus (48). Most of these loci are associated with immune regulation. Therefore, using iPSCs from T1D individuals carrying high-risk genetic susceptibility alleles would allow the factoring in of underlying genetic factors, which play a role in modulating autoimmunity and mechanisms of impaired immune tolerance in T1D, which lead to disease predisposition.

Environmental Factors

There is a complex interplay between genetic disease susceptibility and environmental triggers of autoimmunity. Factors such as viral infections, diet and toxins are believed to be potentiators of islet autoimmunity (49, 50). While an association has been reported with most of these factors, a direct causal relationship has yet to be found. Enteroviruses have been most commonly implicated in disease pathogenesis and are believed to either lead to direct beta cell destruction or to initiate autoimmunity because of molecular mimicry between enteroviral proteins and beta cell antigens (51). Similarly, various dietary factors such as cow's milk protein, gluten exposure and Vitamin D have long been implicated in the pathogenesis of T1D, however, their roles are still not very well defined. The beta cell stress hypothesis postulates that a combination of any of these factors leads to a state of beta cell endoplasmic reticulum stress promoting generation of neoantigens *via* post-translational modification of islet proteins (52–55).

Any *in vitro* model that seeks to study the entire autoimmune pathogenesis of T1D will likely have to incorporate these triggering factors which link beta cell stress to autoimmunity. It can be envisaged that certain factors such as viruses and their role in beta cell infection and stress could easily be studied in an iPSC derived *in vitro* model of T1D.

The Role of Individual Cell Types

Beta Cells

Most protocols designed to promote the *in vitro* differentiation of beta-cells from PSCs rely on recapitulating the key developmental steps, which occur during embryogenesis (56). Therefore, an understanding of the steps of early pancreatic organogenesis is essential. Most of the knowledge of

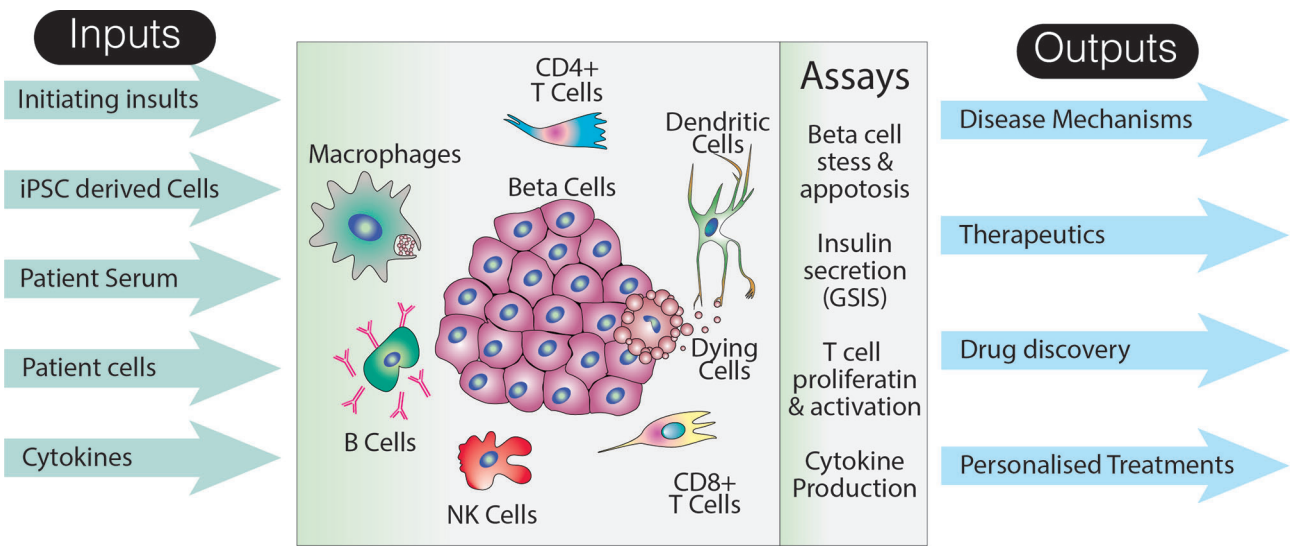

FIGURE 2 | Utility of an idealized model of T1D. The schematic suggests various inputs to the model, how the effects of these inputs could be assayed, as well as long term potential outputs. Note that this representation of the model does not specifically include a host of non-immune and non-endocrine cells which may also impact on the disease pathogenesis. Additionally, although a limited number of assays are shown, analyses specific to particular cell types are likely to increase the breadth and depth of data that could collected from such a system.

developmental biology and signaling pathways involved in pancreatic lineage specification has come from studying rodents (40, 57).

The development of protocols for generating functional endocrine cells has been hampered by a lack of knowledge of pathways involved in final stages of beta cell differentiation. The initial protocols generated cells which though insulin positive, were bi-hormonal and failed to secrete insulin in response to glucose in a glucose-stimulated insulin secretion (GSIS) assay (58–60). However, more recent protocols have described the generation of more mature cells, with development of insulin positive mono-hormonal cells which, show function both *in vitro* and *in vivo*, are glucose responsive, ameliorate diabetes in mice models and are transcriptionally more similar to native beta cells (61–65). Protocol development is an ongoing process, with recent improvements enabling the generation of beta like cells which have a more physiological glucose secretion profile and show appropriate dynamic insulin secretion to high and low glucose challenges (66, 67). However, in spite of these advances, *in vitro* derived beta cells are still ontologically and functionally immature when compared to adult beta cells, with a lower insulin secretion per cell at high glucose, lower glucose stimulation, slower first-phase insulin release and persisting differences in gene expression profiles (68). Studies using single cell RNA-sequencing techniques for transcriptomic profiling of *in vitro* derived beta cells will contribute to a more refined understanding of beta cell maturation pathways and help in development of more evolved beta cell generation protocols (69).

The maturity of the beta cells may indeed be an important factor determining their susceptibility to T cell mediated cell death. Similarly, death induced by cytokines may also be affected by beta cell maturity. It is also conceivable that the degree of functional and transcriptional maturity might also affect the cell's susceptibility to the initial triggering insult which sets of the process of beta cell death and autoimmunity, potentially a viral insult. With advances in the development beta cell differentiation protocols, generation of mature, functional beta cells that are transcriptionally similar to adult beta cells may become possible. Development of functional adult like beta cells is likely to be an important aspect of *in vitro* models attempting to study T1D *in vitro*.

Antigen Presenting Cells

Following an initial insult, macrophages and dendritic cells within the islet and/or draining lymph node are the first responders, phagocytosing cellular debris and processing it for antigen presentation. This is believed the to be the key step in the initiation of autoimmunity and involves the presentation of beta cell autoantigens by professional antigen-presenting cells to CD4+ T cells *via* HLA class II, leading to subsequent T cell activation (15). In studies on pancreata from human subjects with T1D, macrophages have been found to be an important part of the islet infiltrate, thereby underlining their role in the immune events, which precipitate autoimmune diabetes (11, 70). Macrophage depletion and functional inhibition have been shown to reduce the development of autoimmune diabetes in rodent models (71–73). Similarly, NOD mice deficient in CD103+ DCs were found to have a reduced islet infiltration of autoreactive T cells and a corresponding reduction in diabetes incidence (74). Finally, macrophages and DCs may also be involved in causing direct beta cell death by the secretion of proinflammatory cytokines such as interleukin 1-beta (IL1β), tumor necrosis factor alpha (TNFα) and ROS (70, 75). These experiments clearly indicate that antigen presenting cells are likely to play a part in initiating and propagating the T cell mediated autoimmune attack against beta cells.

In an idealized model of T1D, both macrophages and dendritic cells could be generated from iPSCs using established protocols (**Table 2**). In many protocols, PSCs are guided through the sequential stages of hematopoietic development using by stage specific growth factors and cytokines. Induction of mesoderm is achieved by the use of BMP4, activin and FGF2 followed by the addition of VEGF, SCF and FGF to generate hematopoietic precursors. Developing myeloid cells, which are shed from the cultures, are harvested from the supernatant and matured using M-CSF with or without IL3. These cells then pass through an intermediate monocyte stage where CD14+ monocytes can be collected using flow cytometric sorting. Macrophages can then be matured in adherent cultures using high concentrations M-CSF and activated using either Lipopolysaccharide (LPS)/IFNg (classic activation) or IL4 (alternate) activation (86).

In vitro PSC derived macrophages have been shown to have similar phenotypic, functional, and transcriptomic characteristics to peripheral blood monocyte derived macrophages (78, 87, 88), suggesting they could be used to provide the antigen processing and presentation functions thought to be key steps in T1D initiation and maintenance. Ideally, iPSCs would be derived from T1D donors from whom islet antigen specific T cells were also available, enabling the interactions between these two cell types to be assessed in a fully autologous HLA setting. However, as noted below, creating or isolating such T cells is likely to be major obstacle to the generation of an authentic *in vitro* model of T1D.

The developmental identity of *in vitro* derived macrophages however remains to be resolved. Most protocols promoting the *in vitro* hematopoietic differentiation of pluripotent stem cells are believed to create cells resembling those generated from embryonic primitive hematopoiesis rather than those derived from adult definitive hematopoiesis. Indeed, there is evidence that PSC-derived macrophages have a primitive embryonic-type macrophage phenotype, predominantly because they expand in the absence of cMyb, a transcription factor required for definitive hematopoiesis (89). However, differences, if any exist, between embryonic and adult origin macrophages in terms of function remains to be elucidated. Moreover, macrophages of fetal origin continue to exist in adult humans in the form of tissue resident macrophages, which self renew with a minimal contribution from adult blood monocytes (90). Therefore *in vitro* derived macrophages may have a role in modeling the functions of these specialized tissue resident macrophages as well (91).

Protocols for the production of dendritic cells from PSCs are limited and most rely on derivation of cells of the myeloid lineage

TABLE 2 | Generation of T1D relevant immune cells from PSCs.

Cell type	Starting cell	Method	Cell functionality and maturity	Reference
Dendritic cells (DCs), monocytes and macrophages	hiPSC-	OP9 mouse stromal cell co-culture for generation of hematopoietic precursors, followed by further differentiation along the macrophage/DC pathway with the use of GM-CSF/M-CSF, Flt3L, SCF and IL1β	DCs capable of cytokine secretion and antigen presentation and activation of allogenic and autologous HLA matched T cells. Macs showed ability of phagocytosis and antitumor activity	Senju et al. (76)
Monocytes and Macrophages	hiPSC-	Embryoid body formation followed by hematopoietic specification by IL-3/M-CSF combination with high dose M-CSF for terminal differentiation	iPSC Macs capable of phagocytosis pro inflammatory cytokine release on LPS stimulation	Lachmann et al. (77)
	hiPSC-	Monolayer culture on a layer of matrigel, an extra- cellular matrix component by using stage specific hematopoietic cytokines to generate monocytes followed by differentiation to macrophages using high dose M-CSF	iPSC Macs showed capacity of bacterial and tumor cell phagocytosis along with relevant cytokine and chemokine release. Phenotypic, functional, and transcriptomic characteristics to peripheral blood monocyte derived macrophage	Cao et al. (78)
	hiPSC-	Embryoid body formation using rotational cultures followed by hematopoietic differentiation using IL-3, M-CSF,GM-CSF and FLT3-L combination with high dose M-CSF for terminal differentiation to mature macrophages. Activation using IFN-γ	iPSC Macs displayed mature morphology and surface marker profile with ability of phagocytosis and capacity to process complex protein mixtures and present relevant epitopes derived from proinsulin C-peptide to TCRs derived from autologous islet infiltrating T cells leading to their activation.	Joshi et al. (44)
Dendritic cells	hiPSC-	Embryoid body formation followed by guided differentiation using hematopoietic growth factors and final DC differentiation using GM-CSF and IL-4	Generation of CD141+ DCs with ability of phagocytosis and features reminiscent of tolerogenic DCs as evinced by capacity of IL-10 secretion, reduced capacity of immunostimulation and polarization of naïve CD4 cells to Tregs.	Sachamitr et al. (79)
	iPSCs derived from an individual with Sjögren's syndrome	Co-cultured on C3H10T1/2 mouse mesenchymal cells to differentiate into hematopoietic cells	Generation of CD141+ myeloid DCs with ability of phagocytosis. Mature functionality as demonstrated by capacity to stimulate allogenic T cells and present antigen to and activate autoreactive CD4+ T cells.	Iizuka-Koga et al. (80)
Antigen specific T cells	iPSC and hESCs	Directed differentiation and artificial thymic organoids (containing DLL expressing mouse stromal cells). Lentiviral vector introduction of transgenes encoding antigen specific TCRs	Antigen recognition and antigen specific activation CD8+ T cells	Montel-Hagen et al. (81)
	iPSCs from tumor infiltrating CTLs	Mesoderm differentiation followed by co-culture on DLL1 expressing OP9 stromal cells	Antigen recognition and antigen specific activation of CD8+ T cells	Nishimura et al. (82)
	iPSCs from antigen specific lymphocytes	Mesoderm differentiation followed by co-culture on DLL1 expressing OP9 stromal cells	Antigen recognition and antigen specific activation of CD8+ T cells.	Nagano et al. (83)
NK cells	iPSCs from umbilical cord blood	Embryoid body formation, hematopoiesis induction and expansion of NK cells with IL7	*In vitro* and *in vivo* killing of ovarian cancer cell lines.	Hermanson et al. (84)
	iPSCs from peripheral blood	Directed differentiation	Target cell killing and antibody mediated cytotoxicity	Zeng et al. (85)
Antigen specific B cells		N.D		N.D

M-CSF, Macrophage colony stimulating factor; GM-CSF, Granulocyte Macrophage colony stimulating factor; FLT3L, fms like tyrosine kinase 3 receptor ligand; IL-3, Interleukin 3; IL1β, Interleukin 1β; LPS, lipopolysaccharide; IFN-γ, interferon gamma; hESCs, human embryonic stem cells; CTL, cytotoxic T lymphocytes; IL-7, interleukin 7; ND, not done.

as described above, followed by the further differentiation towards a dendritic cell lineage with the use of GM-CSF and IL4, mirroring methods for deriving DCs from peripheral blood. Most protocols describe an intermediate monocyte stage (92–94) following which blood cells in suspension are harvested and matured in media containing GM-CSF and IL4. These immature DCs then undergo a final maturation step with the use of proinflammatory stimuli like LPS, TNFα (94, 95) or IFNγ, IL1β, PGE2 (79, 93).

Functionally, these DCs have been found to have a cytokine profile, chemotaxis ability and capacity for allogenic T cell stimulation, which is reminiscent of peripheral blood derived myeloid DCs (92, 93, 95). Finally, the antigen presenting functions of iPSC-derived DCs have been used to characterize T cell responses in Sjogrens syndrome, thereby demonstrating that the antigen presenting functions of iPSC derived APCs such as dendritic cells can be used to study the repertoire of pathogenic T cells in autoimmune disorders (80).

Lymphocytes in Type 1 Diabetes

T cells are thought to be the primary mediators of beta cell loss in T1D, with cytotoxic CD8+ T lymphocytes mainly responsible for causing beta cell death. The evidence supporting their key role in pathogenesis includes the ability of beta cell specific CD8+ and CD4+ T cell clones to transfer T1D to immunocompromised hosts (96). Furthermore, the use of an anti-CD3 antibody has been shown to reverse T1D in the NOD mouse model (97); a result that has translated to humans with anti-CD3 antibodies shown to preserve beta cell function in recent onset T1D (98) and to delay diabetes progression in secondary prevention trials (99).

CD8+ Cytotoxic T Cells

Cytotoxic T lymphocytes (CTLs) are the most common immune cell found in insulitic lesions in human T1D pancreatic specimens (100). They recognize beta cell autoantigens presented by HLA class I expressed on the beta cell surface. CTLs can lead to beta cell death by a variety of mechanisms including the induction of molecules involved in the granule exocytosis pathway such as perforin, granzyme, or granulysin, as well as increased surface expression of death inducing molecules such as Fas ligand and TNF-related apoptosis inducing ligand (101, 102). Autoreactive CD8+ T cells from insulitic lesions in human T1D pancreatic specimens have been found to react to known islet autoantigens such as insulin, islet amyloid polypeptide (IAPP) and islet- specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), pre-proinsulin, GAD65, pre-proislet amyloid protein, and IA-2, providing direct evidence for involvement of these cells in autoimmune beta cell destruction (14).

CD4+ Helper T Cells

CD4+ T helper cells are a key player in the pathogenesis of Type 1 diabetes mellitus and have been consistently identified in the inflammatory infiltrate of islets from T1D patients (27). The strong association of HLA class II molecules with the genetic disease susceptibility risk also underscores the important role of CD4+ T cells in the pathogenesis of the disease (48). While CD4+ T cells do not lead to direct beta cell killing, they are important effector cells involved in pro-inflammatory cytokine secretion, which amplify and propagate the immune response and lead to activation of immune cells such as macrophages and CD8+ cytotoxic T cells (102).

Characterizing the antigenic repertoire of these autoreactive T cells has been difficult as the frequency of islet antigen-specific T cells is very low in the most readily available tissue sample, blood, and access to T cells in the islets is limited by the availability of pancreatic tissue samples (103). However, the increasing availability of pancreatic specimens from T1D tissue donors has made possible the study of islet infiltrating autoreactive T cells (104–106). Another approach has been to express T cell receptors (TCRs) from islet infiltrating T cells in an immortalized T cell line to provide a readily available and expandable source of T cells for antigenic testing (107). These studies have played a crucial role in understanding the antigenic targets of CD4+ T cells in T1D, which has not only provided novel insights into disease pathogenesis but may also be useful for testing antigen specific therapies for disease prevention (108).

Any model that seeks to include these cell types needs to be cognizant of the importance of specific T cell receptors that recognize islet antigens. Although a number of methods for making T cells from iPSCs have been published, only a handful of these have addressed the production of T cells bearing specific TCRs (**Table 2**). In short, T cells with specific TCRs can be created by "rejuvenating" T cells isolated from tissue donors or by providing a known TCR in the form of a transgene. In the case of the "rejuvenated" T cells, which can be made from iPSCs generated from T cells, care has to be taken to ensure that the TCR expressed by the iPSC derived T cell is identical to that possessed by the starting cell that was reprogrammed to generate the iPSC. Similarly, iPSC derived T cells expressing a TCR encoded by transgenes may also express endogenously encoded TCRs, potentially complicating the interpretation of antigen specific activation studies.

Although the technical issues surrounding the fidelity of TCRs expressed by *in vitro* derived T cells can be addressed, the work required to generate T cells with specific TCRs from iPSC is still significant. For this reason, direct isolation of T cells from T1D donors may provide a more accessible route for examining this aspect of the autoimmune reactions. However, this path also presents its own challenges, including the phenomenon of T cell exhaustion (82, 109, 110) and the issue of whether the TCR repertoire of T cells in circulation reflects that of autoreactive T cells present with the islets (105, 108). Ideally, an *in vitro* model would incorporate T cells isolated from islets of T1D individuals (44, 104, 107), a scenario that would limit the scope of such a model to deceased tissue donors.

B Cells

CD20+ B cell infiltrates have been described in insulitis in human T1D pancreatic specimens (11, 28), however, their exact role in the pathogenesis remains unclear. B cell activation leads to production of autoantibodies against key islet autoantigens that are used as markers for disease onset and as entry points for enrollment in secondary prevention trials (16, 111). However, the conventional understanding is that

antibodies by themselves are not pathogenic in T1D (112). There is conflicting data on the need of B cells in initiating autoimmunity as B lymphocyte depletion with the anti CD20 antibody (rituximab) has been associated with reversal of diabetes in the NOD mouse (113) and preservation of beta cell function in newly diagnosed T1D subjects (114). However, in contrast, a report of T1D development in a child with X linked agammaglobulinemia (115) suggests that B cells are not an absolute necessity for disease causation. Nevertheless, B cells can also function as antigen presenting cells and thus may play a role in disease pathogenesis by activating and diversifying the responses of autoreactive T cells in T1D (112).

In an idealized model of T1D, the dual role for B cells as antigen presenting cells and antibody producers could be addressed separately. Specifically, the potential effects of circulating antibodies directed against islet specific antigens could be examined by including patient serum or purified immunoglobulin fractions as input into the *in vitro* model. On the other hand, B cells themselves could be included as APCs. Currently, reports describing protocols for generating B cells from iPSCs have been scant (116, 117) and the robustness of methods for *in vitro* B cell maturation limited. As such, if B cells are to be incorporated into and *in vitro* model of T1D it is likely these will also need to be initially sourced directly from donors. In a similar scenario to that described above for T cells, this approach is likely to exclude the use of B cells producing antibodies with a known specificity.

As a final point, although generation of all the required cells types for a complete model of T1D is onerous, the fact that blood cells can be effectively cryopreserved means that experiments in which cells are recombined into a single culture can be separated from the process of cell generation.

Natural Killer Cells

Natural Killer (NK) cells are an innate immune cell that plays a critical role in identifying and killing abnormal cells, particular those that are the target of viral infection or have undergone tumorigenic transformation (reviewed in (118). Historically, NK cells have been classified as lineage negative cells that express CD16 in conjunction with either high or low levels of CD56. The designation "Natural killer" is indicative of this class of lymphocyte's capacity to kill cells without the requirement for activation by specific HLA antigen complexes, distinguishing them from conventional cytotoxic CD8+ T cells. Indeed, an important characteristic of NK cells is their ability to recognize and kill cells that fail to display self-HLAs, a property important for their role in tumor surveillance. Similarly, NK cells lack of requirement for antigen specific activation means they are first responders to viral infections, recognizing and destroying cells under stress. In addition to the lysis of abnormal cells, another key characteristic of NK cells is their ability to rapidly produce high levels of numerous cytokines and chemokines, putting them in a position to orchestrate immune attacks, as well as serving as an active participate (118).

Only a limited number of studies have examined the role of NK cells in human T1DM (119). Analysis of peripheral blood samples from individuals with T1DM suggested that those with long-standing disease had an NK population that showed reduced levels of activation [for example, reduced production of IFNg (120) and potentially decreased lytic activity (121). Rodacki et al. suggested this reduced NK activity was more likely a consequence than a cause of T1DM, given its association with long-standing disease. Nevertheless, others have suggested that reduced NK activity might make individuals more susceptible viral insults that may precipitate T1DM in the first instance (119)].

Consistent with their role as first responders to viral infection Dotta et al. (122), identified NK cell infiltrates within the islets of 3 T1DM individuals who also showed evidence of Coxsackie B4 enteroviral infection. The presence of NK cells coincided with non-destructive islet inflammation, suggesting these cells could represent a stepping-stone between and initial insult and an expanding inflammatory reaction.

Current protocols for generating NK cells from iPSCs have focused on those representing the myeloid lineages, characterized by expression of CD56 and CD16 (see **Table 2**). These methods have been primarily developed with a view to using iPSC derived NK cells as anti-tumor therapies (84, 85). Given the role of NK cells in detecting cellular stress, inclusion of this cell type in an *in vitro* model of T1DM could provide information related to beta cell stress, whether that be induced by exogenous stimuli or by the presence of the NK cells themselves.

Patient Derived Serum—Role of Autoantibodies in T1D

The release of autoantigens following the initiation of autoimmune attack on the pancreatic beta cells leads to the formation of antibodies against key islet proteins such as insulin (micro IAA or mIAA), glutamic acid decarboxylase (GAD), islet antigen 2 (IA-2), and zinc transporter 8 (ZnT8) (13, 123). As noted above the role of autoantibodies in T1D is not well defined. It is believed that the antibodies themselves are not pathogenic and do not cause disease by forming immune complexes as has been described for other autoimmune diseases (124). However, they do predict the risk of development of disease and rate of progression of disease (115). One potential important role played by autoantibodies in the T1D disease process is their effect on autoantigen processing and presentation by class II major histocompatibility complexes (125).

Several effector mechanisms render autoantibodies potentially harmful. These include antibody-dependent, cell-mediated cytotoxicity; release of inflammatory mediators through stimulation of Fc receptors on natural killer cells, macrophages, or mast cells; opsonization of islet autoantigen, which promotes phagocytosis by macrophages; and complement activation with subsequent assembly of the membrane-attack complex (126, 127).

Earlier studies demonstrated that sera from patients with T1D can have a cytotoxic effects on cultured rat beta cells (128, 129). Increased complement activation has been seen in serum of patients with recent onset T1D and similarly was found to cause apoptosis in rat islets (130). A study analyzing human pancreatic tissue specimens from the Network for Pancreatic Organ Donors

Diabetes (nPOD) program has also demonstrated evidence of complement activation in the pancreas (131). However, there have been few studies that examine the direct interaction of these antibodies with live beta cells and data on effect of these antibodies on human beta cells is sparse. In the same vein, few experiments have directly addressed the possibility that other serum bound factors may influence beta cell viability or function. An iPSC based model would be a useful platform to study the functional effects of islet autoantibodies/patient serum derived factors on beta cell function and the propagation of the autoimmune process. Indeed, such platforms have been successfully used for modeling autoantibody mediated neuromuscular diseases such as Myasthenia gravis (132).

Model Outputs

Disease Mechanisms

One of the most important uses of this system would be to study disease pathogenesis by *in vitro* assays that could examine beta cell function and immune cell activation

Assessment of Beta Cell Mass and Function

- Beta cell apoptosis assays
- Insulin content of beta cells and Glucose stimulated insulin assay (GSIS)

Immune Cell Activation

- Cytokine production assays
- T cell activation assays
- T cell proliferation assays

In addition to these cell specific assays, single cell RNAseq analysis could be employed to examine how the complex collections of cells respond to changes in their environment or to the presence of other cell types.

Potential Therapeutic Outputs

One application of an *in vitro* immune model of T1D would be to explore interventions that might modify the autoimmune response.

Drug Discovery and Screening

With the availability of patient specific iPSCs it is possible to recapitulate disease pathogenesis *in vitro* and to use this knowledge to guide development of patient specific targeted therapies (133).This can be particularly useful in disorders with a long preclinical phase such as T1D where, at the time of clinical disease onset, a significant proportion of tissue function is already lost (134). Disorders for which iPSCs have been used for drug discovery include spinal muscular atrophy (135), familial-dysautonomia (136) and amyotrophic lateral sclerosis (137). The failure of translation of most therapies, which are found successful in rodent models in human trials, has highlighted specific issues that are crucial to consider when designing future intervention trials. These issues include the significant knowledge gaps that exist in the understanding of human disease and the realization that rodent disease patterns and key physiological responses are significantly different from humans. Indeed, emerging knowledge suggests the disease process itself is very heterogenous in humans and therefore personalized strategies for immune intervention may be needed (138). A human iPSC derived *in vitro* model could account for interindividual variations in disease pattern and also circumvent the problems relating to pathophysiological differences between rodent and human disease.

In most autoimmune diseases the therapeutic interventions can be tested even when the disease state is well established. On the contrary, in T1D, intervention strategies would ideally be instituted at the pre-symptomatic phase where significant residual beta cell mass and function still remain. Therefore, iPSC-based models, which recreate the early disease milieu of T1D, are fertile ground for testing strategies for secondary and tertiary preservation in Type 1 Diabetes. Recent trials have focused on the use of immunomodulatory agents which inhibit T cell activation, cytokine action and promote Treg formation such as the use of anti CD3 antibody, CTLA-4 Ig, Anti thymocyte globulin (ATG), anti TNF alpha and IL-2 (139, 140). Cell based therapies such as tolerogenic DCs, Tregs and cord blood cells are also being studied for induction of immune tolerance in T1D. Hematopoietic stem cells have been used to reset the immune system in human trials and trials utilizing mesenchymal stem cells for immunomodulation are also underway (141). An iPSC model would be an ideal testing platform for pre-clinical trials of these therapeutic agents and provide output data relevant to human disease.

Drug repositioning, that is, uncovering new applications for existing drugs, is another application of iPSC technology. This approach has been investigated for conditions such as skeletal dysplasia's, Alzheimer's disease and amyotrophic lateral sclerosis (142). Drugs such as hydroxychloroquine (an anti-malarial with immunomodulatory activity) and imatinib mesylate (a tyrosine kinase inhibitor used in chronic myeloid leukemia) are currently being tested in beta cell preservation trials (143) after promising results in preclinical studies. iPSC based models provide a unique human platform for drug discovery and testing of novel therapeutic agents and also for validating the efficacy of these therapeutic agents in pre-clinical studies before translation to clinical trials.

POTENTIAL LIMITATIONS

Any model that seeks to recapitulate pathogenic events of human T1D may need to examine all of the cell types that have been implicated in disease causality and progression. At present, this is one limitation of trying to create such a model as robust protocols for generating cells with the correct characteristics are currently not available. As such, rather than trying to incorporate the multitude of environmental, genetic and cellular factors that could potentially affect disease pathogenesis, modeling will need to focus on specific aspects of the disease process that are experimentally tractable. Thus, combinations of iPSC and patient derived native cells will need to be used until more robust protocols for generation of immune cells are available.

In this review, we have not discussed the inclusion of other cell types such as endothelial, epithelial and mesenchymal cells,

which may also play a role in disease pathogenesis. Similarly, studies have identified a role for the autonomic nervous system in the control of both insulin and glucagon release, as well as a regulation of islet mass (144). It would be clearly very challenging to construct an islet-like organoid *in vitro* that could fully account for such modulatory neural inputs.

In addition to the non-endocrine cells mentioned above, the endocrine components of the islet itself constitute a complex mixture of multihormonal cell types. Moreover, some of these cell types, such as alpha cells, may have a role in T1D pathogenesis. The *in vivo* islet environment and cross talk between various endocrine cells is believed to be important for normal islet function and hormone release (145). Although many beta cell differentiation protocols generate other islet cell types including alpha cells (59, 60), the relative proportions of cell types generated are often difficult to control. In this respect, the islet-like milieu recreated *in vitro* will, at best, be an approximation to the rich interconnected environment of the native human islet.

Finally, such a model would also find it difficult to take into account the effects of non-islet derived hormones and growth factors within circulation that may collectively contribute to inflammation and beta cell stress.

Our omission of specific cell types and circulating factors is a reminder that any *in vitro* model cannot fully mimic the subtle multi-systemic interactions that occur *in vivo*. Future models which incorporate multi-lineage organoid cultures may circumvent some of these issues. Similarly, our current conception of an *in vitro* model of T1D does not address the initial loss of tolerance, which portends the onset of autoimmunity. Models for examining this question might require improvements in T cell and thymic epithelium differentiation protocols that will allow the study of T cell selection.

CONCLUSION

The advent of iPSC technology, which brings the possibility of creating diverse human cell types *in vitro*, has provided the opportunity to construct a fully humanized model of T1D that recapitulates human disease pathology. A major impetus for this work has come from recent improvements in protocols for generating iPSC derived beta cells with mature functionality, allowing investigators to generate the cellular target of autoimmunity in the context of specific HLA haplotypes.

The successful creation of a human iPSC based T1D model will allow a more nuanced understanding of the disease process and help investigators design better beta cell preservation strategies. Such models will also capture the heterogeneity of the human disease process and provide a landscape for testing patient tailored therapies. Finally, and most importantly, stem cell models of T1D will lessen our dependence on rodent disease models and pave the way for better translation of preclinical therapeutic strategies to the clinical arena.

AUTHOR CONTRIBUTIONS

KJ reviewed the literature and wrote the paper. FC, ST, SM, AE, and ES were involved with the writing, editing, as well as the key concepts behind the paper. All authors contributed to the article, approved the submitted version, and agree to be accountable for the content of the work.

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Selection for CD26^{-} and CD49A^{+} Cells From Pluripotent Stem Cells-Derived Islet-Like Clusters Improves Therapeutic Activity in Diabetic Mice

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Background: Cell therapy of diabetes aims at restoring the physiological control of blood glucose by transplantation of functional pancreatic islet cells. A potentially unlimited source of cells for such transplantations would be islet cells derived from an *in vitro* differentiation of human pluripotent stem cells (hESC/hiPSC). The islet-like clusters (ILC) produced by the known differentiation protocols contain various cell populations. Among these, the β-cells that express both insulin and the transcription factor Nkx6.1 seem to be the most efficient to restore normoglycemia in diabetes animal models. Our aim was to find markers allowing selection of these efficient cells.

Methods: Functional Cell-Capture Screening (FCCS) was used to identify markers that preferentially capture the cells expressing both insulin and Nkx6.1, from hESC-derived ILC cells. In order to test whether selection for such markers could improve cell therapy in diabetic mouse models, we used ILC produced from a clinical-grade line of hESC by a refined differentiation protocol adapted to up-scalable bioreactors. Re-aggregated MACS sorted cells were encapsulated in microspheres made of alginate modified to reduce foreign body reaction. Implantation was done intraperitoneally in STZ-treated C57BL/6 immuno-competent mice.

Results: CD49A (integrin alpha1) was identified by FCCS as a marker for cells that express insulin (or C-peptide) as well as Nkx6.1 in ILC derived by hESC differentiation. The ILC fraction enriched in CD49A^{+} cells rapidly reduced glycemia when implanted in diabetic mice, whereas mice receiving the CD49A depleted population remained highly diabetic. CD49A-enriched ILC cells also produced higher levels of human C-peptide in the blood of transplanted mice. However, the difference between CD49A-enriched and total ILC cells remained small. Another marker, CD26 (DPP4), was identified by FCCS as binding insulin-expressing cells which are Nkx6.1 negative. Depletion of CD26^{+} cells followed by enrichment for CD49A^{+} cells increased insulin^{+}/Nkx6.1^{+} cells fraction to ~70%. The

CD26⁻/CD49A⁺ enriched ILC exhibited improved function over non-sorted ILC or CD49A⁺ cells in diabetic mice and maintain prolonged blood C-peptide levels.

Conclusions: Refining the composition of ILC differentiated from hPSC by negative selection to remove cells expressing CD26 and positive selection for CD49A expressing cells could enable more effective cell therapy of diabetes.

Keywords: human ESC-derived insulin producing cells, islet-like clusters (ILC), functional cell capture screening, integrin alpha1 (CD49A), DPP4 (CD26), alginate encapsulation, STZ-treated C57BL/6 mice diabetes models

INTRODUCTION

Diabetic conditions due to destruction (in type I) or dysfunction (in type II) of pancreatic islets of Langerhans, have detrimental impacts on the quality of life and lifespan. For the hundred million patients depending on frequent insulin injections for controlling blood glucose levels, islet cells transplantation would have the advantage to restore a physiological regulation of glycemia. The Edmonton protocol (1–3) based on transplantation through the portal vein of human islets obtained from brain dead donors has allowed patients to become insulin-free for significant periods of time. Yet, the availability of such islet donations is too limited to meet the transplantation demand. An alternative, more abundant supply may be generated by differentiation of islet-like clusters (ILC) from large-scale cultures of human pluripotent cells (hPSC). Recently developed multistage protocols of differentiation produce pancreatic islet cell populations including mature β-cells that function in reducing glycemia in animal models of diabetes (4–6). New methods for microencapsulation in modified alginate support long term function after implantation in diabetic animals (7–9).

A drawback is that *in vitro* differentiated ILC contain additional populations of cells, which may not be necessary for islet function, and even may impair the efficacy or the safety of the transplanted cells for future clinical treatment of diabetes. To alleviate this problem, we investigated which cell surface marker could help identify and isolate the ILC cells that have higher capacity to normalize glycemia in diabetes models. We screened a large array of antibodies to cell-surface proteins by the Functional Cell-Capture Screening (FCCS) previously developed to identify cell-surface markers selective for endoderm and non-endoderm populations of differentiating hESC (10). The same procedure was later used to characterize surface markers of cells in post-mortem islets from adult humans (11). The present study was aimed to generate more potent ILC by using antibodies that were identified by the FCCS as selecting for insulin-producing cells in ILC differentiated from human ESC. Starting from a clinical-grade hESC line, we refined a 30-day protocol, in spinner suspension cultures or controlled bioreactors, which converts 3D clusters of these highly pluripotent stem cells into pancreatic islet-like clusters. The clusters were dissociated into single cells and fractionated according to their capacity to bind, or not bind, to two antibodies found by FCCS to capture insulin-producing cells. One antibody against integrin-alpha1 (CD49A) was found to bind the insulin-producing cells that express Nkx6.1, a transcription factor essential for the formation and function of mature β-cell s (12, 13), but to bind also insulin-producing cells lacking Nkx6.1. Another antibody, against Dipeptidyl peptidase-4 (CD26), was found to bind only insulin-producing cells that do not express Nkx6.1. A novel sorting strategy is suggested by first removing cells expressing CD26 and then enriching for CD49A positive cells. Thus, it is possible to increase the *in vivo* therapeutic activity of ILC to normalize glycemia in diabetic mice by two consecutive selections and supports the feasibility of functional enrichment strategies to improve the activity of hPSC-derived ILC for the treatment of diabetes.

Abbreviations: AFP, alpha fetoprotein; BSA, bovine serum albumin; CMRL, CMRL serum free medium; DPP4, dipeptidyl peptidase-4; ES, embryonic stem; FACS, fluorescent activated cell sorting; FBS, fetal bovine serum; FCCS, functional cell capture screening; GCG, glucagon; GLP1R, glucagon-like peptide-1 receptor; GSIS, glucose stimulated insulin secretion; HPRT, hypoxanthine phosphoribosyl transferase; hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; IAPP, islet amyloid polypeptide; ILC, islet-like cluster; ip, intraperitoneal; IPGTT, intraperitoneal glucose tolerance test; ITGA1, integrin alpha1; MACS, magnetic activated cell sorting; MafA, Pancreatic B-Cell-Specific Transcriptional Activator; Nkx6.1, Nk6 homeobox-1;NMR, nuclear magnetic resonance; NPTX2, neuronal pentraxin-2; $PBS^{-/-}$, phosphate buffered saline, no calcium, no magnesium; PCSK1, prohormone convertase; PFA, paraformaldehyde; PPY, pancreatic polypeptide Y; qPCR, quantitative Polymerase Chain Reaction; RT, room temperature; SST, somatostatin; STZ, streptozotocin; TMTD, triazole-thiomorpholine-dioxide; TPH1, tryptophan hydroxylase-1; UCN3, urocortin-3.

METHODS

Human ES Cell Expansion and Differentiation to Islet-Like Clusters

Highly pluripotent, clinical grade, human ES cells HADC-100 (14) (provided by Professor Benjamin Reubinoff, Hadassah Medical School, Jerusalem, Israel) were grown to confluent monolayers in essential E8 medium (Gibco, Cat#A1517001), with addition of penicillin and streptomycin (PS, Gibco, Cat#15140-122) on vitronectin-coated flasks (Gibco, Cat# A14700). Differentiation was performed on cell aggregates formed in spinner flasks during 2 days in dynamic suspension cultures. In brief, 48 h before starting the differentiation protocol (day-2), non-differentiated cells were dissociated with Versene (Gibco, Cat#15040033). Single cells washed with $PBS^{-/-}$ (Gibco, Cat#14190-094), were seeded in 500 ml disposable spinner flasks (Corning, Cat#CZ-3153), filled with 250 ml E8 medium containing 10 μM Rock Inhibitor Y27632 (Cayman Chemical, #10005583-10), at concentration of 0.8–1 × 10^6 cells/ml. The spinner flasks were placed on magnetic stirrer

(DURA-MAG, 9 position stirrer, Chemglass) at speed of 70 rpm in a humidified incubator set at 5% CO_2 and 37°C. This resulted in the formation of ES cell clusters in suspension, as well as in cell proliferation. On day -1, 80% of the E8 medium was replaced. On Day 0, the E8 medium was washed away (15) by letting the aggregates settle for 5 min and removing the supernatant with a pipette. Cells were washed with 250 ml $PBS^{-/-}$; after 3 min stirring in the incubator, $PBS^{-/-}$ was replaced by 250 ml of stage 1 differentiation medium. The media for the seven-stage differentiation protocol, refined on the basis of several published protocols (5, 16–19), are detailed in additional files 1 and 2 (**Tables S1** and **S2**).

For Bioreactors, the hESC aggregation and differentiation was similarly done in the DASbox® mini system (Eppendorf) with online monitoring of culture parameters. Up to four parallel bottles containing 150 ml medium were seeded with 0.8–1 × 10^6 cells/ml 48 h before and washed with PBS just before differentiation as above. Medium changes were done batch-wise in the semi-closed system using peristaltic pumps.

Real-Time Quantitative PCR

RNA was isolated from cells using the RNAeasy micro kit (Qiagen #74004) and purified from genomic DNA with RNase-free DNase kit (Qiagen #79254). The cDNA synthesis was done with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814). Transcript levels were measured by real-time qPCR using Taqman Fast advanced master mix (Applied Biosystems #4444557). The level of each gene was normalized to endogenous HPRT gene, using the $2^{-\Delta\Delta}CT$ method. The probes used for qPCR are listed in **Table S3**. The MARIS procedure (20) is described in Additional file 4.

Flow Cytometry

Samples from settled aggregates during or at the end of the differentiation process were dissociated with Accumax (Sigma, Cat# A7089) at 0.35 ml per 300,000 cells for 8–10 min, after which the enzyme was blocked in 1 ml $PBS^{-/-}$ with 10% FBS, and the cells were centrifuged (350 × g, 3 min). For external cell membrane labeling, cells were washed in $PBS^{-/-}$, and antibodies (e.g. anti-CD49A, as listed in **Table S4**) were directly added to the cell suspension in FACS buffer (0.5% BSA in PBS) followed by incubation at 4°C for 30 min. For internal antigen labeling (e.g. anti-human C-peptide and Nkx6.1, **Table S4**), cells after centrifugation were washed once in 1 ml PBS and fixed in 0.4 ml of 4% paraformaldehyde (PFA, EMS Cat# 15710), for 20 min at 4°C. After two washes with PBS cells were incubated for 1 h at 4°C in blocking solution [PBS with 5% Bovine serum albumin (BSA) and 3% horse serum] containing 0.3% Triton X-100 (Sigma, T6878), and washed once with PBS. The cell pellet suspended in 0.1 ml blocking solution (with 0.1% Triton x-100) containing antibodies, was incubated overnight at 4°C, or for 1 h at room temperature (RT), and washed with PBS. When fluorescent tag-conjugated primary antibodies were used, fluorescence was read after this step in Flow Cytometer-BD FACS Canto II. Otherwise, cells were incubated in blocking solution containing 1:100 dilutions of the fluorescent tag-conjugated secondary antibody and washed with PBS before reading fluorescence.

Functional Cell Capture Screening (FCCS) on Antibody Arrays

Antibody arrays were printed in a Microgrid printer with solid pins (Total array Systems, BioRobotics, Cambridge, UK) on hydrogel-coated slides (Full Moon Biosystems, Sunnyvale, CA, USA) using a panel of 235 monoclonal mouse anti-human antibodies (BD biosciences), each antibody being spotted at five different places in the array, as described before (10, 11) (US patent 2018/0369290 A1, Item 0081). The cell clusters were dissociated using TrypLE Express (Invitrogen Cat#12604) for 4 min, followed by quenching with 10% FBS in PBS, centrifugation, and resuspension in CMRL. The printed area of the array was blocked for 3 min with 1% BSA in PBS solution, before cell seeding, at about 0.5 × 10^6 cells/ml in 0.25–0.5 ml of CMRL medium, supplemented with 2 µl of DNase I (Ambion 2 U/µl) and incubation was for 1 h at 37°C. Excess cells were removed in a large volume of PBS and the arrays were fixed in 4% PFA for 10 min. Cells on the array were permeabilized in PBS, 0.2% Triton X-100 for 20 min, washed twice with PBS, and blocked for 45 min in blocking buffer (2% FBS, 2% BSA, 50 mM glycine in PBS). After blocking, arrays were washed twice with PBS and incubated for 2 h at RT in blocking buffer with 0.1% of Triton X-100 containing the primary antibody guinea-pig anti-insulin (DAKO, A0564). Primary antibodies were removed, and arrays were washed three times with working buffer. Then, secondary antibodies were added in working buffer for 45 min at room temp: cy5 donkey anti-guinea–pig (Jackson ImmunoResearch 706-175 -148). Arrays were washed three times in working buffer and imaged using automated, high content fluorescence microscopy (IXmicro, MDC). Total cells in each spot were counted by phase microscopy and the percent of insulin positive cells was calculated. Three repeats were performed with different batches of ES-derived cells at stage 7 of the differentiation protocol. The significance of the amount of cell binding to surface antibody was evaluated by two sample paired T-test (P value less than 0.05). In another set of experiments, cells were reacted with antibodies against insulin as above but also with antibodies against Pdx1 and against Nkx6.1 (see **Table S4**). The number of cells stained for PDX1 and Insulin, and for insulin and Nkx6.1 was counted.

Magnetic Activated Cell Sorting (MACS)

Cell clusters after stage 7 of differentiation were washed in $PBS^{-/-}$, dissociated with Accumax (10 ml for 25 × 10^6 cells) for 10 min at 37°C and washed with CMRL 2% BSA. Dissociated cell suspension was passed through a 30 µm MACS filter previously washed with $PBS^{-/-}$ and counted using Nucleocounter® NC-200. Part of the clusters was set aside for control (non-dissociated, non-selected cells), seeded at 10^6 cells per ml in ultra-low binding six-well-plates (Corning Cat#CLS3471) and left in the incubator on orbital shaker (NovaShake-B32X) set at 95 rpm for 3 days in medium $CMRL^+$ (**Table S2**), before implantation. Cells were suspended in MACS buffer ($PBS^{-/-}$, 2% BSA, 2 mM EDTA, sterile, degassed), 100 µl per 10^7 cells, for reaction with anti-CD49A-PE (Myltenyi, cat#

130-101-397) using 10 µl per 10^7 cells for 10 min at 4°C, followed by washing with 5 ml MACS buffer. Cells suspended in MACS buffer (80 µl per 10^7 cells) were reacted with 20 µl per 10^7 cells of anti-PE magnetic microbeads (Miltenyi; cat# 5181214192) for 15 min at 4°C. After washing with 5 ml cold MACS buffer and centrifugation, cells were suspended in MACS buffer and applied to pre-separation filters and LS MACS column(s) as recommended by the manufacturer. Prior to implantation, all single cells fractions were re-aggregated in suspension in non-TC treated six-well plates (Corning Cat#CLS3471-24EA) in CMRL$^+$, 10 µM RI Y27632 and 2 µg/ml Laminin (Bio Lamina Cat#MX521CTG), on orbital shaker.

For removal of CD26 positive cells prior to CD49A enrichment, the cells were dissociated as above, incubated for 10 min at 4°C with anti-CD26-PE (cat#302706), (10 µl/10^7 cells, in 100 µl MACS buffer for 10^7 cells), and after washing, reacted with anti-PE microbeads as described above. The mixture, after washing and resuspension in MACS buffer, was applied to LS column(s) and the flow through fraction (CD26 depleted) kept for further fractionation by MACS with anti-CD49A antibody.

Diabetes Induction in Mice and ILC Implantation

Six-to-8-week-old immune-competent mice C57BL/6JOlaHsd (Harlan, Israel) were rendered diabetic by intraperitoneal injection of streptozotocin (STZ) (Sigma, Cat#S0130), using 4 × 50 mg STZ/kg after 6 h daily fasting. Implantation was performed in diabetic mice, defined by blood glucose higher than 250 mg/dl for three consecutive tests. Blood glucose was measured by a glucometer, on tail vein blood. Intraperitoneal Glucose Tolerance Test (IPGTT) was done after fasting the mice overnight by i.p. injection of glucose (2 g/kg). Blood glucose was then monitored during a 2 h period.

For implantation of micro-encapsulated ILC cells (see below), mice were anesthetized by an IP injection of ketamine/xylazine (Sigma, K4138) at 87.5 mg/kg ketamine/12.5 mg/kg xylazine and then mounted on a surgical pad. The skin was prepared by shaving with electric clippers, application of Polydin, and then 70% ethanol solution. An abdominal incision (1 cm), and peritoneal incision (0.5 cm) allowed to insert microencapsulated ILC into the peritoneal cavity of the mouse using a 1 ml sterile plastic tip (about 0.5 ml total volume). The peritoneum and the skin were closed with sutures and cleaned with Polydin. The mice were kept warm by a heating pad till they woke up. The cell doses implanted were between 1.0 and 2 × 10^6 cells, as indicated.

Human C-Peptide ELISA Assays

The levels of human C-peptide, which reflect the levels of insulin secreted by the human ILC, were measured in blood samples collected after anesthesia from mouse retroorbital sinus. To test the insulin/C-peptide response to glucose, mice were withdrawn from food for 12 h, and injected intra-peritoneally with a glucose solution (25%, 41-302-500, Biological Industries). Blood was collected before and 30 min after the glucose injection, centrifuged, and stored at −20°C. ELISA assays were performed using ultra-sensitive ELISA kit for human C-peptide from Mercodia (#10-1141-01) according to instructions.

Preparation of TMTD-Modified Alginate and Microcapsules

Triazole thiomorpholine dioxide (TMTD Y1-Z15) preparation and coupling to alginate PRONOVA UP-MVG alginate (NovaMatrix) were done for Kadimastem, by Recipharm-Israel as described [8, 9]. After verification of the product structure by NMR, purification by filtration, dialysis, and desiccation, elemental analysis revealed that more than 50% alginate guluronic or mannuronic residues were coupled to TMTD. Solutions of 4.6% of TMTD-coupled alginate were used [80% in volume of 5% (w/v) TMTD-coupled UP MVG and 20% of 3% (w/v) UP MVG]. Stage 7 ILC (see **Table S1**), washed with KREBS buffer without Ca^{++} pH 7.4, were mixed with alginate solutions at the concentration of about 10^7 cells/ml in a 5 ml Eppendorf tube. The micro-encapsulator Buchi B395, located in a tissue culture laminar flow hood for sterility, was set up to obtain microcapsules of 1.5 mm diameter with 4.6% alginate polymerized in $CaCl_2$ (100 mM in HEPES pH 7.4).

ILC Immunostaining and Imaging

Stage 7 cell clusters were fixed in 4% PFA and washed in PBS$^{-/-}$. A minimum volume of warm 1% agarose was added to the pellet of clusters. After agarose became solid, the block was embedded in paraffin, and 10 µm thick sections were produced and bound to glass slides. After removal of paraffin by alternative baths of xylene and ethanol, antigen retrieval was performed by heating slides in 10 mM citrate buffer pH 6.0 (ZYTOMED systems), for 15 min in pressure cooker (Bio TintoRetriever). Blocking and permeabilization was done by incubation with PBS containing 5% BSA, 3% horse serum (blocking solution) supplemented with 0.3% Triton X-100 for 1 h at RT. Antibodies against PDX1, C-peptide, Nkx6.1 (**Table S4**) were diluted blocking solution supplemented with 0.1% Triton X-100 and incubated overnight at 4°C in humidified chambers. After two washes in PBS, incubation with secondary antibodies was done for 1 h at room temperature and washes were done similarly. Nuclei were stained with DAPI (1 µg/ml). The slides were mounted with aqueous mounting medium and covered with coverslip. Images were obtained using Nikon Eclipse 80i fluorescence microscope.

RESULTS

Islet-Like Clusters Differentiated From hESC Contain Mature β-Cells

Highly pluripotent hESCs were differentiated according to a seven-stage stepwise protocol carried out in suspension culture conditions (3D), in spinner flasks as well as in controlled bioreactors (as detailed in *Methods*). After stage 7 (days 30–34), 100–200 µm Islet-like clusters (**Figure 1A**) contain hormone-positive cells, especially cells producing insulin (as well as the C-peptide fragment processed from human proinsulin) (**Figure 1B**). These insulin-producing cells typically amount to 60% of the total cells, with about 10% of cells producing glucagon and 2% producing somatostatin (**Figure 1B**). In addition to hormone-producing cells, the hESC-derived ILC still contain precursor cells, since over 90%

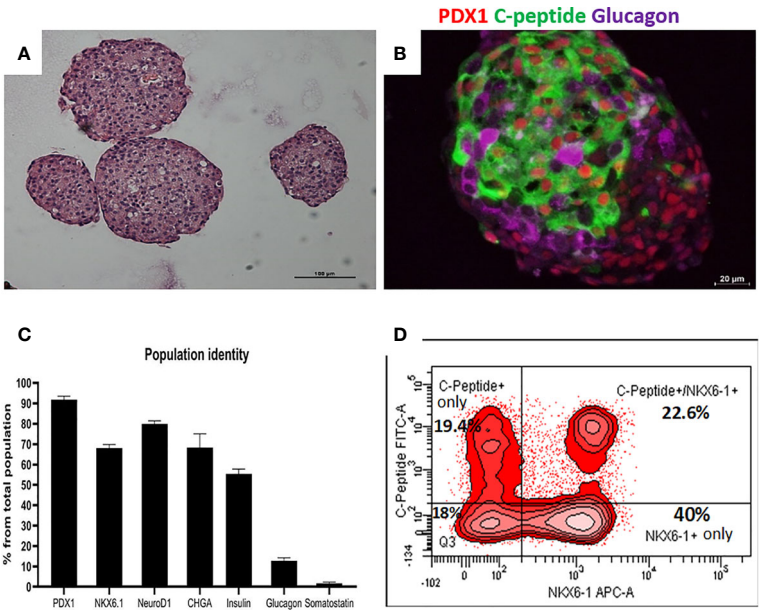

FIGURE 1 | Cell populations identity in Islet-like clusters at the end of the differentiation process. **(A)** H&E of microencapsulated ILC, demonstrating clusters ranging from 100 to 200 μm. **(B)** Immunostaining of an ILC for human C-peptide (green), Glucagon (purple), PDX1 (red, nuclear stain). **(C)** Percentage of ILC cells stained by antibodies to indicated proteins, calculated from flow cytometry analysis of a representative ILC preparations (n = 10, different batches, antibodies listed in **Table S4**). **(D)** Flow-cytometry analysis of dissociated total ILC cells, fixed and stained for human C-peptide and Nkx6.1. The percentage of cells with C-peptide only (no Nkx6.1), with C-peptide and Nkx6.1, with Nkx6.1 only (no C-peptide) is shown. Q3 is the double negative fraction; C-peptide$^-$/Nkx6.1$^-$.

of cells express the key transcription factor for pancreatic development PDX1 and 70–80% express transcription factors important for β-cell function such as Nkx6.1 and NeuroD1 (**Figure 1C**). Nkx6.1 is of particular importance, being essential for development and function of mature β-cells (12, 13), and serving as a marker of mono-hormonal insulin-producing β-cells (21). In the hESC-derived ILC, these β-cells can be identified by flow cytometry (FACS) as double positive for Nkx6.1 and human C-peptide (**Figure 1D**). This C-peptide$^+$/Nkx6.1$^+$ double positive fraction usually represents around 20–30% of the total population, the rest being C-peptide$^+$/Nkx6.1$^-$ (C-pep$^+$ only), C-peptide$^-$/Nkx6.1$^+$ cells (Nkx6.1$^+$ only), and C-peptide$^-$/Nkx6.1$^-$ (Negative, Q3) cells (**Figure 1D**). The four subpopulations were characterized by gene expression (**Figure 2**). After separation by preparative FACS, RNA from each of the fixed and stained cell fractions was extracted and analyzed using the MARIS method (20). The qPCR data relative to the unsorted cells confirmed that the C-peptide$^+$/Nkx6.1$^+$ double positive compartment is enriched for cells that primarily express insulin. On the other hand, the C-peptide-only fraction is enriched for cells expressing Insulin but also Glucagon (GCG), Somatostatin (SST), and Pancreatic Polypeptide (PPY), identifying these cells with the reported polyhormonal precursors (22–24). Among the four sorted cell fractions, the C-peptide$^+$/Nkx6.1$^+$ double positive cells had, in addition to insulin, the highest expression level of the transcription factors MafA and Nkx6.1, of the prohormone convertase PCSK1 and the GLP1 receptor (GLP1R) (**Figure 2**).

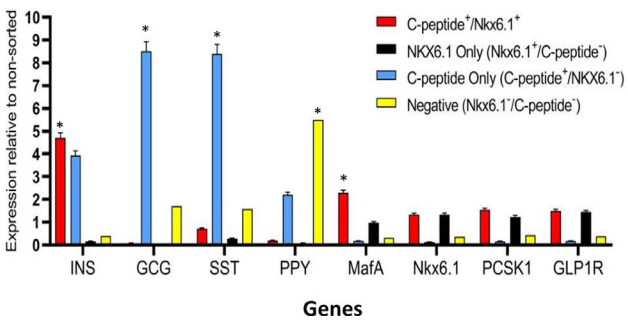

FIGURE 2 | Gene expression analysis of Islet-like clusters stained for C-peptide and NKX6.1. A method for analyzing RNA after intracellular staining (MARIS) was applied to ILC cells. Shortly, dissociated cells were fixed and stained for intracellular C-peptide and NKX6.1 antigens. Following labeling with secondary antibodies, the designated subpopulations were sorted by preparative FACS and RNA from each subpopulation was extracted. The relative expression levels of indicated genes was analyzed by qPCR relative to the non-sorted total ILC cells taken as 1. Results (expressed as mean ± SEM) demonstrated signature genes for specific sorted populations (*significance $p < 0.05$, Student t test): Insulin and MAFA is highly expressed in C-peptide$^+$/Nkx6.1$^+$ population; Glucagon and Somatostatin are highly expressed in C-peptide$^+$/Nkx6.1$^-$ population (poly-hormonal cells fraction), PPY in C-peptide$^-$/Nkx6.1$^-$ population. The expression of GLP1R, NKX6.1, and PCSK1 genes is not significantly enriched in one specific population but notably expressed in similar levels in both C-peptide$^+$/Nkx6.1$^+$ and C-peptide$^-$/Nkx6.1$^+$ populations.

These are characteristics of more mature β-cells. The Nkx6.1-only fraction also showed enrichment for PCSK1, GLP1R, and Nkx6.1 expression, but had lower MafA and very low insulin, suggesting that these are pre-hormonal progenitors. The C-peptide$^-$/Nkx6.1$^-$ population contained cells expressing GCG, SST, and PPY (**Figure 2**), suggesting the presence of maturing α, δ, and PP islet cells, respectively.

Identification of CD49A as a Marker for Mature β-Cell

We used the FCCS platform (10) for identifying antibodies to cell surface proteins that preferentially capture insulin-producing cells (**Figure 3A**). Single cells from dissociated ILC were incubated on the array and antibody–bound cells were then stained for insulin. Out of the 235 antibodies in the array, 61 of them captured some ILC cells. Of these antibodies, anti-CD49A consistently captured insulin$^+$ cells in amounts exceeding 33% of the total cells captured (the median value for all antibodies being 13%). As illustrated in **Figure 3A**, there were other antibodies binding insulin$^+$ cells (e.g. CD99) and several that captured almost only insulin-negative cells (e.g. CD66C, CD73). These latter antibodies may serve for negative selection to remove cells that do not express insulin.

As a marker for positive selection of β-cells, we chose CD49A (Integrin alpha-1) since further experiments showed that this marker is predominantly present in the C-peptide$^+$/Nkx6.1$^+$ double positive subpopulation (**Figure 3B**, gray in upper panel). Magnetic activated cell sorting (MACS) with antibodies to CD49A, was used to fractionate live ILC cells into CD49A enriched and CD49A depleted populations. A marked increase in the percentage of C-peptide$^+$/Nkx6.1$^+$ double positive cells was observed by FACS in the CD49A enriched fraction compared to CD49A depleted fraction (**Figure 3B**, lower panel) or to non-sorted ILC cells (**Table 1**, line 1). On the other hand, there was

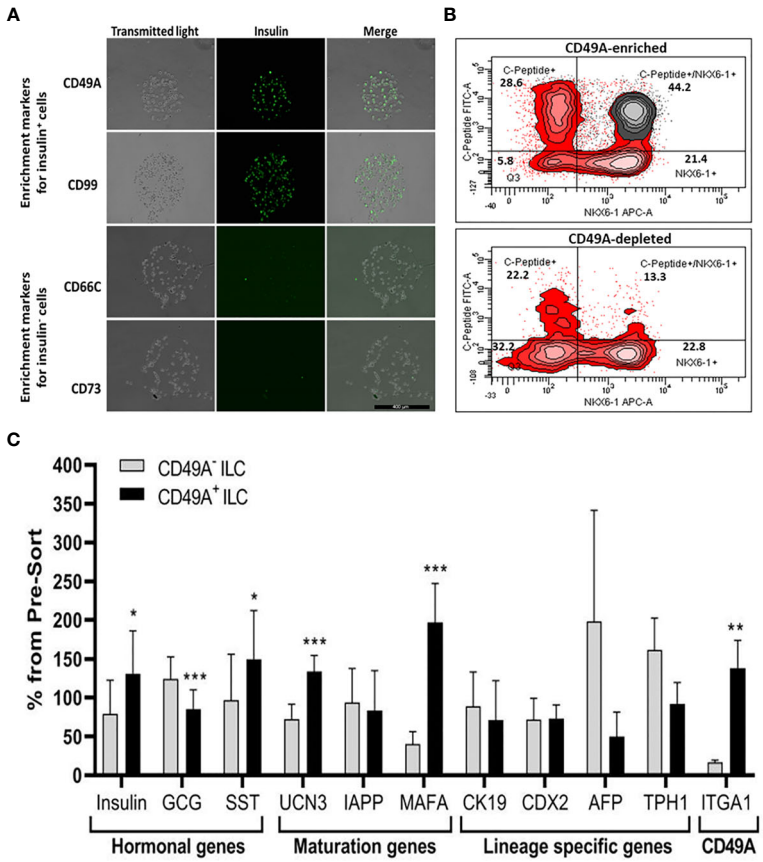

FIGURE 3 | Functional Cell-Capture Screening on antibody array. **(A)** In the array, the spots containing CD49A and CD99 antibodies attach ILC cells that comprise a high proportion of Insulin-positive cells, while spots with CD66c and CD73 antibodies attached cells that do not express insulin. The spots shown were part of arrays of 235 antibodies against cell-surface proteins, reacted with dissociated ILC cells and then stained with anti-insulin antibodies and Cy-5 labeled secondary antibodies. The number of insulin-positive cells was compared to the total cells captured (phase contrast). **(B)** Upper panel: FACS plot of ILC cells selected by MACS for binding to the CD49A antibody (CD49A enriched) and stained for human C-peptide, Nkx6.1, and CD49A, shows that CD49A positive cells (gray) are mainly in the C-peptide$^+$/Nkx6.1$^+$ double positive fraction. Lower panel: same for fraction that did not bind to the MACS CD49A antibody column (CD49A depleted). For comparative percentage of cells in each FACS subpopulation see **Table 1**. **(C)** qPCR analysis of RNA extracted from ILC cells fractionated by MACS shows that the cells binding to CD49A antibodies (CD49A enriched) have higher Insulin, UCN3, and mainly MAFA expression level. Results calculated in comparison to non-sorted cells, taken as basal 100% reference. The results are expressed as mean ± SEM. Student t test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

TABLE 1 | Proportions of C-peptide and Nkx6.1 positive subpopulations in ILC cells selected for binding to CD49A antibodies.

Cell Fraction:	Expt 1			Expt 2		
	Total Non-sorted	CD49A enriched	CD49A depleted	Total Non-sorted	CD49A enriched	CD49A depleted
Cells positive for markers:			Percent of cells			
1. C-peptide/Nkx6.1	17.8	46.2	13.3	22.6	44.2	16.2
2. C-peptide only	24.8	19.7	22.2	25.4	28.6	26.8
3. NKX6.1 only	29.2	10.7	22.8	27.9	15.4	50.4
4. CD49A (overall)	23.8	61.4	9.5	26.7	52.8	10
5. C-peptide/Nkx6.1/CD49A	ND	40.1	5.0	ND	35.9	2.9

Table 1. *The C-peptide/Nkx6.1 double positive population is increased in the cells binding to CD49A antibodies (CD49A-enriched) compared to cells that did not bind (CD49A depleted). Quantitation of FACS plots (as in* ***Figure 2B****) of ILC cells either non-sorted or sorted by MACS with anti-CD49a antibody and further reacted with anti-human C-peptide, anti-Nx6.1, and anti-CD49 antibodies. Two different experiments are shown. ND, not determined.*

almost no change in the C-peptide-only cells (**Table 1**, line 2). Thus, within the CD49A-enriched fraction, the C-peptide$^+$/Nkx6.1$^+$ double positive cells became relatively more abundant than the C-peptide-only (i.e. Nkx6.1 negative) and Nkx6.1-only (i.e C-peptide negative) cells. While the proportion of CD49A$^+$ cells increased to about 50–60% in the enriched fraction, there were still around 10% of CD49A$^+$ cells in the depleted fraction (**Table 1**, line 4). This indicates that even after two passages on a MACS column with anti-CD49A, the separation was not complete. Yet, there may also be heterogeneity in the distribution of CD49A. For example, when the CD49A-enriched fraction was triple stained for CD49A, C-peptide and Nkx6.1, it was found that 81–86% of the C-peptide$^+$/Nkx6.1$^+$ cells were positive for CD49A (compare lines 5 to line 1 in **Table 1**). However, the same comparison shows that most of the double positive cells remaining in the CD49A-depleted fraction did not express CD49A (only 18–38% of them scoring positive for CD49A). This suggests that the C-peptide$^+$/Nkx6.1$^+$ double positive population may be heterogeneous, some with and some without CD49A.

The CD49A-enriched MACS fraction had significant differences in gene expression as compared to the CD49A depleted fraction (**Figure 3C**). After enrichment for CD49A$^+$ cells (confirmed by the increase in ITGA1 transcripts encoding CD49A), there was an increase in mRNA for insulin, urocortin-3 (UCN3), and most significantly for MafA. The expression level of glucagon mRNA was somewhat decreased but, unexpectedly, somatostatin mRNA was slightly increased, suggesting enrichment of the relatively small population of δ-cells. Interestingly, transcripts of a hepatic lineage gene (AFP), of which low amounts still remain in the hESC-derived ILC, are further decreased in the CD49A enriched MACS fraction (**Figure 3C**). In addition, TPH1, a gene of the serotonin synthesis pathway, was also reduced (**Figure 3C**), suggesting that CD49A enrichment removes non β-cells producing serotonin inhibiting insulin secretion (25). Overall, the gene expression data confirm that selection for the CD49A cell surface antigen helps to enrich for functional mature β-cells.

In order to be able to transplant cells in large enough amounts, the selection method needs to perform in large-scale preparations. Cells dissociated from ILC at day 35 of differentiation (~200*10^6 cells), were fractionated by two consecutive passage on MACS columns with anti-CD49A antibodies. The twice retained fraction contained 75 million cells. The quality of this CD49A enriched preparation, evaluated by qPCR, was similar to that of small-scale preparations, with increased Insulin, MafA, and UCN3 mRNA, decreased GCG and AFP mRNAs, relative to non-sorted and CD49A depleted cells. This made it possible to study the *in vivo* activity of the sorted ILC cells to reduce glycemia in diabetes model.

CD49A Selection Separates ILC Cells That Normalize Glycemia in Diabetic Mice From Inactive Cells

Current methods for implantation of human ILC in immunocompetent mice are based on micro-encapsulation in alginate spheres, so as to reduce direct contact of the cells with host immune cells. While the introduction of alginate spheres into the peritoneal cavity of C57BL/6 mice has been shown to elicit foreign body reaction (FBR) and fibrosis even without ILC (8, 9), this reaction can be inhibited by using chemically modified alginate. In particular, long-term functionality was demonstrated for ILC transplants that were encapsulated in low-viscosity SLG20 alginate carrying triazole-thiomorpholine dioxide (TMTD-alginate) and implanted intraperitoneally (i.p.) in mice with streptozotocin (STZ)-induced diabetes (9). In the present study, we used an ultra-pure alginate of medium viscosity (UP MVG alginate) coupled with TMTD for ILC microencapsulation. Microencapsulated ILC, sorted or total, were implanted i.p. into STZ-induced diabetes C57BL/6 mice. The function of non-sorted (Total ILC), CD49A-enriched (CD49A$^+$ ILC), and CD49A-depleted (CD49A$^-$ ILC) cells was compared by measuring of blood human C-peptide levels and effects on glycemia. Mice implanted with CD49A$^-$ ILC fraction cells did not exhibit reduction in BGL levels (**Figure 4A**, purple line), in direct correlation to the low human C-peptide secretion level (**Figure 4B**). Nevertheless, even the low amounts of human insulin secreted improved the viability of CD49A$^-$ ILC implanted mice compared to non-implanted diabetic control, which remained highly hyperglycemic and died around day 40 of the follow-up period. The mice implanted with either non-sorted ILC (Blue line, **Figure 4A**) or CD49A$^+$ enriched ILC (Red line, **Figure 4A**) exhibited a rapid decrease of blood sugar following implantation, reaching within ~6 days to the normoglycemic range (**Figure 4A**), with the CD49A$^+$ enriched ILC exhibited significantly lower BGL values than non-sorted ILC only at the first week. The average non-fasting blood glucose remained

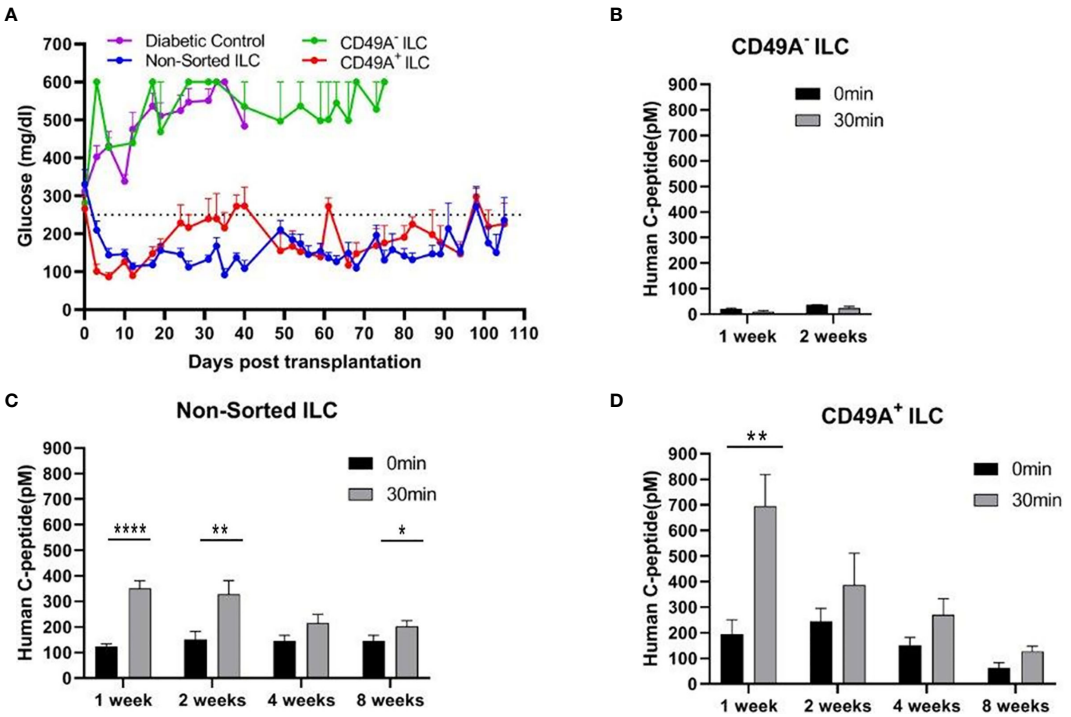

FIGURE 4 | Glycemia of diabetic mice is reduced by CD49A$^+$ enriched ILC cells but not by CD49A depleted cells. **(A)** ILC cells at the end of S7 differentiation protocol were fractionated by MACS column with CD49A antibodies. Cells of the bound fraction (CD49A enriched) and of the unbound (CD49A depleted) were reaggregated for 2 days, encapsulated in TMTD-MVG alginate, and implanted by i.p. route (~2*10^6 cells) into STZ-diabetic mice (n ≥ 5). Non-sorted ILC were similarly implanted. Shortly following transplantation, a reduction of hyperglycemia was observed only in the non-sorted and CD49A$^+$ enriched ILC groups, and blood glucose levels of diabetic mice were normalized for more than 100 days. **(B–D)** Glycemia reduction is correlated to human C-peptide glucose dependent secretion. At designated timepoints, mice were tested for human C-peptide concentration levels in blood. Human C-peptide was measured starting from 7 days post-implantation in the three groups of mice after overnight food deprivation (t = 0, black bars) and 30 min after i.p injection of 2 g/kg glucose (t = 30, gray bars). Average blood C-peptide level in pM ± SEM are shown for each group. The glucose-dependent C-peptide levels are significantly higher at day 7 in CD49A$^+$ ILC treated mice ($p = 0.002$), but at later time points, there is no significant functional difference **(C, D)**. The CD49A$^-$ ILC treated mice differ significantly from CD49A$^+$ enriched and non-sorted treated mice, exhibiting significantly lower levels of human C-peptide **(B)**, suggesting that this ILC population can be depleted ($p = 0.034$) (Student *t* test). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mostly in the normal range (below 250 mg/dl) over a follow-up period of 100 days with some fluctuations. These temporal fluctuations can be attributed to changes in ILC composition *in vivo*. The effect on glycemia was confirmed by detection of serum human c-peptide. Albeit CD49A-enriched ILC produced a ~2 fold higher levels of C-peptide than implants of non-sorted ILC at the first week post implantation (**Figures 4C, D**), eventually at later stages, the *in vivo* function results were similar between the groups. Both, non-sorted and CD-49A-enriched, exhibited stimulated human c-peptide secretion 30 min after glucose injection already 1-week post transplantation, demonstrating typical β-cells response to glucose. Overall, these data establish that selection for the CD49A surface marker identifies and separates the ILC cells that control glycemia from inactive ones. On the other hand, the data did not show a clear functional difference between the CD49A-enriched ILC to the non-sorted ILC (**Figure 4A**) during the analysis period. The conclusion from these experiments was that MACS sorting for CD49A by itself was not sufficient to markedly improve the therapeutic activity in the diabetes model, in line with the equivalent levels in the amount of human C-peptide found in the blood of the mice.

Combining Negative Selection for CD26 With Positive Selection for CD49A

Selection for the CD49A marker increases the percentage of C-peptide$^+$/Nkx6.1$^+$ double positive cells but does not remove the C-peptide-only fraction (**Figure 3B** upper panel and **Table 1**). To improve the selection, we attempted to identify surface markers of ILC cells expressing both Insulin and Nkx6.1 simultaneously. In a new FCCS, each spot of the antibody array was evaluated for capture of insulin$^+$ cells and then for the percentage of Nkx6.1$^+$ cells among them. We were unable to find an antibody that captured exclusively C-peptide$^+$/Nkx6.1$^+$ double positive cells, but observed that in the *in vitro* differentiated ILC, an antibody to CD26 (Dipeptidyl peptidase-4, DPP4), captured efficiently insulin$^+$ cells (70% of all the captured cells), which only 3.5% of it had Nkx6.1 (**Table S6**). The presence of CD26 on these cells was unexpected since CD26 has been described as a marker of ductal cells and glucagon

producing α-cells, but not of insulin-producing β-cells in natural islets isolated from pancreas (26–28). Our finding that in hESC-derived ILC cells, CD26 binds the cells expressing insulin but not Nkx6.1 (**Figure 5**, gray in the uppermost panel) offered the possibility to remove these cells and enrich for cells positive for Nkx6.1 and insulin (or C-peptide). Indeed, MACS selection with anti-CD26 showed that in the unbound cell fraction (CD26 depleted) the C-peptide$^+$/Nkx6.1$^-$ cells were markedly reduced whereas the C-peptide$^+$/Nkx6.1$^+$ double positive cells were increased (**Figure 5** and **Table 2**). By a subsequent selection for the CD49A marker (**Figure 5**, lower left panel), the percentage of double positive cells raised and could reach 70% in the CD26 depleted/CD49A enriched fraction (**Table 2** line 1). Gene expression analysis of the CD26$^-$/CD49A$^+$ ILC cells fraction demonstrated significant differences in gene expression as compared to the CD49A+ enriched population (**Figure 6**). The prior removal of CD26$^+$ population resulted in an increase mRNA for insulin, and most significantly for MafA. The expression level of glucagon mRNA was decreased by 70%, and somatostatin mRNA continued to increase, suggesting additional enrichment of the relatively small population of δ-cells. Moreover, depletion of CD26$^+$ cells further decreased AFP mRNA levels by ~90% from non-sorted cells (*vs.* 50% decrease by CD49A$^+$ enrichment). Overall, the gene expression data confirm that the preparative depletion of CD26$^+$ cells from ILC and subsequent selection for the CD49A$^+$ cells augment the functional mature β-cell phenotype characteristics.

To evaluate their activity for diabetes therapy, implants of CD26-depleted/CD49A-enriched ILC were compared to total non-sorted ILC. We observed that even when the dose of cells implanted was reduced to 1 million cells, the CD26 depleted/CD49A enriched ILC still showed efficiency to rapidly reduce glycemia and maintain it in the normal level (**Figure 7A**, green squares). Under these conditions, the non-sorted ILC did not bring down glycemia to normal levels, but only reduced it slightly and maintained it at an intermediate diabetic state (**Figure 7A**, red circles). The CD49A depleted fraction obtained from the CD26 depleted cells, did not maintain the glycemia, which rose to highly diabetic levels (**Figure 7A**, blue triangles). The levels of human C-peptide in the blood of mice implanted with CD26 depleted/CD49A enriched ILC were higher than with the other types of ILC, throughout the follow-up period of 8 weeks (**Figure 7B**). This was observed in fasting mice and after stimulation of C-peptide secretion by glucose. The increased therapeutic activity of the CD26 depleted/CD49A enriched ILC cells over non-sorted total cells was also demonstrated by an intraperitoneal glucose tolerance test (IPGTT) on day 46 after implantation (**Figure 7C**), as the area under the curve being reduced by 50% ($p = 0.046$).

DISCUSSION

Our aim was to fractionate the cell populations produced by differentiation of human pluripotent stem cells into pancreatic islet-like clusters, in order to select the insulin-producing cells which are most active to normalize glycemia in diabetes models. To find markers for selection, we used a functional cell capture screening (FCCS) assay on a microarray of large set of antibodies to cell surface proteins (10). In a first series of screens, we identified an antibody to integrin-alpha1 (CD49A, ITGA1) as efficiently binding insulin-producing cells. We show that by binding to anti-CD49A, one can separate cells that reduce glycemia in diabetic mice from cells completely lacking this capacity, although they express insulin. However, MACS selection with CD49A antibodies was not sufficient to clearly improve the therapeutic activity over that of non-sorted ILC. We, therefore, made a new series of screens in which we compared the

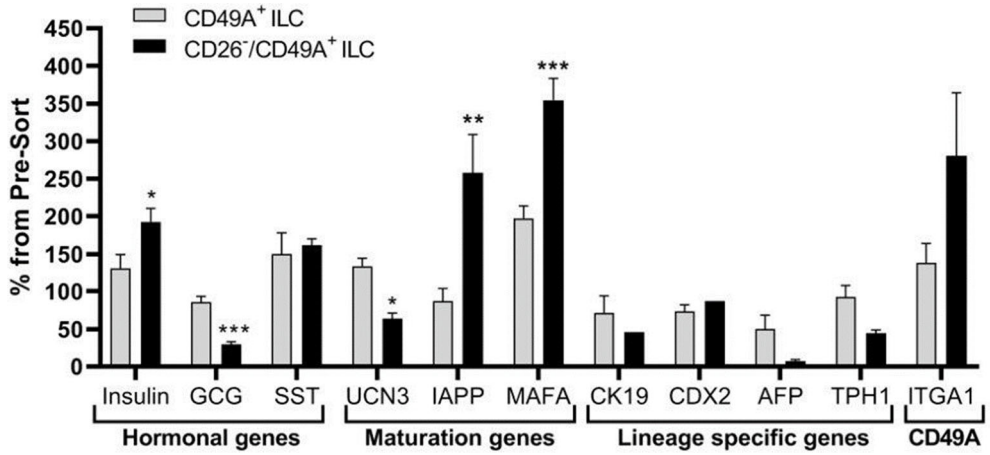

FIGURE 5 | CD26 depletion followed by CD49A selection improves mature β-cells phenotype *in vitro*. qPCR analysis of RNA extracted from ILC cells fractionated by CD26$^-$/CD49A$^+$ MACS ($n \geq 6$ different batches) shows that the depletion/selection strategy yields an improved gene expression phenotype with increased expression of mature islet signature genes as MAFA, Insulin, and IAPP compared to CD49A enrichment alone. In addition, the removal of CD26 results in significantly lower expression levels of Glucagon and AFP mRNAs. Results calculated in comparison to non-sorted cells, taken as basal 100% reference. The results are expressed as mean ± SEM. Student t test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

TABLE 2 | Proportions of C-peptide and Nkx6.1 positive subpopulations in ILC cells double selected with antibodies to CD26 and to CD49A.

Expt 1 Cell fraction	**Total Non-sorted**	**CD26 enriched**	**CD26 depleted**	**CD26 depleted CD49A enriched**	**CD26 depleted CD49A depleted**
Cells positive for:	Percent of cells				
1. C-peptide and NKX6.1	24.2	19.7	33.8	71.5	16
2. C-peptide only	22.9	43.0	4.8	10.7	4.0
3. NKX6.1 only	35.8	18.0	52.8	12.1	69.5
4. Negative (Q3)	17.1	19.3	8.5	5.7	10.5
Expt 2					
Cell fraction	Total non-sorted	Total reaggregated	CD26 enriched	CD26 depleted	CD26 depleted CD49A enriched	CD26 depleted CD49A depleted
Cell positive for:	Percent of cells				
1. C-peptide and NKX6.1	22.5	32.6	6.1	33.7	66.6	21.2
2. C-peptide only	20.2	21.7	43.8	8.4	11.4	7.0
3. NKX6.1 only	13.3	21.6	6	24.3	13.3	42.7
4. Negative (Q3)	44.1	24.2	44.1	33.6	8.7	29.1

The C-peptide/Nkx6.1 double positive cells predominate in CD26 depleted/CD49 enriched fraction. After double MACS sorting and staining for human C-peptide and Nkx6.1 cells were analyzed in FACS plots as illustrated in ***Figure 5****. Quantitation from fractionation of two independent batches are shown in two experiments. In experiment 2, non-sorted ILC cells after dissociation and reaggregation without fractionation are also shown.*

antibodies for capture of insulin-producing cells and of Nkx6.1$^+$ cells together. We found that an antibody to dipeptidyl peptidase-4 (CD26, DPP4) bound ILC cells expressing insulin (and human C-peptide) but lacking Nkx6.1, and did no bind C-peptide$^+$/Nkx6.1$^+$ cells (**Figure 5**). By depletion of CD26$^+$ cells followed by enrichment for CD49A$^+$ cells, ILC populations containing 60–70% C-peptide$^+$/Nkx6.1$^+$ cells could be obtained. Such purified ILC fractions had an improved efficacy to reduce glycemia and secrete human C-peptide, relative to non-sorted ILC, or cells selected only for CD49A.

CD49A, the Integrin alpha1 chain, had been reported to have a function in the development of β-cells in human fetal and adult pancreas since, in combination with Integrin-β1, it forms the primary collagen-binding receptor (in particular for Collagen IV) and thereby contributes to β-cell adhesion and motility, as well as to insulin secretion (29). In recent studies using single-cell RNA Seq analysis of gene expression (30, 31) in the various cell populations formed by differentiating hPSC toward islet-like cells, Veres et al. (31) found that CD49A (ITGA1) is mostly (but not exclusively) a marker of β-cells expressing the INS, Nkx6.1, NPTX2, PCSK1 genes. These authors reported that MACS with CD49A antibodies can isolate these β-cells at 60–80% purity and that, *in vitro*, glucose-stimulated insulin secretion (GSIS) was improved in these cells. Our approach was different and based on screening of a large array of antibodies against membrane proteins to identify antibodies that bound insulin-producing hESC-derived β-like cells. CD49A emerged from this screen and we proceeded to study the *in vivo* therapeutic activity of cells selected for this marker in models of STZ-induced diabetes in C57BL/6 mice.

CD26 had been reported to be a marker of ductal cells and of alpha cells, but not of β-cells, in islets isolated from human pancreas (26, 27). However, we unexpectedly found that antibodies to CD26 efficiently bound insulin-producing ILC cells lacking Nkx6.1. This finding allowed us to further purify the hESC-derived ILC cells by depletion for CD26 and enrichment for CD49A and thereby obtain ILC with improved anti-diabetic activity in STZ-treated diabetic mice. The correlation between the cell types seen in the selected fractions and the anti-diabetic activity is interesting to investigate. Overall, our data support the assumption that it is the abundance of C-peptide$^+$/Nkx6.1$^+$ double positive cells which contributes most to the therapeutic activity. In the total non-sorted ILC used in the different experiments, the percentage of double positive cells was in the range of 18–24%, which increased to 34–35% after removal of cells binding to anti-CD26 antibodies and to 60–71% after further purification of the cells binding to CD49A. If the CD49A selection was done alone, only 44–46% double positive were obtained. Hence, the combined CD26 and CD49A selection is better and this is what was observed in term of therapeutic activity in diabetic mice. Similarly, compared to non-sorted cells, the combined CD26 and CD49A selection gave a larger increase in the levels of insulin and MafA mRNA, supporting the effect of enrichment on functional maturation phenotype. Besides enriching for more active β-cells, the purification also removes unnecessary cells. Thus, depleting CD26$^+$ cells removed the insulin-producing C-peptide$^+$ cells lacking Nkx6.1, which correspond to polyhormonal progenitors that do not seem to contribute to β-cell functions *in vivo* an even to become alpha cells (22) delta cells (13). The beneficial or detrimental role of the other cell types in the ILC remains to be clarified. For example, the CD49A-enrichment also removes cells expressing TPH1 (**Figure 2C**), which probably correspond to cells producing serotonin (entero-chromaffin cells) that inhibit insulin secretion (25). Removing CD26$^+$ cells may reduce the amount of DPP4 enzyme which degrades GLP-1, an important stimulator of β-cell function (32). So, removing these populations could eliminate cells that do not participate in the control of glycemia or disturb this control. In addition, and not less important, the purification helps reducing the number of *in vitro* differentiated ILC cells that need to be implanted for cell therapy of diabetes. Our double selection, depleting CD26$^+$ cells and enriching for CD49A$^+$ cells, removes

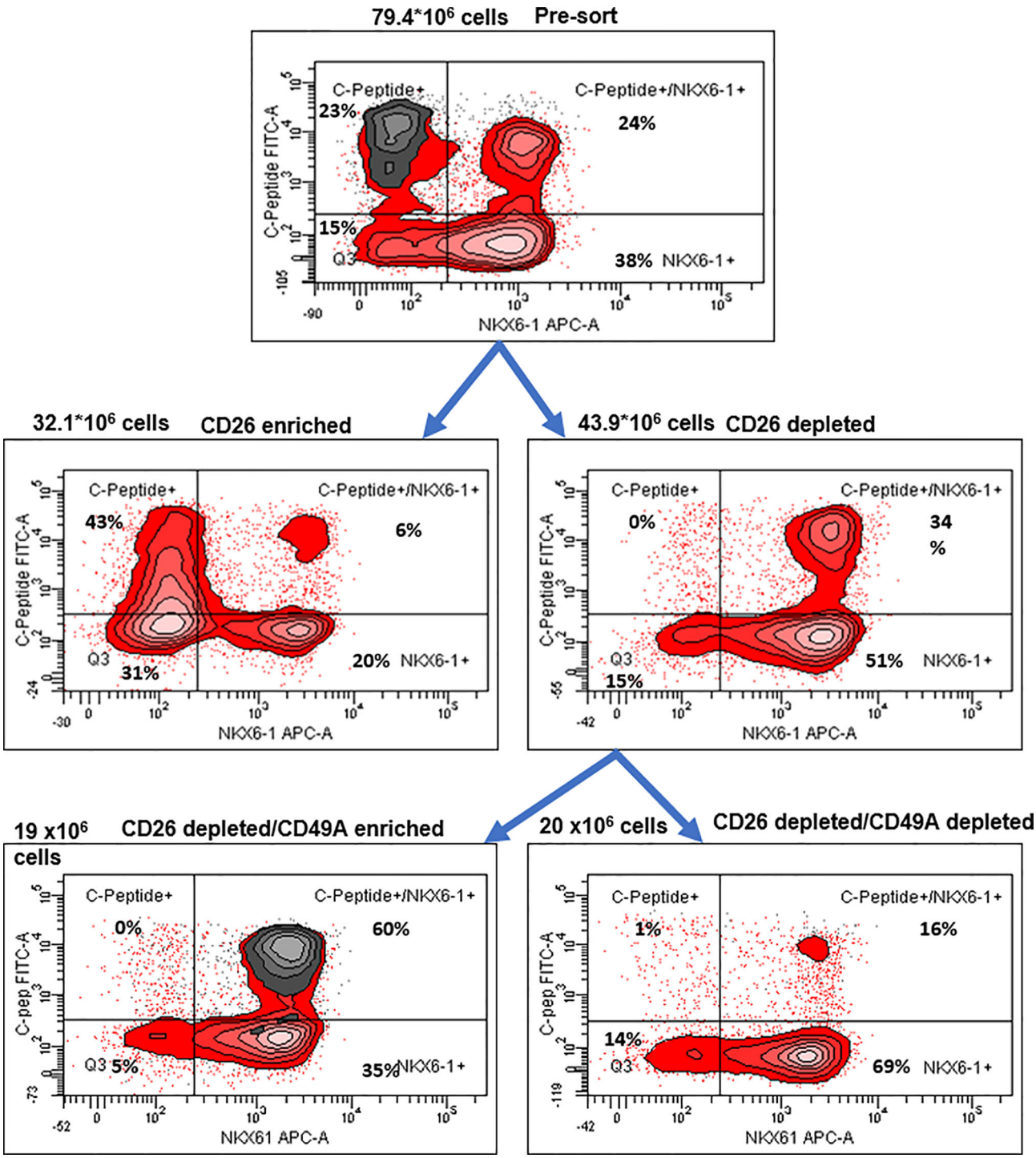

FIGURE 6 | Preparative MACS selection using novel sorting strategy. After selection by MACS with CD26 antibodies (middle two panels), the CD26 depleted cells have lost the C-peptide only fraction (right panel). After subsequent selection of the CD26 depleted fraction by MACS with CD49A antibodies (lower two panels), the C-peptide/Nkx6.1 double positive fraction is increased (lower left panel with CD49A stain in gray). For comparative percentage of cells in each FACS subpopulation see **Table 2**. The total number of cells at each step is shown next to the panels.

many cells (**Figure 5**) and allowed to reduce the number of cells needed for normalizing glycemia in our experiments with diabetic mice. This reduction could be of practical importance to decrease the volume of hPSC-derived ILC that will be required to treat human diabetic patients. Performing MACS purification requires dissociation of the differentiated islet-like clusters produced in our

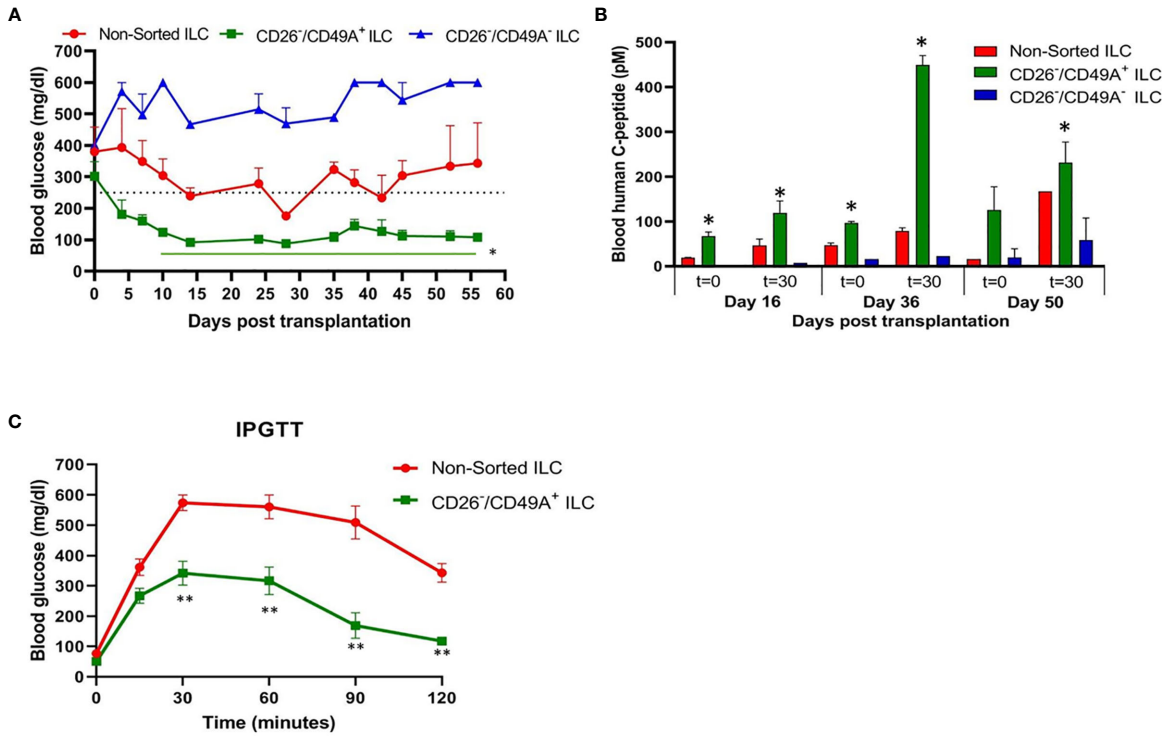

FIGURE 7 | Improved activity of CD26 depleted/CD49A enriched ILC to normalize glycemia in diabetic mice. **(A)** CD26 depleted/CD49A enriched ILC cells (squares with green line) implanted in diabetic C57BL/6 mice, reduce blood glucose better than non-sorted cells (red circles) or than CD26 depleted/CD49A depleted cells (dark blue triangles). After a first MACS with CD26 antibodies, the CD26⁻ flow-through cells were separated by MACS with CD49A antibodies. The non-sorted and the sorted cell fractions were reaggregated into ILC prior to micro-encapsulation in TMTD-MVG alginate and, from each, a dose of 1×10^6 cells was implanted i.p. in C57BL/6 mice ($n \geq 5$). Average blood glucose ± SEM is shown. Starting from day 10, the average blood glucose results of CD26⁻/CD49A⁺ group is significantly lower than non-sorted cells (Student t test. *$p < 0.05$, horizontal green line). **(B)** In the same mice, blood C-peptide was measured at indicated days, after overnight food deprivation (t = 0) and 30 min after i.p. injection of 2 g/kg glucose (t = 30). Average blood levels of human C-peptide in pM ± SEM are shown for mice with implants of ILC either non-sorted (red), CD26 depleted/CD49A enriched (green), or CD26 depleted/CD49A depleted (blue). The significantly higher values for each timepoint are marked (Student t test. *$p < 0.05$). **(C)** Intraperitoneal Glucose Tolerance Test (IPGTT) shows that CD26 depleted/CD49A enriched ILC cells (squares with green line) reduce more the diabetic state than non-sorted ILC cells (red circles). On day 45 after implantation the mice were injected i.p with 2 g/kg glucose and blood glucose was measured at different times for a 2-h period. The results are expressed as BGL mean ± SEM. Student t test. **$p < 0.01$ from t = 30 onwards. The AUC with the CD26 depleted/CD49A enriched cells was 50% lower than that with the non-sorted re-aggregated cells ($p = 0.046$).

3D-suspension cultures. The dissociated cells, MACS-fractionated or not, were reaggregated by a few days of culture before implantation. This procedure does not alter the composition of the ILC, nor their activity in diabetes models (33). For implantation, we encapsulated the ILC in medium-viscosity MVG alginate coupled with TMTD, and not the very low-viscosity SLG20 alginate as used by Vegas et al. (9). When encapsulated in TMTD-MVG microspheres (of 1.5 mm diameter) and implanted in the peritoneal cavity of the diabetic mice, the total and the purified ILC caused a rapid decrease in glycemia starting soon after implantation. There was no 3–6 month delay as seen when non-mature progenitors are implanted (12). This indicates that the ILC fully differentiated *in vitro*, provide the functionally mature β and other cell types needed to restore and maintain glycemia in the normal range, shortly after implantation. A reservation is that since the MACS fractionation is not complete, the purified fractions are likely to still contain other cell types that influence the therapeutic effect. Indeed, the presence of glucagon-producing alpha-like cells is probably important to prevent hypoglycemic events. The aim is to implant cell clusters that mimic the activities of natural islets, and not pure β-cells. The FCCS assay had previously shown that CD56 and CD9 are markers of β-cell s in islets from human post-mortem donations (11). With our hESC-derived ILC, CD56 was also high on the list of antibodies binding insulin-producing cells, while CD9 was not found (not shown). The CD200 marker reported to isolate endocrine cells at the pancreatic progenitor stage of hESC differentiation (22) was also in our FCCS (not shown). The FCCS assay identified additional antibodies that may be candidates to select cells active in restoring and maintaining normoglycemia in diabetics, and/or remove inactive cells by negative sorting (data not shown). Development of better means of large-scale cell sorting with combinations of antibodies, such as shown here, appears as an important endeavor to produce the amounts of ILC needed for treating the many millions of insulin-dependent diabetic patients by regenerative cell therapy.

CONCLUSIONS

In vitro differentiation of islet-like clusters from human pluripotent stem cells represents a potentially unlimited source of cells that could restore physiological control of blood glucose in diabetic patients requiring insulin. The CD49A surface protein (integrin-alpha1) was identified as a selective marker of the ILC cells that are active to normalize glycemia in a diabetic mice model. Removal of cells expressing CD26 (DPP4) prior to enrichment of $CD49A^+$ cells further improved their therapeutic activity and reduced the number of ILC cells needed to normalize glycemia. The ILC purification described appears as a promising strategy to improve cell therapy of diabetes with hPSC-derived ILC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by National Council for Animal Experimentation, MOH, Israel.

AUTHOR CONTRIBUTIONS

KM, JC and MR directed the research work and wrote the manuscript. OE, MW and YS conceived and contributed the Functional Cell Capture Screening. DB, AB, KY, MZ, IT, AE and EV performed the experiments. AL, KM supervised the animal experimentation. AH and JI were senior advisors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.635405/full#supplementary-material

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REVIEW
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Human Pluripotent Stem Cells Go Diabetic: A Glimpse on Monogenic Variants

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Diabetes, as one of the major diseases in industrial countries, affects over 350 million people worldwide. Type 1 (T1D) and type 2 diabetes (T2D) are the most common forms with both types having invariable genetic influence. It is accepted that a subset of all diabetes patients, generally estimated to account for 1–2% of all diabetic cases, is attributed to mutations in single genes. As only a subset of these genes has been identified and fully characterized, there is a dramatic need to understand the pathophysiological impact of genetic determinants on β-cell function and pancreatic development but also on cell replacement therapies. Pluripotent stem cells differentiated along the pancreatic lineage provide a valuable research platform to study such genes. This review summarizes current perspectives in applying this platform to study monogenic diabetes variants.

Keywords: pluripotent stem cells, diabetes, monogenic variants, Maturity Onset of Diabetes in the Young, type 2 diabetes, type 1 diabetes

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INTRODUCTION

Diabetes, as one of the major diseases in industrial countries, affects over 350 million people worldwide. Type 1 (T1D) and type 2 diabetes (T2D) are the most common forms. T2D accounts for most diabetes cases and is a multifactorial metabolic disease where insulin deficiency is caused by insulin resistance in target organs and pancreatic β-cell failure. The current diabetes classifications are insufficient to explain the large clinical and biological variability of diabetes, suggesting an unrecognized level of heterogeneity (1). T1D is described as a chronic autoimmune disease against insulin-producing β-cells leading to hyperglycemia. T1D results from the combination of multiple factors, including environment, genes, and a prominent role of the immune system. Genetic studies have long recognized that mutations of the human leukocyte antigens (HLAs) within the Major Histocompatibility Complex (MHC) represent major genetic risk factors in T1D (2, 3). More recently, genome-wide association studies (GWAS) and candidate gene approaches have identified

Abbreviations: CRISPR, Clustered regularly interspaced short palindromic repeats; ER, Endoplasmatic reticulum; GWAS, Genome-wide association studies; hESC, Human embryonic stem cell; HLA, Human leucocyte antigen; hPSC, Human pluripotent stem cell; iPSC, Induced pluripotent stem cell; JOD, Juvenile-onset diabetes; KO, Knockout; MHC, Major histocompatibility complex; MODY, Maturity Onset of Diabetes in the Young; PNDM, Permanent neonatal diabetes mellitus; SNP, Single nucleotide polymorphism; T1D, Type 1 diabetes; T2D, Type 2 diabetes.

more than 50 other loci contributing to T1D risk, including *INS, PTPN22, CTLA4*, and *GLIS3* genes (4, 5). In addition, mutations in several genes, such as *AIRE, FOXP3, and STAT* (6, 7), may cause rare monogenic forms of autoimmune diabetes.

Similarly, genetic studies of T2D identified many single nucleotide polymorphisms (SNPs) associated with T2D risk, which are located near several functionally relevant genes such as *PPARG* (8), *WFS1* (9), *KCNJ11* (10), *KLF14* (9), *ANK1* (11), *INS* (12), *HNF1A* (9), *HNF1B* (13), and *GLIS3* (14). In addition to genetic predisposition, environmental factors and epigenetic changes are influencing the pathophysiology of T2D, which may contribute to the additional variance in susceptibility.

Overall, genetic studies of T1D and T2D resulted in the identification of many disease-associated variants, most of which, with the exception of the HLA locus for T1D, contribute to a small increase in disease risk (5, 15, 16). These studies have provided valuable information on putative genes and the mechanisms involved in diabetes. For example, many genes identified by T2D GWAS are expressed in human islets (17) and may regulate β-cell mass and function (18). While a large proportion of T1D susceptibility genes are surprisingly not related to the immune system (19), studies from D. Eizirik's group have also shown that >60% of these genes are expressed in β-cells, and their expression is affected upon exposure to cytokines, viruses, and double-stranded RNA, a by-product of viral infection, in human and rodent β-cells (19–21). Altogether, these studies suggest an essential role of mechanisms acting at the level of β-cells in the etiology of both T1D and T2D (19–21).

Interestingly, several of these genes are involved in both monogenic diabetes (rare variants) and multifactorial diabetes (frequent variants). This is the case of the insulin gene (*INS*, monogenic neonatal diabetes, Maturity Onset of Diabetes in the Young (MODY) and multifactorial T1D), as well as *KCNJ11, WFS1, HNF1A*, *HNF1B* (neonatal, syndromic, or MODY monogenic diabetes, and multifactorial T2D) and *GLIS3* (neonatal monogenic diabetes and multifactorial T1D and T2D). This plethora of genes involved in common multifactorial and rare monogenic forms of diabetes suggests that some disease mechanisms and biological pathways may be shared between different forms of diabetes. The identification and detailed study of genes responsible for monogenic diabetes are therefore extremely valuable to investigate important genes and pathways involved in both monogenic diabetes and common forms of diabetes. Noteworthy, it has become evident most recently that a subset of all diabetes patients, generally estimated to account for 1–5% of all diabetic cases, is attributed to mutations in single genes (22, 23).

As only a subset of these genes has been identified and fully characterized, there is a dramatic need to understand the pathophysiological impact of genetic determinants on β-cell function and pancreatic development but also on cell replacement therapies. Although islet transplantation can lead to insulin-independency of diabetic patients for 5 years or longer, this therapeutic option is only accessible for a rare number of patients due to the limited number of cadaveric human islets and complex handling (24). On the other hand, the use of human pluripotent stem cells [hPSCs, induced pluripotent (iPSC) and embryonic stem cells (hESC)] may bypass this need by generating mature β-cells *in vitro* upon improving the current protocols of β-cell generation.

PSCs have been used as a relevant model system to elucidate pathophysiological mechanisms in diseases such as diabetes, blood disorders, defined neurological disorders, and genetic liver disease (25–27). Induced pluripotent stem cells (iPSCs) allow dissecting monogenic human disease mechanisms (28) as well as mechanisms of genetically complex human disorders such as schizophrenia (29). This opens promising perspectives in both regenerative medicine but also in drug development to screen for innovative, "druggable" targets (30) and to develop *ex vivo* gene-targeting therapies (28). Given the still high intra- and interpatient variability of patient-derived iPSCs, controls are the key for a precise analysis (31). Recent advances in the development of genomic editing tools such as the Zinc-finger or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology have further revolutionized this research field. Now researchers can precisely modify a human pluripotent stem cell genome with (i) high efficiency, (ii) on a single-base resolution, (iii) without altering the pluripotent capacity, and (iv) with negligible off-target effects to provide isogenic controls and to facilitate data interpretation. In turn, these recent tools represent novel state-of-the-art disease-in-a-dish models and will pioneer research fields aiming to understand also the mechanisms underlying monogenic diseases (32–35). Human pancreatic disease modeling is highly dependent on reliable and efficient differentiation protocols for human PSCs. We and others have recently challenged the currently existing protocols (36), first, to optimize the step toward pancreatic progenitor cells (37), second, to drive maturation in a 3D environment (38–40), and third, to increase yields of true monohormonal β-cells (41–44). In turn, optimized differentiation platforms now allow for appropriately modeling complex pancreatic diseases such as diabetes (45). A schematic overview of currently available disease modeling tools for diabetes employing hPSCs is presented in **Figure 1**.

MATURITY-ONSET DIABETES OF THE YOUNG

So far, 14 subtypes of maturity-onset diabetes of the young (MODY) have been described to be caused by mainly heterozygous dominant mutations in genes for pancreas-specific transcription factors as well as enzymes, hormones, and ion channels (46, 47). These mutations impair endocrine function at various levels ranging from alterations in development, glucose sensing, synthesis, and storage of insulin to inappropriate secretion of insulin in β-cells. The most frequently identified mutations are located in the *HNF4A* gene (MODY1) (48) with a frequency of 4–10% (49–51), in the *GCK* gene (MODY2) (52) with 30–60% (49–51, 53) and in the *HNF1A* gene (MODY3) (54) with 30–50% (49–51) depending on the study population. MODY1 patients are particularly characterized by defective glucose-stimulated insulin secretion possibly caused by disrupted gene expression playing a role in glucose transport

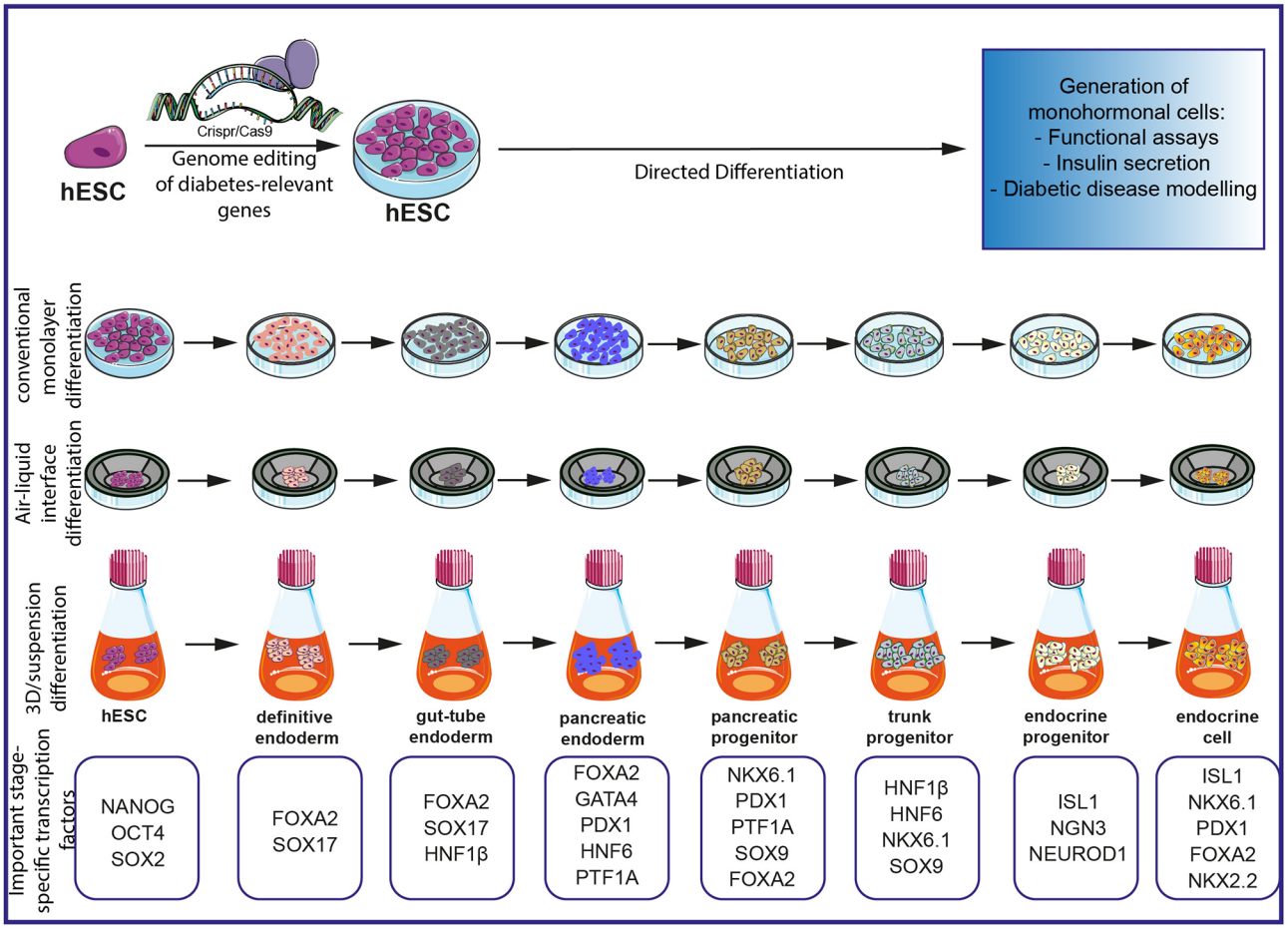

FIGURE 1 | Schematic overview of currently existing protocols for the investigation of diabetes on an hPSC-based platform. Gene editing with CRISPR/Cas9 allows precise editing of diabetes-relevant genes and generation of hESC for further differentiation experiments. Different differentiation protocols allow the generation of monohormonal cells by passing through different milestones during embryonic development. Important stage-specific transcription factors are indicated below the schematics. Subsequent analysis of monohormonal β-cells, including insulin secretions assays, can be performed and generate hypotheses about the influence of specific genetic variants. The figure was modified from Smart Servier Medical Art (https://smart.servier.com/) under a Creative Common Attribution 3.0 Generic License.

and glycolysis (48, 55, 56). MODY2 due to glucokinase deficiency often results in mild hyperglycemia during early life (57). These patients have a defect in glucose-stimulated insulin secretion caused by impaired glucose sensitivity in β-cells. MODY3 patients develop β-cell dysfunction and hyperglycemia caused by impairment of glucose-dependent insulin secretion (58).

In addition, rare MODY cases [accounting for up to 6% of all MODY forms (49)] have been diagnosed with mutations in *PDX1* (59), *HNF1B* (60), *NEUROD1* (61, 62), *KLF11* (63), *CEL* (64), *PAX4* (65), *INS* (66), *BLK* (67), *ABCC8* (68), *KCNJ11* (69), and *APPL1* (70) (known as MODY4-14).

mpaired functions of proteins caused by pathogenic variants can vary depending on the nature of the mutation, therefore causing a spectrum of clinical manifestations. Patients harboring heterozygous *HNF1B* mutations suffer from MODY, but may also feature pancreas exocrine dysfunction as well as kidney and liver abnormalities (71) and vaginal and uterine malformation (72). Few specified cases of *NEUROD1* mutations are characterized mainly by early onset diabetes (61, 62, 73), but patients with neurological defects such as pituitary gland hypoplasia, growth hormone deficiency, epilepsy, and intellectual disability have also been described (74, 75). *CEL* mutations cause early onset diabetes associated with exocrine pancreatic dysfunction and chronic pancreatitis (76, 77). In addition to the diabetic phenotype in patients with *PAX4* mutations, diabetic complications such as retinopathy and nephropathy have been observed (65, 78). MODY-causing *INS* mutations have been associated with early onset diabetes as well as ketoacidosis in some cases (79), whereas rare cases with *BLK* mutations have also been associated with overweight (67).

No other clear clinical manifestation besides diabetes has been described for patients with heterozygous mutations in *KLF11* (MODY7), *ABCC8* (MODY12), *KCNJ11 (MODY13)*, and APPL1 (MODY14). A summary of confirmed MODY-causing mutations as well as of other prominent clinical features is presented in **Table 1**.

TABLE 1 | Different MODY forms, including their frequencies, affected genes, and potential other prominent clinical manifestations are presented.

MODY form	Affected gene	Frequency	Potential prominent additional clinical manifestations besides diabetes and its complications	Affected gene investigated using hESC	Affected gene investigated using hiPSC
MODY1	*HNF4A*	4–10%	Not relevant	No	Yes (80–82)
MODY2	*GCK*	30–60%	Not relevant	No	Yes (80, 83)
MODY3	*HNF1A*	30–50%	Not relevant	Yes (84, 85)	Yes (80)
MODY4	*PDX1*	Rare	Pancreatic agenesis and miscarriages	Yes (86)	Yes (87–89)
MODY5	*HNF1B*	Rare	Exocrine pancreatic dysfunction, kidney and liver abnormalities, vaginal aplasia, and uterus hypoplasia	No	Yes (80, 90, 91)
MODY6	*NEUROD1*	Rare	Neurological defects including pituitary hypoplasia, growth hormone deficiency, epilepsy, and intellectual disability	No	No
MODY7	*KLF11*	Rare	Nothing else described	No	No
MODY8	*CEL*	Rare	Exocrine pancreatic dysfunction, chronic pancreatitis	No	Yes (80)
MODY9	*PAX4*	Rare	Not relevant	Yes (92)	No
MODY10	*INS*	Rare	Not relevant	No	Yes (93)
MODY11	*BLK*	Rare	Overweight	No	No
MODY12	*ABCC8*	Rare	Nothing else described	Yes (94, 95)	No
MODY13	*KCNJ11*	Rare	Nothing else described	Yes (96)	Yes (97)
MODY14	*APPL1*	Rare	Nothing else described	No	No

Furthermore, a statement about the current research of the respective mutations or MODY forms, including hESC and hiPSC, is included.

Noteworthy, the homozygous status for mutations in several MODY genes has been found to lead to extreme clinical presentations, contrasting with the less severe early onset diabetes observed in heterozygous carriers. For example, homozygous mutations in the *PDX1* gene result in early onset diabetes associated with pancreatic agenesis and maternal miscarriages (59, 98).

A subset of patients with MODY-like phenotype doesn't carry any mutation in the known MODY genes, suggesting the involvement of additional genes. The identification of these additional genes responsible for rare MODY forms is now facilitated by the availability of large databases of diabetic cases and control cohorts that enable increased efficiency to detect novel genes with rare contributing variants (including MODY-like effects) (99), compared to earlier studies with smaller sample size (100). In addition, the availability of large databases of control subjects (*e.g.*, gnomAD, TOPMED) provides now the possibility to estimate the frequency of rare coding variants in candidate genes, hence allowing for efficient association and burden-testing for rare monogenic contributions, such as MODY. Consequently, recent studies identified *RFX6* as a novel MODY gene (101) and *WFS1, PPARG*, and *GLIS3* have recently been proposed as potential candidates for these rare MODY forms (101–103).

Taken into consideration the overlap in genes involved in common multifactorial and rare monogenic forms of diabetes, the specific analysis of monogenic pathogenic variants can therefore reveal novel interaction partners and gene targets that might be helpful to better understand the mechanisms involved in the onset of T1D and T2D.

PERMANENT NEONATAL DIABETES MELLITUS

Permanent neonatal diabetes mellitus (PNDM) is the second form of monogenic diabetes. It is characterized by hyperglycemia and partial or complete insulin deficiency in patients in the first 6 months postnatal (104, 105). Moreover, patients with PNDM may suffer from intrauterine growth retardation, glycosuria, ketoacidosis, failure to thrive as well as various clinical features depending on the gene. Mutations in more than 20 genes with monogenic contribution important for β-cell development have been identified to cause PNDM (46). Treatment of PNDM includes oral sulfonylureas or insulin therapy and may require pancreatic enzyme replacement for infants with pancreatic aplasia or hypoplasia.

Some genes, including *ABCC8* (106), *GCK* (107), *INS* (108), *KCNJ11* (109), and *PDX1* (110) may alternatively cause PNMD or MODY, with various severity and clinical features depending on the gene, nature of the mutation, and genotype (homozygous or heterozygous). Common variants in these genes may also be associated with multifactorial T1D or T2D.

In addition to diabetes, *KCNJ11*-PNMD patients may also have neurological features such as developmental delay and epilepsy (DEND syndrome) (111). Similarly, pathogenic homozygous *PDX1*-PNDM patients have pancreatic agenesis and pancreatic hypoplasia leading to exocrine pancreatic insufficiency (110, 112). Pancreatic agenesis is furthermore caused by homozygous mutations in another pancreatic transcription factor, *PTF1A* (113). Here, PNDM patients additionally suffer from severe intrauterine growth retardation, cerebellar agenesis, and neurological dysfunction.

In addition, PNDM may manifest in the context of specific syndromes. Homozygous mutations in *EIF2AK3* cause Wolcott–Rallison syndrome, characterized by PNDM, exocrine pancreas dysfunction, and abnormalities such as liver failure, developmental delay, and epiphyseal dysplasia (114). Inactivating *GATA4* variants can induce pancreatic agenesis or hypoplasia, causing PNDM but also lead to extrapancreatic symptoms such as cardiac and neurodevelopmental abnormalities (115). Similarly, *GATA6* mutations cause pancreatic agenesis leading to PNDM, together with abnormalities of the heart, biliary tract, and gut development

(116). Homozygous mutations in *GLIS3* cause PNDM together with congenital hypothyroidism associated with congenital glaucoma, hepatic fibrosis, and polycystic kidneys (117). In addition to neonatal diabetes, *NEUROD1* mutations cause cerebellar hypoplasia, sensorineural deafness, and visual impairment (118), whereas *NEUROG3* mutations affect intestinal development leading to congenital malabsorptive diarrhea (119–121). Mutations in *PAX6*, encoding a transcription factor involved in β-cell development as well as eye and brain development, cause neonatal diabetes combined with abnormalities of the central nervous system and visual system (*e.g.* microencephaly, optic nerve defects, microphthalmia) (122). In addition to PNDM, mutations in the transcription factor *RFX6* cause pancreatic hypoplasia, intestinal atresia, and gall bladder hypoplasia (123, 124). Patients with Wolfram syndrome caused by mutations in *WFS1* suffer from early onset diabetes as well as optic atrophy, deafness, ataxia, and dementia (125). Other neonatal diabetes syndromes have been described for mutations in *SLC19A2* (associated with thiamin-responsive megaloblastic anemia, neurological disorders, cardiac abnormalities, and deafness) (126, 127), *MNX1* (associated with growth retardation, delayed central nervous system development, hypoplastic lungs, renal maldevelopment, skeletal dysplasia) (128), *NKX2.2* (further leading to growth retardation, delayed central nervous system development, constipation) (128), and *IER3IP1* (additional microcephaly, CNS maldevelopment) (129). Furthermore, some mutations in the glucose transporter *SLC2A2* can cause neonatal diabetes prior to the Fanconi–Bickel syndrome associated with glycosuria, galactosemia, aminoaciduria, proteinuria, hepatomegaly, as well as glucose and galactose intolerance (130, 131). Franco et al. recently showed that mutations in *YIPF5* cause neonatal diabetes associated with microcephaly and epilepsy (132).

Interestingly, an overlapping phenotype between PNDM and autoimmune T1D was observed for a patient with an activating mutation in the *STAT3* gene (133). Although the autoimmune-mediated destruction of β-cells was prominent Saarimäki-Vire et al. revealed an additional mechanism (PNDM) due to the observed pancreatic hypoplasia (134).

An overview of all described genes leading to the development of PNDM if affected by mutations is presented in **Table 2**.

MODELING PANCREATIC ENDOCRINE DEVELOPMENT

The detailed pathomechanisms of monogenic diabetes are not yet fully understood since mouse models do not completely recapitulate the human disease phenotype (121, 144, 145), and patient samples such as β-cells have very limited availability. Moreover, animal models with a specific knockout of MODY genes show species-specific differences that do not entirely recapitulate the patient phenotype (146–150). Therefore, even more suitable disease models are crucial to develop an adequate therapy.

In the recent years, human pluripotent stem cells have been deployed as a suitable human model system. On the one hand, human embryonic stem cells (hESCs) can be subjected to a directed differentiation protocol to investigate different mechanisms during differentiation of mature pancreatic β-cells. Additionally, patient-specific induced pluripotent stem cells (iPSCs) can be generated from materials such as fibroblasts and keratinocytes, allowing to address various genetic backgrounds of patients. Of note, gene-mutated iPSCs show high heterogeneity in terms of differentiation efficiency and are best controlled with isogenic, repaired lines. Furthermore, patient-specific iPSCs are a useful tool for biobanking because of their unlimited expansion capacity. Subsequently, these cells are differentiated *in vitro* into disease-relevant cell types such as pancreatic endocrine cells or β-cells.

To better understand the importance of certain genes in the maturation of β-cells, genetic engineering may be performed with hESCs and iPSCs. Here, state-of-the-art gene-editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the more recent clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas allow the generation of specific point mutations or gene knockouts (KOs). A potential option of genetic engineering might involve gene correction in iPSCs. This allows the generation of autologous β-cells for transplantation that may circumvent immune reaction and donor scarcity.

The generation of pancreatic endocrine cells is achieved by different differentiation protocols. Established protocols try to mimic signaling pathways of *in vivo* embryonic developmental stages by involving different/various combinations of growth factors, cytokines, and small molecules known/reported to guide the stem cells through stages of definitive endoderm, gut-tube endoderm, pancreatic endoderm, pancreatic progenitors, and endocrine progenitors to finally yield mature mono-hormonal endocrine cells (**Figure 1**).

Overall differentiation models of hESCs and hiPSCs provide a versatile tool to study the influence of genetic disorders on β-cell development in the human pancreas as well as the embryonic development of the human pancreas itself. Different published protocols whose concepts are described below allow the investigation of multiple facets of β-cell maturation during different steps of embryonic development. However, the procedure itself may have a huge influence on the phenotype of differentiated cells. This might lead to the bias that a "good" differentiation protocol can overcome the inherent genotypic features, which would rather underestimate the phenotypic features of a certain genotype. This is, for example, the case for a *GATA6*-mutant iPSC cell line with a severe phenotypic loss of endoderm and β-cell differentiation capacity in a differentiation condition involving low levels of retinoic acid. On the other hand, high levels of retinoic acid mask this phenotype (136). The same principle might apply to a "poor" differentiation protocol that may overestimate the phenotypic properties of a certain genotype.

GENERATION OF MATURE PANCREATIC β-CELLS REQUIRES COMPLEX AND SOPHISTICATED DIFFERENTIATION PROTOCOLS

During the last decade, differentiation protocols have been adapted to achieve a more mature state of *in vitro* differentiated β-cells. Since

TABLE 2 | Overview of mutations in genes that can lead to PNDM.

Affected gene in PNDM	Affected gene also described for MODY	Part of a syndromic phenotype	Potential prominent additional clinical manifestations	Affected gene investigated in hESC	Affected gene investigated in hiPSC
ABCC8	MODY12	No	Nothing else described	Yes (94, 95)	No
EIF2AK3	No	Yes, Wolcott-Rallison syndrome	Exocrine dysfunction, acute liver failure, developmental delay, epiphyseal dysplasia	No	No
GATA4	No	Yes	Pancreatic agenesis, cardiac, and neurodevelopmental abnormalities	No	No
GATA6	No	Yes	Pancreatic agenesis, abnormalities of heart, biliary tract, and gut development	Yes (135, 136)	Yes (135, 136)
GCK	MODY2	No	Not relevant	No	Yes (80, 83)
GLIS3	Potentially	Yes	Congenital hypothyroidism, congenital glaucoma, hepatic fibrosis, polycystic kidneys, pancreatic exocrine insufficiency, kidney, liver, and biliary dysfunction	Yes (86)	No
IER3IP1	No	Yes	Microcephaly, CNS maldevelopment	No	No
INS	MODY10	No	Not relevant	No	Yes (93, 137, 138)
KCNJ11	MODY13	No	Nothing else described	Yes (96)	Yes (97)
MNX1	No	Yes	Growth retardation, delayed central nervous system development, hypoplastic lungs, renal maldevelopment, skeletal dysplasia	Yes (86)	No
NEUROD1	MODY6	Yes	Cerebellar hypoplasia, sensorineural deafness, visual impairment	No	No
NEUROG3/ NGN3	No	Yes	Intestinal maldevelopment with malabsorptive diarrhea	Yes (86, 139)	No
NKX2.2	No	Yes	growth retardation, delayed central nervous system development, constipation	No	No
PAX6	No	Yes	Abnormalities of the central nervous system and visual system including microencephaly, optic nerve defects, microphthalmia	No	No
PDX1	MODY4	Yes	Pancreatic agenesis and miscarriages	Yes (86)	Yes (87–89)
PTF1A	No	Yes	Intrauterine growth retardation, pancreatic agenesis, cerebellar agenesis, and neurological dysfunction	Yes (86)	No
RFX6	Potentially	Yes, Mitchell–Riley syndrome	Pancreatic hypoplasia, intestinal atresia, and gall bladder hypoplasia	Yes (86)	Yes (140)
SLC19A2	No	Yes, Thiamine-responsive megaloblastic anemia	Megaloblastic anemia, hearing loss, neurological disorders, cardiac abnormalities	No	No
SLC2A2	No	Yes, Fanconi–Bickel syndrome	Glycosuria, galactosemia, aminoaciduria, proteinuria, hepatomegaly, glucose intolerance, galactose intolerance (130, 131)	No	Yes (141)
STAT3	No	Potentially correlated to autoimmune diabetes	Strong autoimmune component of diabetes, pancreatic hypoplasia	No	Yes (134)
YIPF5	No	Yes	Microcephaly, epilepsy	Yes (132)	Yes (132)
WFS1	Potentially	Yes, Wolfram syndrome	Optic atrophy, deafness, ataxia, and dementia	No	Yes (142, 143)

Additional information of mutations leading to syndromic phenotypes and their characteristics is included. Further statements regarding the relationship to MODY forms and feasibility of hPSC to model the disease development and progression in terms of PNDM are included.

earlier pancreatic endocrine differentiation protocols in monolayers yield mainly an immature or heterogeneous population of polyhormonal cells lacking robust insulin secretion in response to glucose stimulation, a prerequisite of β-cells (36, 151), novel *in vitro* approaches including different culture conditions have been established. Recent protocols include a transition to 3D culture using a suspension culture system with spinner flasks and orbital shaker generating endocrine spheres (41, 42). Alternatively, a switch from the initial culture in monolayer to an air–liquid interphase culture stage promoting basal and apical cell polarity generates even more functionally mature β-cells (152) (**Figure 1**).

Air–liquid interphase culture systems require spotting the cells from the pancreatic endoderm stage on filters. Upon formation of small cell clusters, the differentiation is further improved as measured by *NGN3* and insulin expression (43). This transition might help to mimic the natural 3D environment and cell orientation within the developing tissue, thus promoting *in vitro* differentiation (153).

Further progress in β-cell maturation was also achieved by reaggregating immature cells into enriched β-cell clusters using an insulin-driven fluorescence reporter (154). Veres et al. combined cellular reaggregation and β-cell purification using CD49a to enrich endocrine cells and promote functional maturation of β-cells able to maintain their identity for several weeks in culture (155).

An alternative approach is to enrich precursor cells in the differentiation process. A recent study demonstrated that enrichment of anterior definitive endoderm with CD177 results in a more homogenous pancreatic progenitor population and subsequent better functional maturation (156).

Moreover, besides optimizing technical conditions for differentiation, the modulation of signaling pathways and

cytoskeleton is a promising mean to increase the β-cell yield. Inhibition of certain pathways such as ROCKII and WNT using specific inhibitors promoted maturation (157, 158). Hogrebe et al. investigated the role of the actin cytoskeleton in promoting the expression of pancreas-specific transcription factors such as NEUROG3 during differentiation (159). The manipulation of actin polymerization during early developmental stages influences the expression of transcription factors important for the specification of lineage fate in pancreatic progenitors. Depolymerization of the cytoskeleton during endocrine induction further improved the functionality of derived β-cells, also allowing for a planar differentiation protocol.

Another strategy to improve differentiation efficacy is to modulate the basic content of cell culture media. Two studies explored metabolic changes during β-cell maturation (160, 161). Adaptation of nutrient-sensing *via* mTORC1 signaling during the transition from fetal to adult pancreatic islets can be recapitulated by reduced amino acid content in differentiation media, further advancing cellular insulin content and glucose-stimulated insulin secretion (160). Similarly, epigenomic characterization of primary and *in vitro* differentiated pancreatic cells revealed that entrainment to cycles of fasting and feeding leads to circadian control of genes important for energy and insulin metabolism, further improving β-cell function (161).

Protocol improvements resulted in more mature β-cells and faster reversal of diabetes after transplantation in mice. However, manifold successful approaches show that regulation of human pancreas development is still not fully understood, and various adaptations to endocrine differentiation protocols are difficult to compare because they use different cell lines and culture methods as well as slightly different functional assays. Therefore, more research is necessary to determine the appropriate combination of culture methods, cytokine and small molecule cocktails, purification markers, and metabolic modifications, generating a protocol that robustly produces the desired pancreatic cell types.

For potential clinical use, several questions/issues regarding the composition of transplanted cells containing only β-cells or more than one endocrine cell type, the best transplantation site, and whether transplanted cells benefit from co-transplantation with other cell types such as mesenchymal stem cells need to be answered. Moreover, long-term survival and functionality of transplanted cells exceeding the life span of mice have to be addressed. Additionally, the possibility of teratoma formation from the remaining progenitor cells even after prolonged time has to be eliminated. Encapsulation of cells in suitable biomaterials such as alginates or synthetic polymer hydrogels might not only reduce the risk of tumor formation but also provide protection from the immune rejection of the host, removing the need for lifelong immunosuppression.

Although the latest research significantly improved our knowledge about transcriptional regulation, signaling pathways as well as metabolic adaptation during *in vitro* differentiation and maturation of β-cells and paved the way for future clinical use, more research is necessary until *in vitro*-generated pancreatic endocrine cells can be used as potential diabetes therapy.

PLURIPOTENT STEM CELL MODELS TO UNDERSTAND MONOGENIC DIABETES

Although iPSC could be successfully generated from T1D and T2D patients, complex autoimmune reactions, environmental influence, as well as multifactorial genetic factors hampered the intimate recapitulation of pathogenesis (162–164). Despite the complexity of T1D and T2D pathogenesis, some recent approaches have been performed to model T1D using pluripotent stem cells. Co-culture studies of iPSCs derived from T1D patients together with immune cells are one such way to model the mechanisms of T1D *in vitro* (165). Yet, it has to be kept in mind that this kind of model requires additional prerequisites such as environmental factors and complex composition of different immune cell types as recently reviewed by Joshi et al. (166). Modeling T2D *in vitro* is far more complex as many more different pathogenic mechanisms can cause or even interact to promote T2D development, including multiple genetic and environmental factors. This fact makes it even harder to investigate T2D by pluripotent stem cell-based approaches solely *in vitro*.

Whereas modeling T1D and T2D using PSCs remains challenging due to their complex nature, monogenic alterations leading to a MODY or PNMD diabetes phenotype are ideal to be investigated by PSC-based approaches. The role of specific variants of the respective genes has already been investigated using pancreatic differentiation of pluripotent stem cells. Compared to various genetic and environmental aspects contributing to other diabetes types, single mutations present in monogenic diabetes allow tighter control of the observed phenotypes. Deciphered mechanisms for the development of MODY, which were uncovered using hPSC-based systems, are presented in **Figure 2** (MODY1, 2, 3, 4, 5, 10, 13).

Teo et al. showed that karyotypically normal iPSC expressing pluripotency markers and able to differentiate in all three germ layers could be derived from different MODY patients (MODY1, MODY2, MODY3, MODY5, and MODY8) (80) and can serve as a tool to study the role of the respective genes in pancreatic development. A more detailed study of these MODY1 iPSCs with premature HNF4A protein truncation revealed impaired foregut and hepatopancreatic progenitor development. These events were associated with HNF4A mislocalization and reduced expression of target genes such as the *FOXA* gene family, *HNF1B, PDX1, GATA4*, and *RFX6* (81). In turn, impaired activation of target genes disturbs β-cell gene signatures. Prior to that study, iPSCs from MODY1 patients with a nonsense mutation were characterized (82). Here, the patient phenotype was caused by a reduction in levels of functional HNF4A accompanied by increased expression of pancreatic transcription factors and pancreatic hormones as a compensatory mechanism (82).

Understanding the role of transcription factor HNF1A in MODY3, human ESC lacking one or both alleles have been differentiated to study endocrine development (84). HNF1A deficiency increased expression of markers for α-cells but reduced expression of β-cell markers suggesting a role in endocrine hormone expression. In addition, HNF1A is required for insulin secretion, in line with hyperglycemia observed in patients. Moreover, mutated

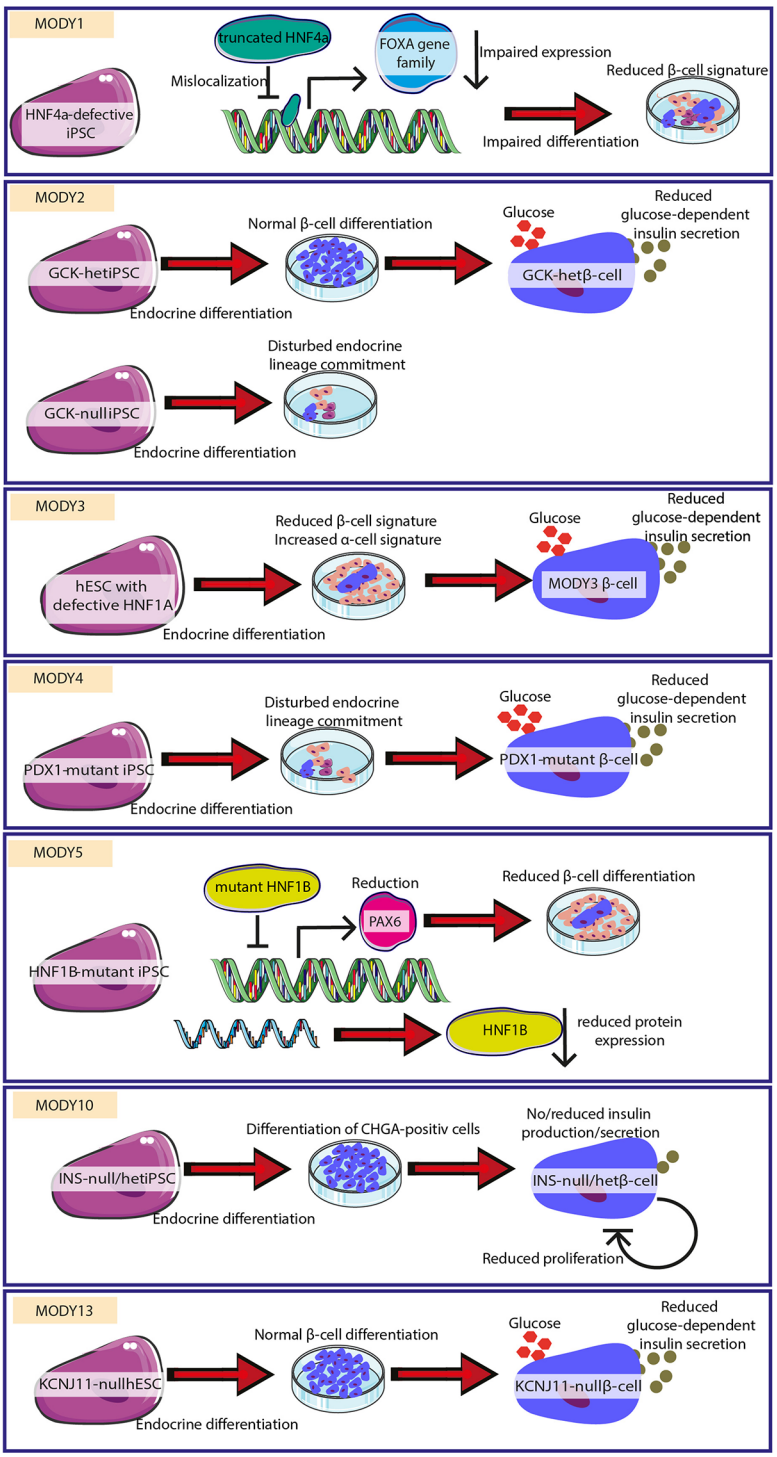

FIGURE 2 | Successful uncovering of pathomechanisms for different MODY forms. Different MODY forms were modeled by employing hESC/hiPSC with respective mutations. Mechanisms leading to monogenic diabetes could be delineated/characterized. In MODY1, mutated HNF4a leads to reduced FOXA gene family expression and impaired β-cell signature. MODY2 is characterized by reduced differentiation or reduced glucose-dependent insulin secretion. MODY3 is caused by reduced β-cell differentiation and insulin secretion. MODY4 shows reduced endocrine lineage entrance and impaired insulin secretion. MODY5 is caused by diminished β-cell differentiation. MODY10 is highlighted by lacking production and secretion of insulin. MODY13 is characterized by impaired glucose-dependent insulin secretion. The figure was modified from Smart Servier Medical Art (https://smart.servier.com/) under a Creative Common Attribution 3.0 Generic License.

cells show metabolic defects in glycolysis and mitochondrial respiration, also typical for T2D. This observation, together with the finding that frequent *HNF1A* variants are associated with T2D, suggests a link between mechanisms identified in MODY and in common T2D. Furthermore, iPSCs from a patient harboring heterozygous *HNF1A* mutation generated by non-integrative viral transduction, show a normal karyotype, and express pluripotency factors (85). These cells can be used for further functional analysis of this specific mutation.

Relevant defects in the *GATA6* gene disrupt the endoderm differentiation by decreased cell survival. Later, the pancreas specification and β-cell function were identified using iPSC and genome-edited ESC (135, 136). By circumventing the developmental block at the endoderm stage, cell lines with *GATA6* mutations were differentiated with low dose retinoic acid to mimic severe patient phenotype. These cells failed to show normal insulin secretion after glucose stimulation and harbor defective insulin processing (136).

Fibroblasts from patients with heterozygous point mutations in the *PDX1* transactivation domain were successfully reprogrammed to iPSCs and can be used to study diabetes-associated pathomechanisms (87–89). Further pancreatic differentiation reveals that mutations in the *PDX1* transactivation domain disturb the pancreatic endocrine lineage development and result in impairment of the glucose-responsive function of β-cells (88).

The analysis of patient-derived iPSCs with *HNF1B* mutations (MODY5) suffering from early onset diabetes and pancreatic hypoplasia revealed a compensatory increase in markers of definitive endoderm and pancreatic transcription factor expression such as PDX1 (90). Additionally, downregulation of transcription factor PAX6, important for islet development (167), may result in the observed patient phenotype (90). Furthermore, iPSCs were generated from a Japanese MODY5 patient with a truncated *HNF1B* variant in order to account for differences in insulin sensitivity and insulin response depending on the genetic background (91). Yabe et al. compared iPSCs derived from healthy and patient skin fibroblasts and detected degradation of mutant mRNA by the nonsense-mediated decay pathway in differentiated patient-derived iPSCs (91).

A more systematic analysis of pancreatic transcription factors *PDX1*, *RFX6*, *PTF1A*, *GLIS3*, *MNX1*, *NGN3*, *HES1*, and *ARX*, partly identified in monogenic variants of MODY and PNDM, characterized the transcriptional control and corresponding defects at several developmental stages (86). This study highlights especially the role of RFX6 in controlling pancreatic progenitor numbers and differences of NEUROG3 requirement in humans and mice. Mutations in *WFS1*, causing Wolfram syndrome, lead to chronic endoplasmic reticulum stress activating the unfolded protein response, which impairs survival of β-cells and neurons (168–170). This could be recapitulated using iPSC with *WFS1* variants (142). A recent publication from Maxwell et al. also characterized iPSC from a Wolfram syndrome patient (143). Patient-specific iPSCs harboring a pathogenic variant of *WFS1* were corrected using CRISPR/Cas9 technology. This study used a differentiation protocol with cytoskeletal modification, which significantly improved differentiation efficiency compared to previously tested suspension culture in these cell lines (159). Corrected cells showed higher WFS1 expression and robust insulin secretion, probably benefiting from reduced ER stress and improved mitochondrial respiratory capacity in endocrine cells (143). In turn, further maturation of *in vitro* generated β-cells allows better identification of effects also in later stages, additionally providing potential use for β-cell replacement therapy.

Homozygous mutations in the insulin gene (*INS*) are known to lead to PNDM. Pancreatic differentiation of patient iPSC results in CHGA-positive endocrine cells expressing β-cell markers NKX6.1, PDX1, and MAFA but lacking insulin expression (93). Gene correction rescued the phenotype and prevented diabetes in a streptozotocin mouse model, providing a future tool for patient cell therapy. Another study involved iPSCs generated from patients with neonatal diabetes and heterozygous insulin mutations disturbing proper proinsulin folding (137). Patient-derived iPSCs show normal pancreatic differentiation comparable to corrected isogenic iPSC but have reduced insulin expression. Moreover, *INS* mutation increases ER stress and hampers proliferation of β-cells but without increased apoptosis promoting diabetes development in patients. In addition, fibroblasts from a PNDM patient harboring an intronic *INS* mutation have been efficiently generated and may serve as a diabetes model to characterize the expected aberrant splicing (138).

Another study characterized iPSCs from MODY2 patients with a heterozygous *GCK* mutation (83). Similar to control cells, these iPSCs differentiated into insulin-producing β-cells but showed reduced insulin secretion in response to glucose stimulation. In addition, iPSCs with two inactive *GCK* alleles also showed reduced differentiation efficiency recapitulating the functional impairment observed in patients and mouse models.

After the identification of *YIPF5* mutations causing a novel PNDM syndrome, Franco et al. characterized *in vitro* differentiated patient-derived iPSCs harboring a homozygous *YIPF5* mutation as well as genome-edited ESC in addition to a β-cell line (132). Functional impairment of YIPF5, responsible for trafficking between the endoplasmic reticulum and the Golgi apparatus, caused proinsulin retention at the ER resulting in ER stress-induced apoptosis and β-cell failure and, thus, diabetes.

In order to understand the role of activating mutations in *STAT3* during pancreatic development, iPSCs derived from patient fibroblasts were subjected to pancreatic differentiation and revealed a premature endocrine differentiation later preferentially forming α-cells which is in line with the observed phenotype of the patient (134). This defect results from enhanced nuclear localization of the mutant protein and NEUROG3 activation and could be rescued by correction of the *STAT3* mutation.

Inactivating mutations in *ABCC8* resulting in excess insulin secretion have been successfully employed for modeling congenital hyperinsulinism using ABCC8-deficient ESC (94, 95). In contrast, activating mutations in *ABCC8* have been described in diabetes (171). Thus, using the hPSC-systems, a better characterization of the components of the β-cell ATP-sensitive potassium channel may be obtained to understand the function of β-cells and associated pathomechanisms such as diabetes (caused by activating mutations) or the *vice-versa* effect of congenital hyperinsulinism (caused by inactivating mutations).

In the context of T2D susceptibility genes identified in GWAS, Zeng et al. generated ESC with null alleles for *KCNJ11*, also associated with MODY13 (96). Although the loss of KCNJ11 does not affect *in vitro* differentiation towards β-like cells and insulin production, these cells show impaired glucose-stimulated insulin secretion (96). In addition to hESCs with *KCNJ11* mutations, iPSCs have been generated from peripheral blood mononuclear cells with a heterozygous activating mutation in *KCNJ11* and are available for mechanistic studies as well as drug testing in differentiated pancreatic cells (97).

Skin fibroblasts from patients with Michell–Riley syndrome were used to generate iPSCs harboring a homozygous nonsense mutation in *RFX6* (140). Pancreatic differentiation revealed an impaired formation of pancreatic endoderm and thus, supports the impaired formation of endocrine cells in the pancreas in line with the patient phenotype.

In order to better understand the impact of different pathogenic *NEUROG3* variants, Zhang et al. expressed NEUROG3 mutant proteins at physiological levels in *NEUROG3* knockout ESC during pancreatic and intestinal differentiation determining the ability to rescue the generation of endocrine cells (139). Depending on the variant, expression resulted either in the decreased or abolished formation of pancreatic endocrine cells recapitulating the respective patient phenotype. Moreover, these effects could be retraced to be caused by impairment of NEUROG3 protein stability, DNA-binding affinity, and protein dimerization. Those features can differ in various tissues, a fact that emphasizes the importance of considering the relationship between protein structure and function.

Adenoviral *PAX4* overexpression during pancreatic differentiation of ESCs results in decreased glucagon-positive cells promoting the formation of monohormonal insulin-positive cells supporting its role in cell fate specification (92). This suggests a crucial role of intact *PAX4* in the development of healthy monohormonal insulin-positive cells.

A recent study reported the generation of iPSCs with a homozygous mutation in the *SLC2A2* gene (141). Peripheral mononuclear blood cells from a patient suffering from Fanconi–Bickel syndrome accompanied by early onset diabetes were reprogrammed using a non-integrating Sendai virus vector. These cells can be used to study the pathogenesis associated with defects in the GLUT2 glucose transporter in pancreatic β-cells.

In addition to KO mouse models, genome-engineered and patient-specific hPSCs have helped to get more insight into developmental and mechanistic processes as well as transcriptional networks (81). Not only do they provide additional information but sometimes even highlight the species-specific differences that make them even more crucial for a better understanding of monogenic diabetes.

A conditional *Hnf4a* KO in mice did not result in a diabetic phenotype but revealed that expression of the potassium channel subunit Kir6.2 regulating insulin secretion is promoted by *Hnf4a* (148). Patient-specific iPSCs inform about changes in transcriptional network (81). Similarly, *Hnf1a* mouse models with heterozygous KO present without developing diabetes, whereas a homozygous KO impairs β-cell function by reducing the insulin secretion. Stem cell-based models provided further details characterizing the developmental, transcriptional, and metabolic role of HNF1a (84). Furthermore, loss of *Wfs1* in mice showed impairment of β-cell development and function with a mild diabetic phenotype (149). Patient-specific iPSCs provided deeper insight into endoplasmatic reticulum stress and were used to test a possible therapeutic approach (142). Since GATA6 haploinsufficiency resulting in pancreatic agenesis in patients cannot be recapitulated in mice (172, 173), effects of GATA6 gene dosage on pancreatic differentiation *in vitro* helped in understanding the clinical presentation of different patients (135). Furthermore, hPSC models facilitate the characterization of disease-specific variants. For example, a *Hnf1b* heterozygous KO in mice is not associated with a diabetic phenotype (150), but patient-specific iPSC carrying a heterozygous variant of *HNF1B* helped to explain the MODY5 phenotype (90). In mice, *Neurog3* is essential for the development of the endocrine pancreas (144), but the disease phenotype slightly differs in humans (121). Expression of different disease-associated *NEUROG3* variants during *in vitro* differentiation helped in explaining various phenotypes in patients (139).

Taken together, a comparison of human and mouse model systems can provide further insight into the role of specific genes but also highlights the species-specific differences concerning, for example, transcription factor activity. That explains why human PSC-based models are crucially needed to compensate for those specific differences.

Therefore, genome editing in PSC or even patient-specific hiPSC provide a versatile approach to study developmental and functional effects of selected diabetes genes and variants and complement or even contradict data obtained from mouse models.

So far, many stem-cell-based models exist that characterize monogenic mutations resulting in early-onset diabetes. These models nicely elucidate the diabetic patient phenotype and help in understanding the common pathways in β-cell development and function. Altogether, improved screening for pathogenic variants in combination with thorough functional analysis will be the first step to precision medicine in diabetes therapy.

CONCLUSION/OUTLOOK

In vitro pancreatic differentiation of pluripotent stem cells is a powerful tool to better understand pancreatic development and the specific role of the involved transcription factors. Identification and characterization of specific variants in monogenic diabetes help in characterizing the complex transcriptional network and in overcoming phenotypic differences between patients and corresponding mouse models. In addition, these model systems provide the basis for drug development and testing that could benefit both patients of monogenic and multifactorial diabetes. Ideally, iPSCs of genetically disordered persons could be repaired and serve as a major source for tissue engineering and regeneration, *e.g.* β-cells in the case of monogenic diabetes.

Yet, some major roadblocks need to be kept in mind before translating genetically repaired iPSCs into clinics. First,

epigenetic modifications of iPSCs which are derived from their originating tissue, might reduce their differentiation capacity and subsequent function as well as immune tolerance after autologous transplantation (174). Furthermore, genetic aberrations after reprogramming might bear tumorigenic potential and thus provoke carcinogenesis in the transplanted iPSC-derived tissues (174). Additionally, iPSC-derived tissues need to be manufactured according to SOPs and GMP guidelines which need lots of effort to implement those prerequisites into standard clinical care (175).

Altogether, these studies further urge the involvement of pluripotent stem cells in deciphering the underlying pathomechanisms as well as the affected genes, particularly when monogenic diabetes displays discrete clinical phenotypes and needs specific treatment depending on the subtype.

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All authors wrote the paper. MM constructed figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Genome Editing Human Pluripotent Stem Cells to Model β-Cell Disease and Unmask Novel Genetic Modifiers

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A mechanistic understanding of the genetic basis of complex diseases such as diabetes mellitus remain elusive due in large part to the activity of genetic disease modifiers that impact the penetrance and/or presentation of disease phenotypes. In the face of such complexity, rare forms of diabetes that result from single-gene mutations (monogenic diabetes) can be used to model the contribution of individual genetic factors to pancreatic β-cell dysfunction and the breakdown of glucose homeostasis. Here we review the contribution of protein coding and non-protein coding genetic disease modifiers to the pathogenesis of diabetes subtypes, as well as how recent technological advances in the generation, differentiation, and genome editing of human pluripotent stem cells (hPSC) enable the development of cell-based disease models. Finally, we describe a disease modifier discovery platform that utilizes these technologies to identify novel genetic modifiers using induced pluripotent stem cells (iPSC) derived from patients with monogenic diabetes caused by heterozygous mutations.

Keywords: IPS (induced pluripotent stem) cell, pluripotent stem cell (PSC), beta cell (β cell), diabetes, disease modifier, MODY (mature onset diabetes of the young), candidate gene approach, GWAS (genome-wide association study)

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INTRODUCTION

Diabetes mellitus is a worldwide healthcare problem that is rapidly increasing in prevalence. In the United States alone, over 10% of the population is affected, with approximately 1.5 million Americans newly diagnosed with diabetes each year (1). Particularly troubling is the dramatic increase in the incidence of diabetes in children, the consequences of which are expected to lead to increased complications and comorbidity as adults (2, 3). With obesity rates projected to increase in the United States over the upcoming decades, there is little chance that the trend of increasing diabetes prevalence will reverse itself (4). In addition to decreasing quality of life, diabetes is associated with significant morbidity and mortality, including retinopathy, neuropathy, cardiovascular and kidney disease (5). Diabetes also puts individuals at risk of having more complicated courses of common illnesses, with recent studies documenting increased morbidity and mortality in patients with diabetes who contracted COVID-19 (6–8).

Though often referred to as a single condition, diabetes is likely many overlapping diseases, with most stemming from pancreatic β-cell dysfunction and the disruption of glucose homeostasis due to abnormal

insulin secretion and/or responsiveness (9). The two most common forms of diabetes, type I (T1D) and type II (T2D), are associated with the eventual loss of insulin-secreting pancreatic β-cells, which can occur either early (T1D) or late (T2D) in disease progression. In the case of T1D, autoimmune destruction results in β-cell death and subsequent insulinopenia, although there is increasing support for the role of β-cell stress in T1D onset (10, 11); T2D is characterized by a combination of peripheral insulin resistance and inadequate β-cell compensation, resulting in a metabolic syndrome that leads to eventual β-cell exhaustion and loss of β-cell mass (12, 13). T1D and T2D display a multifactorial etiology on both a population and individual level, likely motivated by a complex combination of genetic, epigenetic, and environmental factors (14). Furthermore, there is substantial heterogeneity within the underlying β-cell pathophysiology of each of these disorders (9).

Apart from the two major types, there are also 14 known types of monogenic diabetes, historically called MODYs (mature onset diabetes of the young), which are caused by single gene mutations that result in β-cell dysfunction (15–18). Monogenic diabetes is typically underrecognized and underdiagnosed, with identified subtypes likely making up 2-5% of all diabetes cases while additional causative genes undoubtably remain to be discovered (17). To complicate matters further, a number of MODY genes have been associated with the development of T1D and T2D (19–21), suggesting that the pathophysiology of the diabetes subtypes can often overlap. For example, there are additional genes that can cause neonatal diabetes through downstream impacts on pancreatic development, such as GATA6 and NKX2.2, which are not traditionally included as MODYs, although overlap in disease pathology occurs depending on the timing of their presentation (22). There are also numerous monogenic syndromes that have diabetes as a component in all or some affected individuals, including cystic fibrosis and Friedreich's ataxia. While the underlying pathogenesis of monogenic and neonatal diabetes predominantly involves intrinsic defects of the β-cell, syndrome-associated forms of diabetes may result from both peripheral and β-cell defects, with the latter being understudied in many cases (15, 16). Better studies of all forms of diabetes are necessary to understanding the underlying pathophysiology of this complex disease.

The Need for Human β-Cell Models in Diabetes Research

For decades, studies using mouse models greatly advanced our knowledge of diabetes (23). Mice are inexpensive relative to larger animal models (i.e., non-human primates), recapitulate human disease more closely than more basal organismal models, and were genetically-manipulatable even before the invention of modern genome editing techniques. As a result, mouse models continue to be incredibly valuable for the study of whole-body physiology, capable of providing complex metabolic readouts of multiorgan systems, as is the case in oral glucose tolerance tests and hyperinsulinemic euglycemic clamp studies. However, while mouse models play an essential role in the study of diabetes, important differences in rodent versus human physiology have sometimes limited the translatability of rodent datasets to the complex presentation of the human diabetes subtypes. Therefore, the development of *in vitro* human β-cell models will provide an important adjunct to *in vivo* rodent models in the study of diabetes.

While a variety of T1D and T2D mouse models exist, none have been able to comprehensively mimic human diabetes (23–25). For T1D, both genetic and chemically-induced models are used, with the latter resulting in β-cell destruction and insulinopenia, but neither method allowing for the study of the autoimmune processes that drive disease pathophysiology (24, 25). Even mouse models with an autoimmune component do not exactly resemble T1D due to interspecies differences, including well described mechanisms for the regulation of immune cell activation, homing, and target cell interactions (26). As a result, diabetes manifests differently in the two species: for example, the commonly-used non-obese diabetic (NOD) mouse strain exhibits pronounced insulitis and rapid β-cell destruction, while β-cell loss in human T1D is associated with gradual and milder infiltration of islets (27). In the case of T2D, there are a myriad of diet-induced obesity and genetic models that can be used (23, 25). However, the complex polygenic nature of human obesity can be difficult to model using inbred mice strains, and observed effects of sex, age, and epigenetic factors on diet-induced obesity may not be the same between species, though these differences may provide some insight into genetic loci that result in phenotypic variation (23).

While mouse models have yielded significant insights into monogenic causes of diabetes, they do not always fully recapitulate the human disease. One example involves GATA6 and GATA4, members of the GATA family of transcription factors, which are the most highly expressed isoforms in the pancreas. Heterozygous, largely *de novo*, mutations in GATA6 are the most common cause of pancreatic agenesis, resulting in neonatal diabetes as described by multiple groups (28–37). Though less common, GATA4 haploinsufficiency can also result in neonatal and early childhood-onset diabetes (38, 39). However, in mice, GATA4 and GATA6 appear to be completely redundant isoforms, as the loss of a single allele of either *GATA4* or *GATA6* does not appear to impact pancreatic development or glucose homeostasis, and the loss of three of the four Gata4/6 alleles is needed to recapitulate the human phenotype (40, 41). Another example is the most common form of monogenic diabetes, MODY3, caused by heterozygous mutations in the transcription factor HNF1α. Mice with heterozygous mutations in *HNF1A* are healthy (42) and mice with *HNF1A* null mutations can have a diabetic phenotype, but with significant variability dependent on genetic background (43). These results suggest that there are complex, human-specific genome-phenotype interactions that must be additionally investigated using human models. Therefore, the combined and complementary use of both *in vitro* human β-cell models and *in vivo* rodent models will greatly advance our knowledge of the pathophysiology of diabetes.

The Emergence of the Stem Cell-Derived β-Cell as a Model

While mouse models have and certainly will continue to advance our knowledge of diabetes, β-cell-centric research in diabetes has unfortunately been hindered by the lack of human models. Most immortalized β-cell lines are rodent-derived, though several human lines, including the EndoC-βH1 (and subsequent βH2 and βH3) line, are becoming more widely used (44, 45). However, β-cell lines can exhibit differences from *in vivo* human β-cells due to their immortalized status, can be difficult to genetically manipulate, and cannot be used to study β-cell development. While the use of cadaveric human islets in research has greatly expanded due to the success of programs such as the Network for Pancreatic Organ Donors with Diabetes (https://www.jdrfnpod.org) and the Integrated Islet Distribution Program (https://iidp.coh.org), these resources unfortunately remain scarce and precocious. Furthermore, the genetic, epigenetic, and environmental characteristics of donors are largely unknown, while islets themselves cannot be genetically manipulated efficiently.

To address these limitations, great strides have been made over the last two decades in the development of human pluripotent stem cells (hPSC), a term which includes both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) [reviewed in (46, 47)]. Efficient methods for the production of hPSCs have completely changed the face of biomedical research and have opened new avenues of therapeutic development for a multitude of diseases. The subsequent development of techniques to differentiate hPSCs into pancreatic β-cells have enormous potential to contribute to the study of diabetes. Built upon foundational research within mouse developmental biology [reviewed in (48)], modern techniques of stem cell differentiation leverage known inductive signals that drive development *in vivo* by replicating these signals temporally and spatially to drive development *in vitro* [reviewed in (49)]. The first lab-guided hPSC differentiation protocols were developed to generate definitive endoderm (50), followed quickly by protocols capable of driving hPSCs towards pancreatic progenitors and endocrine cells (51). While these protocols initially required that pancreatic progenitors be transplanted into mice to mature into functional β-cells, current protocols can achieve functionality *in vitro* without transplantation (52–54) This field has become robust with technical advancements being published regularly by laboratories around the world, resulting in the generation of β-like cells that are closer and closer to their natural counterparts, though continued optimization of functionality is required (55–62).

With advances in directed *in vitro* differentiation, stem cell-derived β-cells provide a tremendous opportunity to study pancreatic development and endocrine diseases in a human model system, particularly when combined with recent advances in genome editing technology. Using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology (63, 64) (see *Section 2*), targeted mutations in hESC lines can be made, generating mutant and control isogenic lines that avoid confounding results due to differing genetic backgrounds. In addition, iPSCs can be generated from reprogramed patient donor blood or skin fibroblasts (46, 47), resulting in a unique platform within which to study the specific contribution of single mutations to β-cell development and/or function. While these systems certainly have caveats, including expense, labor-intensiveness, and lab-to-lab variability, the expanding use of stem cell-derived β-cells stands to drive our knowledge of diabetes pathophysiology forward beyond the prior limitations of mouse and cell line models. In this review, we will review the current methods of genome editing in hPSCs, discuss how this can be applied to the evaluation of candidate genes in the study of diabetes, and examine the use of stem cell-derived β-cells as a platform for the identification of novel genetic modifiers of diabetes.

GENOME EDITING IN hPSCs

The development of genome editing technologies capable of selectively targeting sites within the human genome has revolutionized our ability to investigate the genetic underpinnings of disease. In the case of diabetes, ESCs and patient-derived iPSCs from multiple genetic backgrounds can now be genetically edited and paired with lab-guided differentiation protocols to build powerful and scalable cell-based models of multiple diabetes subtypes (65, 66). Genome modifications in each system are achieved through nuclease localization with a target sequence, the induction of a double stranded DNA break (DSB), and the activation of endogenous cellular DNA repair mechanisms such as homologous recombination (HR) and non-homologous end-joining (NHEJ) (67, 68). Several types of gene modification can be accomplished through these mechanisms, including (1) 'gene disruption' through the addition/subtraction of short nucleotide sequences and frame shift mutation induction (2), 'gene correction' through targeted base substitutions that restore gene function using a homologous donor DNA construct as a template, and (3) 'gene addition' through the introduction of a complete transgene into a specific locus. Here we briefly review several of the most popular methods for the selective editing of hPSCs, each of which exhibit advantages and disadvantages when editing specific cell types (69).

Zinc Finger Nuclease (ZFN) and Transcription Activator-Like Effector Nuclease (TALEN)

Zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) are structurally similar in that they both rely on the C-terminal *Fok*I endonuclease domain to generate DSBs within a targeted sequence (70–72). ZFN architecture combines multiple zinc finger protein DNA-binding domains (motifs) (73) with the nuclease domain of the *Fok*I restriction enzyme that performs optimally when targeting long (12-18 bp) and unique sequences within the eukaryotic genome (74). In contrast, TALENs employ multiple

transcription activator-like effector (TALE) DNA binding domains – a class of proteins isolated from the *Xanthomonas* bacteria that have evolved to alter the transcription of host plants (75). In either case, the two distinct regions of the nuclease each perform a unique function, with zinc finger motifs or TALEs binding to DNA while the *Fok*I nuclease domain induces a DSB within a target sequence upon dimerization (76, 77).

While structurally similar, there are distinct advantages and disadvantages of each system. Typically, ZFN-based platforms afford greater flexibility in targeting, while also allowing for independent optimization of the two subunits for simplified retargeting (78). Drawbacks of ZFNs include the cost of application, a suite of complex design constraints that must account for context-dependent interactions between fingers (79), and a higher prevalence off-target effects and translocations than other methods (80). In contrast, the highly conserved stretches of 33-35 amino acids (AA) that TALEN-based approaches employ addresses many of the design complexity concerns of ZFNs, while maintaining high cleavage activity rates, a broad targeting range specificity, and improved cytotoxicity (81). However, TALEN-based approaches have been shown to produce off-target effects and suffer from dramatically lower efficiencies when targeting sequences that are methylated or do not include thymidine (82, 83). Comprehensive reviews that provide specific recommendations for the design and reproducible integration of ZFN (78, 84) and TALEN-based (85) genome editing approaching in hESCs and iPSCs are available elsewhere.

Clustered Regularly Interspaced-Short Palindromic Repeats (CRISPR)/Cas

(CRISPR)/Cas-based gene editing platforms have become incredibly popular tools to modify the genome of hPSCs since the introduction of the technology in 2012 (86). Based on the adaptive immune system of bacteria and archaea, CRISPR was made possible by the discovery of DNA fragments within the *E. coli* genome from past viral and bacteriophage invaders known as clustered regularly interspaced short palindromic repeats (CRISPRs). Unlike ZFNs and TALENs which use proteins, CRISPR loci are transcribed during viral infections to produce an RNA-guided DNA endonuclease that selectively binds and cuts invading viral DNA (87). CRISPR sequences exhibit a repetitive pattern, wherein short DNA sequences (24-48 bp) are followed by their reverse complement and a protospacer that matches part of the viral genome. Through coordination with RNase III, CRISPR-associated (Cas) proteins, and trans-activating CRISPR RNA (tracrRNA), long RNA transcribed from CRISPR loci are cleaved into short, spacer-derived RNA (crRNA) (88). TracrRNA and crRNA then act together to guide the Cas9 protein to a target cut site located within the genome of an invading virus, causing a DSB [for a review, see (89)].

When used in genome-editing platforms, tracrRNA and crRNA can now be combined into a single "guide RNA" (gRNA) molecule (86) and administered with the Cas9 protein to selectively cut target DNA sequences (90). Multiple CRISPR/Cas9 systems have been developed specifically for hPSCs that are capable of editing or replacing genome sequences (91, 92) and are quickly replacing TALEN-based systems due to their ease of generation, efficiency, and cytocompatibility (93, 94). Drawbacks of CRISPR-based methods include the re-cutting of target regions after DSB repair and the prevalence of erroneous insertion or deletions (indels) on the non-targeted allele, making the generation of single allele edits difficult. Recent protocols, including one from our laboratory (95), address these issues through the use of two single stranded oligonucleotide repair templates, with one expressing the desired sequence change and the other maintaining the normal sequence. These repair templates also contain silent mutations that prevent gRNA recognition and re-cutting, facilitating the selective editing of a single allele with an average efficiency of close to 10%. In addition, off target cutting of CRISPR/Cas9 at other sites in the genome is also an issue but it can be mitigated. First, in the hPSC system, off target cutting is less prevalent than in somatic cells, mostly likely due to the fact the pluripotent stem cells are very sensitive to DNA damage and cells that have undergone multiple DNA cuts are less likely to survive (96). Second, careful design and testing of potential off target sites can also be used to minimize the impact of off target cutting. Overall, genome editing technologies in hPSCs are advancing to the stage where virtually all coding mutations can be repaired or introduced in a single allele manner, an important advance given the majority of monogenic genetic diseases of the pancreas are caused by heterozygous mutations.

THE ROLE OF CANDIDATE GENE AND GWAS APPROACHES IN THE STUDY OF DIABETES

The availability of standardized laboratory protocols for the generation of glucose responsive β-cells from hPSCs that have undergone selective genome editing has the potential to dramatically increase our knowledge of genes that contribute to diabetes. Traditionally, the functional contribution of genes to disease states has come from the deletion or mutation of a single gene target. The use of candidate genes, chosen as they are known clinically to cause disease, has been employed for the study of monogenic diabetes. However, given the increasing ease of genome and exome sequencing, comparisons of genetic variants between diabetic and non-diabetic populations through GWAS analysis using genome sequencing has generated a large list of variants associated with all forms of diabetes, most of which are in non-coding regions of the genome (97–100). Newly developed stem cell platforms can be used to target these variants, initially by targeted mutation of the gene thought to be regulated by a given variant (101). The direct targeting of a non-coding variant has been studied in neonatal diabetes caused by GATA6 (102), and similar approaches could also be used for variants associated with more common causes of diabetes. Care will need to be taken as it is possible that most non-coding variants may have a small impact on gene expression and disease penetrance on their own. While we are still in the

early stages of utilizing these approaches in stem cell-derived β-cells to interrogate the roles of specific genes in β-cell development and function, we predict that this will become more commonplace and contribute to our understanding of β-cell physiology and disease.

Studying Known Causes of Monogenic Diabetes

There are dozens of types of diabetes caused by single gene mutations, including monogenic, neonatal and syndrome-associated diabetes (15–17, 103–105). Monogenic forms of diabetes are often caused by heterozygous coding mutations within genes that influence β-cell function [reviewed in (106)]. Many forms of monogenic diabetes have been recognized for decades and more gene causes are likely to be found in the upcoming years with the increasing use of exome and genome sequencing (17, 18). In addition, many of the genes associated with monogenic diabetes have numerous reported mutations with potentially different consequences on protein function and therefore on β-cell dysfunction (107–109). Stem cell-derived β-cells may provide a platform in which to investigate some of these polymorphisms, and may help to provide some insight into genotype-phenotype correlations.

The modeling of monogenic diabetes using stem cell-derived β-cells has been extensively described. To date, hPSC lines have been made with mutations in HNF1A (110–114), HNF1B (112, 115, 116), HNF4A (112, 117–119), PDX1 (107, 120, 121), KCNJ11 (122), GCK (112), and CEL (112). Of the studies listed, only two used genome-editing to make mutant hESC lines (113, 121), with the remainder generating patient-derived iPSC lines. While some studies simply described the derivation of iPSC lines from patient samples, most publications included lab-guided differentiations to β-cells and subsequent studies on β-cell gene expression and biology (111, 113–115, 117–119, 121).

MODY3, caused by heterozygous mutations in the transcription factor HNF1α, is the most common form of monogenic diabetes (15, 103) and is currently the most extensively-researched monogenic form of diabetes using stem cell-derived β-cells. HNF1α has been of particular interest because of its additional association with T1D and T2D in several large population studies (19–21, 123). Several studies on the role of HNF1A has been performed in mice, but mouse models with heterozygous deletion of *HNF1A* do not have diabetes and thus have provided limited insight into MODY3 (124). To date, three publications from different groups have modeled HNF1A-deficiency in stem cell derived β-cells, with two employing patient-derived iPSC lines (111, 114) and the other using hESC lines (113). These studies have yielded significant insights into the complex role HNF1α plays in controlling β-cell development, metabolism and function and have discovered new downstream targets of this transcription factor that had not previously been identified in mouse studies.

While the underlying pathogenesis of monogenic diabetes results from intrinsic β-cell defects, the role of β-cell dysfunction in many syndrome-associated forms of diabetes, such as cystic fibrosis (CF)-related diabetes, is largely unknown (105). There is significant interest in these fields to generate syndrome-related iPSC lines for use in multiple areas, but this will ultimately aid in the study of rare causes of diabetes by providing accessible resource lines. Groups have already generated stem cell derived β-cells to model β-cell dysfunction in Wolfram syndrome (125, 126) and Friedreich's ataxia (127). In addition, CF iPSC lines have been made and differentiated in the pancreatic ductal endothelium to study the effects of CF-related pancreatic exocrine function (128). These lines and others generated for non-diabetes study can always be used to produce stem cell derived β-cells and advance our knowledge of these understudied forms of diabetes.

Another avenue of study using stem cell derived β-cells as a model is to focus on genes that are thought to play a role in β-cell development, identity, or function but that may not have been described as monogenic causes of disease. In an impressive paper by Zhu and colleagues, researchers used genome editing to generate hPSCs knockout lines to further probe the role of 8 known pancreatic transcription factors, including PDX1, RFX6, PTF1A, GLIS3, MNX1, NGN3, HES1 and ARX (121). Many of these factors had only been studied previously in rodent models and, through lab-directed differentiation, their role in human β-cell development and function could be interrogated for the first time. This reverse candidate approach using stem cell derived β-cells will provide a significant basis for future advances.

As genome editing techniques improve and become more well-established, the field is turning more to the use of isogenic lines in which to study the contribution of a specific genes on β-cell physiology. This involves making targeted mutations in hESC lines or correcting mutations in patient-derived iPSC lines, generating mutant and control isogenic lines that avoid confounding results due to differing genetic backgrounds. In all studies above using patient-derived iPSC lines to study monogenic diabetes, the mutant stem cell-derived β-cells were compared to unaffected family members or unrelated wild type iPSC lines and not isogenic controls (111, 114, 115, 117–119). With the increasing use of CRISPR/Cas9 technology, we advocate the use of at least 2 pairs of isogenic lines, examining a single clone for each, for interrogating the impact of a given genome alteration. Alternatively, if using a single stem cell line, the examination of several genome edited clones has been suggested by leading stem cell journals such as *Stem Cell Reports* (Information for authors). We would argue that 2 isogenic pairs is superior because it controls for both artifact due to an acquired mutation in a single genome edited clone as well as confirm any phenotype is general enough to be seen in 2 independent genetic backgrounds. The use of a single edited clone per isogenic pair we feel is a good tradeoff between the effort required to differentiate and analyze these clones while still minimizing the impacts of clonal artifacts.

Leveraging GWAS to Identify and Validate Candidate Genes in Diabetes

The cause of T1D and T2D is likely a complex combination of genetic, epigenetic and environmental factors (14). In addition, there is substantial heterogeneity within each of these disorders,

so that the disease-causing combination in each affected individual is slightly different (9). Therefore, a single gene-to-disease strategy is not always effective in the study of T1D and T2D. Technological advancements in next generation sequencing, combined with the targeted efforts of several consortia, continue to expand the size and scope of available genomic datasets from diabetic patients (129–131). Previously, the identification of diabetes-linked genes was the product of quantitative trait mapping (QTL), obtained through the cross of genetically engineered mice (132–134). Over the past decade as the cost and availability of sequencing technology has improved, GWAS have identified more than 60 loci for T1D (135) and more than 240 associated with T2D (136), with the hereditability of each now explaining approximately 15% (137) and 25-80% (138) of the disease-risk for each subtype, respectively.

The explosion of available GWAS datasets for both T1D and T2D can be leveraged by using stem cell derived β-cells. Using this technique, novel genes that are revealed by GWAS, individual exome, or genome sequencing associated with diabetic populations can be targeted for study either in isolation or as part of co-cultures where interactions between adipocytes, immune cells, critical biological components and β-cells are replicated *in vitro* (139–141). Through the use of stem cell derived β-cells, these systems can ascertain whether a specific locus causes β-cell-intrinsic dysfunction, while also probing the contributions of the surrounding environment. For example, polymorphisms in the human leukocyte antigen (HLA) DR and DQ alleles increase T1D-risk by altering T-cell binding [reviewed in (142)], which is predictably a β-cell-extrinsic effect that can be observed in cell culture studies. Alternatively, some polymorphisms in the insulin (INS) gene, a β-cell-specific gene, have been described to influence T1D risk due to changing insulin mRNA production in the thymus altering immune tolerance (143, 144). However, other mutations in INS lead to neonatal diabetes, thought to be caused by β-cell death due to increased cell stress from misfolded insulin protein [reviewed in (145)]. While the difference between polymorphisms and mutations may be determined by their prevalence in the population, modeling these gene differences in stem cell derived β-cells may prove useful for understanding their significance.

While GWAS studies can yield a potential target gene which could be directly involved in disease, sometimes they identify an associated region of unclear significance. GWAS comparing the islets of diabetic and non-diabetic individuals suggest that most T2D-associated variants do not reside in coding regions (146, 147). In order to understand the role of these variants, iPSC banks from T1D, T2D and non-diabetic patients can be used to probe these differences on a multigene scale. Multiple iPSCs from T1D and T2D patients have been made (148–151), and there are consortia and foundations that are focused on making larger banks of available diabetes and non-diabetes iPSC lines, including the Human Islet Research Network (HIRN, https://hirnetwork.org/hpap-overview) and the New York Stem Cell Foundation (NYSCF, https://nyscf.org/research-institute/repository-stem-cell-search/). Several groups have also recently performed lab-directed differentiations on patient-derived iPSC lines to generate stem cell derived β-cells to examine broad molecular and functional differences (150, 151). Unlike the need for the generation of isogenic lines in the study of monogenic diabetes, making banks of T1D, T2D, and non-diabetic stem cell derived β-cells can be used to study many factors contributing to β-cell pathophysiology in diabetes. One caveat of the use of non-isogeneic lines is that differences in genetic background amongst disease and control lines leads to tremendous variability in phenotype and necessitates large sample numbers to dissect the underlying biology.

GENETIC DISEASE MODIFIERS AND DIABETES

The way genetic factors interact with disease can be highly variable. Even in canonical examples of monogenic Mendelian diseases such as cystic fibrosis and sickle-cell anemia where a disease endophenotype is linked to a single mutation (152), fraternal twins that reside within the same household can present vastly discordant phenotypes (153). The results of longitudinal twin studies add to a growing body of clinical evidence that underscores the importance of 'disease modifiers' that alter the penetrance, expressivity, rate of progression, and/or presence of disease endophenotypes through the modification of disease-linked genes (154, 155). While the terminology used to describe the mechanisms of oligogenic inheritance continues to evolve, for the purposes of this review we have chosen to classify genetic disease modifiers within two groups based on their mode of action, or as either 'protein coding' or 'non-protein coding'.

Protein coding disease modifiers typically affect the phenotypic expression of a disease through mutations in the coding sequence of intact genes, leading to changes in protein function (156). These changes can be either sufficient to elicit a diseased state (i.e., a frame shift mutation within a coding sequence of an important functional protein), or can affect the molecular expression of other disease-linked genes through alterations in regulatory DNA such as promoters and enhancers (e.g. modifier genes) (157). In contrast, non-protein coding disease modifiers include non-coding regulatory elements and non-coding RNAs (ncRNA) (158). In either case, disease modifiers can act to either enhance, silence, or modify the expression of genes that can modify the activity of important proteins whose dysregulation result in changes in the penetrance, expressivity, and/or presence of a disease endophenotype.

Diabetes is a complex disease wherein patients express significant heterogeneity in the progression, clinical phenotype, and response to treatment. T1D and T2D show clear evidence of a genetic component and familial reoccurrence (159, 160), with observed associations with lifestyle, obesity, and cancer playing a particularly significant role in T2D (161). In the face of this variability, it is important to note that the direct influence of specific mutations within protein-coding regions on the etiology of diabetes have been described (162, 163). However, while allelic variants have been shown to confer an increased risk of

T1D/T2D, other subtypes of diabetes, such as monogenic diabetes, are causally linked to single mutations, as described above. Apart from changes in coding sequences, there is substantial evidence that disease progression and severity of all forms of diabetes results from the interaction of multiple non-coding genetic, epigenetic, and environmental factors, which act in concert to cause β-cell dysregulation and islet dysfunction (164, 165).

The influence of protein coding and non-protein coding disease modifiers on each of the diabetes subtypes remain active areas of research. In the case of monogenic diabetes, modifier genes have been shown to explain some degree of clinical variability (166), while several studies suggest that non-coding disease modifiers influence the development of gestational diabetes (167, 168). In this section, we provide a brief overview of the two classes of disease modifiers, discussing known associations with the diabetes subtypes when available. To facilitate the further discovery of such mechanisms, we then outline a disease modifier discovery platform that leverages recent advancements in RNA sequencing, genome editing, and laboratory-guided stem cell differentiation to identify genetic disease modifiers of genetic disease caused by heterozygous mutations, using monogenic diabetes as a model. Given the limited availability of research on this topic, it is our hope that the platform outlined here will support the discovery of novel protein coding and non-protein coding disease modifiers that can help explain the heterogeneity observed in diabetes subtypes.

Protein Coding Disease Modifiers of Diabetes

The influence of modifier genes and allelic heterogeneity on human disorders has been the subject of an ongoing discussion within medicine for over a century (169), with multiple parallel avenues of investigation within genetics (e.g. epistasis, oligogenic inheritance) dedicated to understanding the effect of one gene/allele on the phenotypic expression of a second gene/allele (154, 155). In the case of diabetes, there is a growing body of evidence that some subtypes may be the result of oligogenic inheritance, wherein the underlying etiology of the disorder is primarily genetic, but still requires the synergistic action of several genetic modifiers at disparate disease-linked loci (156, 170, 171). In this continuum between classical Mendelian and complex traits, possible protein coding disease modifiers include allelic heterogeneity that results from mutations within disease-linked loci, the activity of modifier genes that regulate others with important roles in glucose homeostasis, and the presence/absence of single nucleotide polymorphisms (SNPs) that are either necessary or sufficient to change the presence, penetrance, expressivity/heritability, or rate of progression of a disease.

As GWAS datasets expand to include sampling of diabetic patients from varied ethnic backgrounds that present different endophenotypes, the technology is poised to assist in the identification of candidate disease-linked genes and SNPs that reside within 'modifier loci' (172). While hundreds of candidate genes that are linked with increased diabetes-risk have been identified, the mechanisms underlying their action often remain unclear. One of the first identified and best understood examples of how genetic protein coding disease modifiers modulate the phenotypic expression of diabetes are the multiple identified polymorphisms within the base pair sequence of the HLA region of chromosome 6p21.3 on T1D (173). In this case, variation within the coding sequence of the HLA DR and DQ alleles produce changes in the amino acid sequence of cell surface receptors, altering their binding affinity to T-cells and increasing T1D-risk [reviewed in (142)].

Apart from polymorphisms in the HLA region, coding mutations within genes that encode important pancreatic transcription factors (TFs) have also been shown to modify the phenotypic expression of diabetes (162, 163). Coding mutations within the TCF7L2 (174), PDX1 (107), HNF1A (108), HNF4A (175), and TM6SF2 (176) genes can result in the dysregulation of blood glucose homeostasis by altering TF expression or imparting direct functional consequences on β-cell or islet function through alterations in a TF's amino acid sequence. For example, hundreds of missense mutations within PDX1 coding regions have been identified, with mutations within the transactivation domain reducing gene activation and impairing both β-cell development and function (107). In the case of HNF1A, 11 rare coding variants have been identified that result in a >40% reduction in transcription and are strongly associated with monogenic diabetes (MODY3) in the general population (108, 109). Similarly, a number of coding SNPs can impart T1D and T2D susceptibility within groups with a shared ancestral heritage, including SNPs in the SUMO4 (177) and MGEA5 (178) genes, identified within Japanese and Mexican populations, respectively.

Non-Protein Coding Disease Modifiers of Diabetes

Recent advances in targeted RNA sequencing technology (RNA Seq, RNA CaptureSeq) have greatly expanded our understanding of transcriptomics (179), underscoring the potential importance of regulatory elements in the control of disease-linked genes (180–182). Rather than coding for a protein directly, non-coding RNA (ncRNA) regulate the transcriptional or post-transcriptional production and modification of proteins. ncRNA make up 98% of the transcripts in the human genome, can be either trans- or cis-acting, and are classified into groups according to their length, morphology, and function (179, 183). 'Short' ncRNAs are less than 200 bp in length and perform a diversity of functions during gene regulation, protein synthesis, and the post-translational modification of proteins. Short ncRNA include nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), micro-RNAs (miRNAs), and transfer RNA (tRNA), to name a only a few (184). In contrast, long non-coding RNAs (lncRNAs) are 200 bps or longer and are generally only involved in the regulation of gene transcription and epigenetic regulation, although in some rare occasions they may produce peptides (185).

To date, the influence of non-protein coding disease modifiers on the pathogenesis of diabetes remains underdefined, providing an exciting avenue for future research. GWAS comparing the

islets of diabetic and non-diabetic individuals suggest that most T2D-associated variants do not reside in coding regions (146, 147), adding to a growing, yet sparse, body of evidence that glucose homeostasis is heavily controlled by the activity of non-coding regulatory elements (186). For example, thousands of miRNAs and lncRNAs have been isolated from islets (136), with preliminary evidence suggesting that some miRNA are required for islet development in mice (187) and β-cell function (188, 189). LncRNA in particular have been linked to several important processes in diabetes (190), with overexpression resulting in enhanced cell proliferation and fibrosis in the early state of diabetic nephropathy [LncRNA CYP4B1-PS1-001 (191)].

Non-coding single nucleotide polymorphisms (ncSNPs), or variations in a single DNA base pair that code for non-coding regulatory elements, can also act as disease modifiers of diabetes (182). More than 90% of disease-associated SNPs are located within non-coding regions, resulting in possible functional variants of promoters, enhancers, and ncRNA genes (192). Through this mechanism, ncSNPs within important regulatory regions can alter the splicing, binding, degradation, or sequence of a ncRNA, which in turn can modulate the activity of multiple regulatory elements that act to control other cellular processes, such as transcription factor binding and chromatin states (193, 194). As an example, a recent study from our laboratory that used genome editing to knock-out *HNF1A* in hESCs identified a human-specific lncRNA (*LINKA*) that is a target of HNF1A and is necessary for normal mitochondrial respiration within stem cell-derived β-cells (113). Given that there is recent evidence that islet-specific lncRNA and transcription factors co-regulate genes associated with enhancer clusters (195, 196), we expect that additional studies that expand upon the functional consequences on ncSNPs and the potential targets of lncRNA in human islets have great potential to explain some of the clinical heterogeneity each diabetes subtype (197, 198).

Patient iPSCs as a Diabetes Disease Modifier Discovery Platform for Monogenic Diabetes

The discovery of genetic modifiers of diabetes have been slowed by the complex presentation of the diabetes subtypes, with the cause of each existing on a multi-dimensional continuum of genetic, epigenetic, and environmental factors (14). However, while progress has been hindered in some areas, success has been achieved over the past two decades within monogenic diabetes, with advancements in molecular genetics enabling the definition of discrete etiological disease subtypes that can inform preventative treatments through precision medicine (199). As discussed in section 3, monogenic forms of diabetes result from coding mutations in single identified genes which cause β-cell-intrinsic dysfunction. This has allowed for targeted studies focused on elucidating the role of the specific causative gene in β-cell development and function. However, disease penetrance and presentation can vary among individuals with the same underlying pathogenic mutation, suggesting that additional factors can influence the genotype-phenotype association (15–18).

The multifaceted nature of monogenic diabetes provides a unique opportunity to model gene-phenotype relationships that contribute to endophenotypes seen in the more common forms of the diabetes and aid in the discovery of novel disease modifiers (200, 201). Recent technological advancements in sequencing technology, genome editing (see *Section 2*), and the generation and guided-differentiation of iPSCs (see *Section 1.2*) now provide the foundation for an iPSC-based discovery platform that can identify novel protein coding and non-protein coding genetic elements that modify the presentation and penetrance of endophenotypes. Presented in **Figure 1**, genetic disease modifier discovery begins with the identification of a heterozygous coding mutation that results in monogenic diabetes. Coding variant identification can be done on demand through partial or whole genome sequencing given available information regarding candidate genes (97), through exome sequencing (98), or by leveraging publicly available GWAS datasets that compare non-diabetic and diabetic patients (99) (see *Section 3*). A useful database for monogenic diabetes includes the products of the DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium (https://www.diagram-consortium.org/).

Upon candidate allele or gene selection, the next step within the disease modifier discovery platform is the production of iPSC lines from diabetic patients with the desired coding mutation (step 2, **Figure 1**). Due to recent technological advancements, iPSCs can easily be generated from adult cells that are harvested from blood or skin tissue (112, 202, 203). Once generated, endodermal cells with the desired coding mutation can be produced from iPSCs through exposure to the inductive signals that drive *in vivo* development (48, 204). To this end, several stepwise protocols that move cells through the multiple stages of pancreatic development *in vitro* over a few weeks have been developed (52–54). This process represents a relatively efficient method for the generation of pancreatic β-cells using tissue from multiple donors that share the identified coding mutation but have varied genetic backgrounds that result in different genetic modifiers, a distinct advantage when addressing observed heterogeneity in phenotypic expression.

After the generation of stable iPSC lines, the next step within the disease modifier discovery platform is to selectively correct the identified coding mutation and compare the resulting gene mRNA and protein expression before and after correction (step 3, **Figure 1**). As described in section 2, there are a number of genome editing technologies available to accomplish line correction, although CRISPR/cas9-mediated systems are becoming the most frequency used within stem cell models (63, 64). The goal of model generation is to compare corrected and non-corrected lines to non-diabetic controls, to which the mRNA and protein expression of the corrected gene can be measured (step 4, **Figure 1**). Throughout this process, one of two outcomes may be observed. *Outcome 1: the correction of the observed coding mutation can result in the complete normalization of gene/protein expression*. This result implies that the coding mutation was completely responsible for the decrease in gene expression and/or function (step 5A, **Figure 1**).

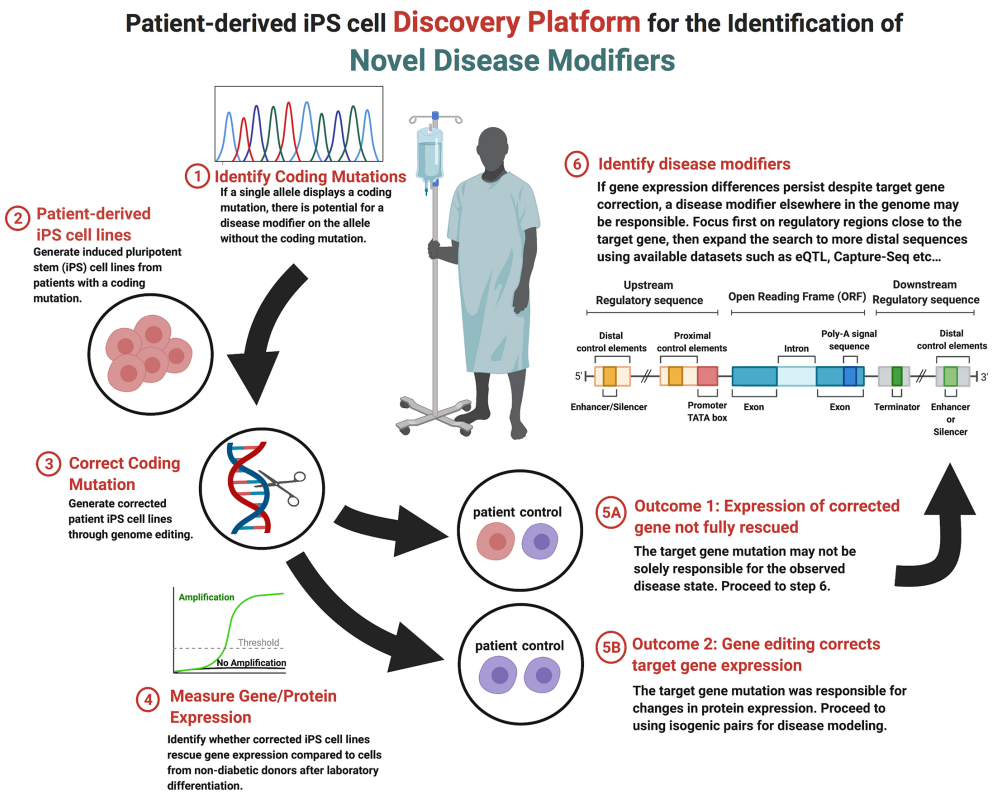

FIGURE 1 | Stepwise flow diagram of the process of genetic disease modifier discovery.

Outcome 2: the expression of the monogenic diabetes gene is not rescued to the levels observed in non-diabetic control cells. In this case, expression is possibly being regulated by a modifier elsewhere in the genome (step 5B, **Figure 1**). It is possible that certain coding mutations may disrupt protein function while not impacting mRNA or protein expression. Such a mutation can still be studied with this platform as regulatory region variants which decrease gene expression may still be detectable when comparing the patient iPSC cell line to control lines.

In the event that the candidate gene expression is not normalized by selective correction of the coding mutation, the target gene may be under the regulatory control of one or more disease modifiers (step 6, **Figure 1**). Disease modifiers can reside either proximally or distally with respect to the coding mutation, as well as either upstream or downstream from the affected gene, making their location difficult to determine. An effective search strategy can be to focus on proximal regulatory regions near the gene of interest first, although if this approach doesn't prove fruitful then there are a number of computational approaches that are specifically designed for the identification of regulatory elements [reviewed in (205)]. Similarly, the sequencing and chromatin mapping efforts of the ENCODE (https://www.encodeproject.org/) (206), Epigenome Roadmap Consortia (https://egg2.wustl.edu/roadmap/web_portal/) (207), and Common Metabolic Diseases Knowledge Portal (https://hugeamp.org) have provided extensive annotation of coding and non-coding regions within the human genome, as well as the likelihood of variants to impact metabolic disease.

Through the use of public databases, it is now possible to determine likely regulatory regions of the target gene of interest that can then be interrogated by targeted sequencing of patient iPS cell lines that could not be completely rescued by correction of the coding mutation. If variants are discovered, they can be studied by genome editing in the context of coding mutations or in isolation to determine the impact on expression of the target gene. For example, this strategy has been successfully used to study pancreas agenesis caused by mutations in GATA6 within our laboratory (102). A non-coding SNP was discovered in a patient iPS cell line that regulated expression of GATA6 during pancreas development *in vitro* and when interrogated in a cohort of patients with the disorder was confirmed to be a disease modifier. This strategy is especially useful in studying variants that impact genetic disease caused by heterozygous coding mutations. Variants that may have only a small influence on gene expression and no impact on disease alone can synergize with heterozygous coding mutations to bring target gene expression below a critical threshold needed for function. This platform does have some limitations including the requirement to focus of monogenic heterozygous disorders and may not be scalable to examine large numbers of genes with current differentiation technologies. We suggest that this methodology could be applied secondarily to any heterozygous iPSC disease

modeling project that entails the creation of isogenic corrected lines with minimal additional effort.

CONCLUSION AND FUTURE OUTLOOK

The recent development of techniques to differentiate hPSCs into pancreatic β-cells has opened up new pathways to study the pathogenesis of diabetes. These human-centric models, combined with rapidly advancing genome editing techniques, provide incredibly powerful and scalable platforms in which to study the contribution of genetic elements to β-cell function, while also addressing the limitation of mouse models. Furthermore, the use of hPSCs provide unique opportunities in which to accomplish the targeted study of β-cell dysfunction as well as provide a platform to discover protein coding and non-protein coding genetic modifiers. Given recent evidence that large numbers of disease-linked variants do not reside in coding regions and the presence of variants can be population-specific, iPSC platforms that use patient-derived tissue hold great promise for the discovery of novel genetic disease modifiers that may help to explain the variability seen across and within diabetes subtypes.

While hPSC-based platforms represent a great leap forward in our ability to study β-cell function, there are caveats to their use that must be taken into account. hPSC derived β-cell generation and culture is labor-intensive, requiring approximately 40 days of differentiation and maturation. Additionally, though they do have some degree of insulin secretion in response to glucose and other secretagogues, significant uncertainty regarding their functionality and maturity still exists (66, 208–211). Ideally, these protocols need to be optimized to support the efficiency and accuracy of discovery platforms utilizing stem cell-derived β-cells. However, further fine-tuning of the established differentiation protocols will drive us closer to an ideal *ex vivo* human model of pancreatic β-cells. In addition to improving β-cell function, protocols need to be improved so that they are more universally successful, as certain hPSC lines can more easily differentiated into β-cells than others using current protocols.

The development of more universally-applicable protocols is required as the use of patient iPSC lines expands. There has been a recent flurry of publications that promise improved protocols with better function and wider applicability, and advances will continue to build on those already made (57, 212). Finally, generation of islet cells in platforms combining different stem cell-derived cell types will allow for more complex modeling of the genetic and environmental factors driving all forms of diabetes. Improving our knowledge of pancreatic β-cells function and development in humans is essential for the development of treatments for the millions of people affected by diabetes.

AUTHOR CONTRIBUTIONS

MG and KL conducted the literature review, designed figures, and wrote the manuscript. MG, KL, and PG conceived of the topic and edited the text. All authors contributed to the article and approved the submitted version.

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REVIEW
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Monogenic Diabetes Modeling: *In Vitro* Pancreatic Differentiation From Human Pluripotent Stem Cells Gains Momentum

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The occurrence of diabetes mellitus is characterized by pancreatic β cell loss and chronic hyperglycemia. While Type 1 and Type 2 diabetes are the most common types, rarer forms involve mutations affecting a single gene. This characteristic has made monogenic diabetes an interesting disease group to model *in vitro* using human pluripotent stem cells (hPSCs). By altering the genotype of the original hPSCs or by deriving human induced pluripotent stem cells (hiPSCs) from patients with monogenic diabetes, changes in the outcome of the *in vitro* differentiation protocol can be analyzed in detail to infer the regulatory mechanisms affected by the disease-associated genes. This approach has been so far applied to a diversity of genes/diseases and uncovered new mechanisms. The focus of the present review is to discuss the latest findings obtained by modeling monogenic diabetes using hPSC-derived pancreatic cells generated *in vitro*. We will specifically focus on the interpretation of these studies, the advantages and limitations of the models used, and the future perspectives for improvement.

Keywords: pancreas, beta cell, human, pluripotent stem cell, monogenic, modeling, diabetes, *in vitro* differentiation

INTRODUCTION

Diabetes mellitus (DM) is characterized by pancreatic β cell loss and chronic hyperglycemia. Type 1 diabetes (T1D) is caused by the autoimmune reaction against β cells (1), and Type 2 diabetes originates from insulin resistance and β cell overload (2–4). In addition, rarer monogenic forms of diabetes account for approximately 1–5% of diabetes cases, depending on the population studied (5, 6). Over 30 subtypes of monogenic diabetes have been identified to date, each having a characteristic phenotype and a specific pattern of inheritance (6, 7). The identification of genes implicated in the pathogenesis of monogenic diabetes, including components of the insulin secretory pathway and transcription factors, has provided important insights into human pancreas and β cell development and function.

Monogenic diabetes is caused by either splice-site, non-sense, missense, or frame-shift mutations, and more rarely partial or full deletions, affecting a single gene (8–14). The disease

phenotype and associated extra-pancreatic features vary depending on the affected gene (15). These characteristics have made monogenic diabetes an interesting disease subtype to model using human pluripotent stem cells (hPSCs). Indeed, hPSCs can be differentiated into pancreatic cells following key steps of differentiation induced by well-established combinations of growth factors and small molecules, thereby respecting a natural path of development [recently reviewed in (16, 17)]. Thus, differentiation of human induced pluripotent stem cells (hiPSCs) either derived from patients with monogenic diabetes or genetically edited to carry the mutation of interest can be used to study the potential regulatory mechanisms affected by each of the disease-associated genes (**Figure 1**). Applying this approach to a diversity of genes has led to the discovery of new mechanisms associated with specific regulators of pancreatic development. The focus of this review is to discuss the latest findings obtained by modeling monogenic diabetes using hPSC-derived pancreatic cells generated *in vitro*. We will focus on the interpretation of these studies, the advantages and limitations of the models used, and the future perspectives for improvement. Of note, the reader is referred to recent reviews concerning: 1) the state-of-the-art knowledge in pancreatic β cell development in mice and humans (18–21); 2) the tools for hPSC genome editing (22, 23); 3) a comparison of the *in vitro* pancreatic differentiation protocols including the latest advances to achieve functional β cells from hPSCs (16, 17, 24); 4) analyzing the intrinsic variation in the protocol outcomes from different sources of hPSCs (25, 26); and 5) the use of *in vitro* pancreatic differentiation from hPSCs to discover new mechanisms underlying human pancreas development (24, 27), or to model other types of diabetes (23, 28–31). The later also summarize findings on monogenic diabetes modeling. Our review adds up on top of these by exclusively focusing on the modeling of monogenic diabetes, discussing in more detail the different approaches taken and including extremely recent works which provide insightful information for the interpretation of the results published so far.

HUMAN PANCREAS DEVELOPMENT

Lessons Learned From Mice and Current Challenges

Pancreas development begins with the establishment of the pancreatic bud containing multipotent pancreatic progenitor cells (MPCs) at ~E8.5 in the mouse (19, 20) or ~29 days post conception in humans (20, 21, 32) and progresses until E18.5 in the mouse (19, 20) or 24 weeks post conception (wpc) estimated in humans (20, 21, 33–35). By this time, most of the pancreatic progenitor cells are terminally fate-committed. The MPCs are capable of differentiating along the three main lineages of the adult pancreas, namely the ductal, the exocrine (comprising acinar cells that secrete digestive enzymes), and the endocrine (including the β cells that produce insulin, but also the α, δ, γ, and PP cells) (19, 36, 37). As the pancreas develops, MPCs differentiate into acinar or endocrine-ductal bipotent progenitor

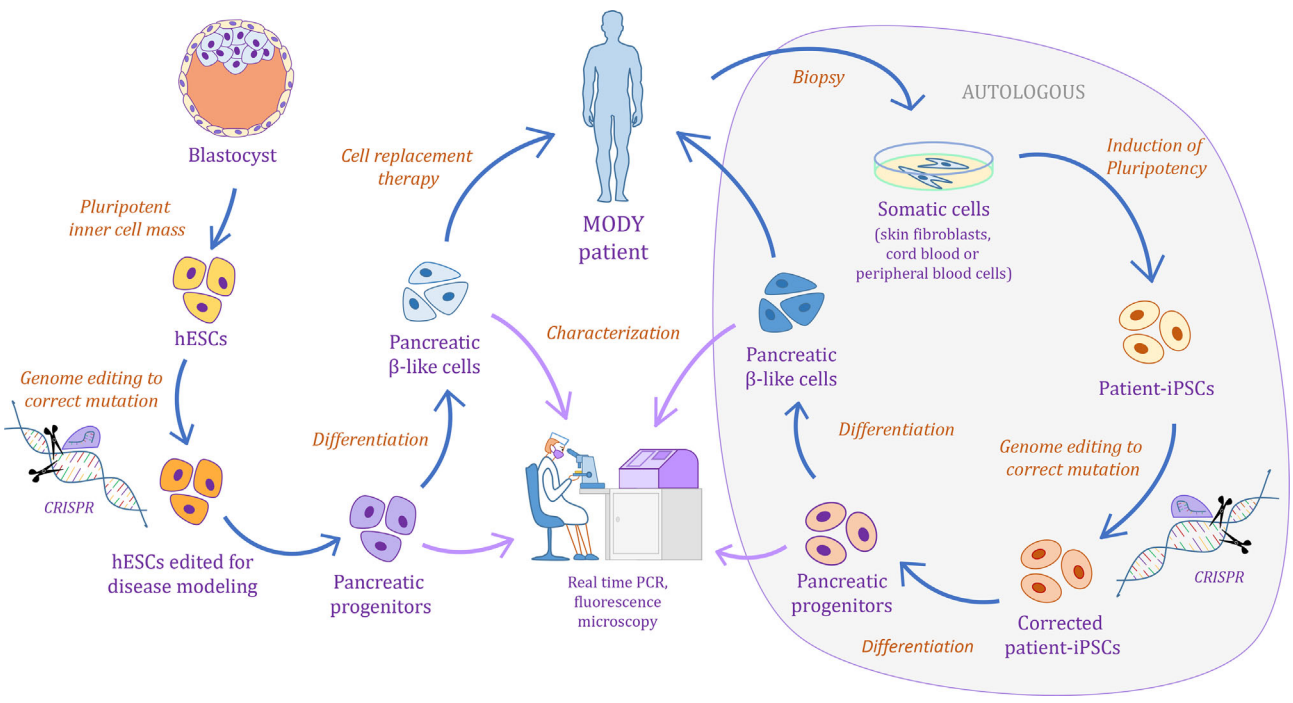

FIGURE 1 | Schematic illustrating the pipeline for monogenic diabetes disease modeling using human pluripotent stem cells.

(BP) cells, and eventually to endocrine-committed progenitors (EPs) that will give rise to β cells. Importantly, recent single cell RNA-seq (scRNA-seq) studies have described the transcriptional profiles that characterize these pancreatic progenitor cell stages in the mouse and identified additional progenitor cell sub stages (38–42). Thus, we can now rely on a precise transcriptomic fingerprint for several progenitors that arise during mouse pancreas development.

Cumulative knowledge has revealed the role and stage-specific functions of signaling pathways in the pancreatic developmental program, including Wnt, TGF-β, Notch, FGF and, more recently, the Hippo pathway (19, 36, 37, 43–46). The pancreatic mesenchyme has also been shown to play important roles during development, by fine-tuning the crosstalk with the pancreatic epithelium through these pathways (47–50). This knowledge has been exploited to develop protocols to differentiate human embryonic stem cells (hESCs) into the pancreatic lineage (51–55), opening the possibility to produce large quantities of β cells for cell-based therapies and also providing a new avenue for research in human pancreas development.

Differentiation protocols currently available to produce β cells from hPSCs take advantage of the cell signaling events that occur during fetal development (18–21). Despite this knowledge-based approach, the generation of fully functional β cells *in vitro* has remained elusive. We thus refer to the cells produced *in vitro* so far as β-like cells. This limitation could be in part explained by the fact that the function of these pathways during pancreas development has been mainly studied in rodents, and it has recently been reported that human pancreas development could differ in several aspects (20, 21, 32, 56, 57). We are still lacking systematic studies comparing islet development between human and mouse, although recent reports are moving forward to address this gap (57–60). It is expected that a deeper understanding of these inter-species differences in islet development will probably be critical for the production of fully functional and mature β cells from hPSCs. As well, this will allow a more precise dissection of the molecular mechanisms driving diabetes predisposition by genetic mutations and/or external stimuli. Noteworthy, recent protocols allow the derivation of monohormonal insulin-producing cells expressing key β cell transcription factors, including *PDX1*, *NKX6.1*, and *MAFA* (61–65). Fine tuning of such protocols could further improve the glucose-response of *in vitro* derived β-like cells (66–69) without the need to involve a step of cell transplantation in mice to ensure proper maturation.

Lessons Learned From *In Vitro* Human PSC Pancreatic Differentiation

The use of hPSCs as *in vitro* model system to study human pancreatic development has gained momentum and several important discoveries have been made using this approach [recently reviewed in (27)].

A Role for TEAD and YAP in Pancreas Development

By comparing the transcriptomes and key epigenomic features of MPCs derived *in vitro* from hESCs with human fetal primary pancreatic tissue of six wpc embryos, we were able to show that *in vitro* derived cells closely recapitulated the main expression profile and regulatory landscape of their *in vivo* counterparts (45). Furthermore, this epigenomic characterization was extended to include ChIP-seq profiling of several transcription factors by taking advantage of the *in vitro* system. A combined analysis of these data led to the discovery that TEAD1 was an integral component of the enhancer network in human embryonic pancreatic progenitors (45, 46). The relevance of TEAD protein binding for the activation of MPC enhancers was mechanistically validated using the platform provided by hPSC-derived pancreatic cells *in vitro*. We concluded that, while highly tissue specific enhancers were defined by co-binding of pancreas-specific transcription factors, TEAD proteins conferred these regions the ability to be regulated by YAP (an effector of the Hippo pathway) during human pancreas development. These results were consistent with reports for a role of the Hippo pathway in mouse pancreas development (43, 44). More recent reports support these findings by showing that YAP and other components of the Hippo pathway are active and highly enriched within the SOX9+/PTF1A+ progenitor cells of the human fetal pancreas (58).

The human *in vitro* differentiation system has provided additional mechanistic evidence for the relevance of the Hippo pathway in pancreas differentiation by showing that YAP links extracellular matrix-mediated mechano-signals to regulate gene expression. Integration of such signaling plays a key role in the fate choice of bipotent pancreatic progenitors, whereby YAP downregulation favors endocrine cell commitment (70). In agreement, sustained *in vitro* YAP activation impairs β cell differentiation while inhibition of YAP enhances differentiation of functional β cells derived from hPSCs (71). Combined together, these results illustrate how *in vitro* pancreatic differentiation can help in the discovery of new regulatory mechanisms that are relevant for human β cell development.

A Role for Polycomb Group-Mediated Repression in Pancreas Development

Mapping the dynamic changes in histone modifications and chromatin accessibility across different stages of the *in vitro* pancreatic differentiation protocol has provided important insights into the suitability and current limitations of this model (72–75). It has to be noted that, despite successful applications, the *in vitro* pancreatic differentiation system does not exactly replicate all the epigenomic features of their *in vivo* counterparts. Sander and colleagues have profiled selected chromatin modifications and the transcriptome of these cells, at different stages of the pancreatic endocrine differentiation protocol using hESCs (72). They showed that removal of Polycomb group (PcG)-mediated repression on stage-specific genes was a key mechanism for the induction of developmental regulators in the *in vitro* system, consistent with the *in vivo* relevance of this mechanism in mouse endocrine pancreas development (76–78). However, they also reported that elimination of PcG-mediated repression on endocrine-specific genes was not fully recapitulated by the *in vitro* derived endocrine cells. This was particularly evident at genes involved in organ morphogenesis, underscoring a current limitation

(*i.e.* the lack of tissue-specific contextual cell signaling) of the *in vitro* protocols for studying some aspects of *in vivo* development. Noteworthy, these experiments were performed using 2D *in vitro* differentiation protocols which have been shown to be less efficient to produce functional β-like cells than three-dimensional suspension culture systems (61–63, 66–68). This "second generation" protocols could be more efficient in allowing the proper deposition of epigenetic marks.

Distinct Progenitor Cell Populations Could Differentiate Into Monohormonal β Cells

Another example of the complexity of the *in vitro* endocrine differentiation process was provided by Petersen et al. who profiled single-cell transcripts by qRT-PCR at selected stages of the protocol used to produce β-like cells from hPSCs (79). This analysis identified two distinct progenitor cell populations with the potential to differentiate into monohormonal β-like cells. *NKX6.1* expression prior or after the onset of *NEUROG3* (the gene coding for the EP transcription factor marker NGN3) was the main difference between these progenitors. Building up on these results, Ramond et al. performed a combined analysis of single-cell qRT-PCR datasets obtained from pancreatic progenitor and endocrine cells from *in vitro* and *in vivo* samples (59, 60). Their observations suggest that these distinct progenitor cell populations identified *in vitro* could indeed exist during *in vivo* development. This finding contrasts with knowledge gained from mouse studies where Nkx6 factors systematically specify endocrine cell fate upstream of Ngn3 in the MPC stage (80, 81). Still, the function and characteristics of these two populations of progenitors in the developing human pancreas remain to be fully elucidated.

Taking advantage of novel single-cell technologies can help to match some of the transcriptional signatures from *in vivo* pancreatic progenitor cell stages identified in the mouse (38–41), with pancreatic progenitors derived from hPSCs (38, 66, 82). However, such exercise remains challenging due to inter-species differences and also the impact of the *in vitro* culture. New tools are being quickly developed to address this limitation, making the bioinformatic analyses of these data an exciting area of research (83, 84). More recently, the first scRNA-seq experiments using human embryonic pancreas from 15.2 and 17.1 wpc have been reported (58, 85). Integration of these scRNA-seq datasets with those derived from the mouse embryonic pancreas will help to identify differences and similarities in the transcriptional fingerprints of the distinct pancreatic progenitor cell types. This will ultimately contribute to validate the identity of pancreatic progenitors produced from hPSCs.

MODELING MONOGENIC DIABETES WITH HUMAN PLURIPOTENT STEM CELLS

The most frequently affected maturity-onset diabetes of the young (MODY) genes include the enzyme glucokinase (*GCK*, MODY2) (86, 87) and the transcription factor genes hepatic nuclear factor 1 alpha (*HNF1A*, MODY3) (88), hepatic nuclear factor 4 alpha (*HNF4A*, MODY1) (89), and hepatic nuclear factor 1 beta (*HNF1B*, MODY5) (90). Other MODY genes include *PDX1* (MODY4), *NEUROD1* (MODY6), *KLF11* (MODY7), *CEL* (MODY8), *PAX4* (MODY9), *INS* (MODY10), *BLK* (MODY11), *ABCC8* (MODY12), *KCNJ11* (MODY13), *APPL1* (MODY14) (91). On the other hand, homozygous mutations at several lineage determining transcription factors, such as *PTF1A*, *PDX1*, *NEUROG3*, *RFX6*, *NEUROD1*, *MNX1*, *NKX2.2* and *GLIS3* result in permanent neonatal diabetes mellitus (PNDM) in humans (13, 92–100). Interestingly, heterozygous mutations in these genes rarely result in diabetes in mice, thereby suggesting an important divergence in the activity or function for these factors between human and mouse (19, 21, 101). The importance of haploinsufficiency and the mechanisms by which a decrease in transcription factor activity causes a disease in humans is poorly understood, mostly due to the lack of an appropriate model system. As an example, MODY5 diabetes (*HNF1B*-associated) can be induced by a diversity of mutations including several splice-site, non-sense, missense, and frame-shift mutations or whole gene deletions, all of which result in a diabetes (102). The heterozygous mutation in mouse has no effect on pancreatic development, while homozygous mutation blocks foregut specification thereby masking its downstream function in the differentiation of MPCs. Ultimately, haploinsufficiency may reflect the functional effects of different gene anomalies, stochastic variation in temporal gene expression during early development or additional genetic and/or environmental modifiers that may influence the disease phenotype (102–104).

As mentioned above, mouse models often do not recapitulate the disease phenotype associated with heterozygous mutations of *HNF1A*, *HNF4A*, or *HNF1B* in humans. The genetic discrepancy between the mouse and monogenic diabetes gene haploinsufficient patients and the difficulty in accessing patient samples have reinforced the interest in using hPSCs (**Figure 1**). Genome-editing tools combined with directed differentiation of hPSCs offer a unique platform for generating patient-specific disease models to elucidate novel genes and molecular pathways that underlie monogenic diseases with complex traits, such as diabetes, and ultimately lead to the development of novel therapeutic strategies [recently reviewed in (22, 25)]. Several studies in the last decade have used genetically engineered hPSC culture systems for differentiation into pancreatic cells to further expand our understanding of the roles of various genes associated with monogenic diabetes. Their findings are summarized in **Tables 1**, **2**, and these will be discussed in more detail next.

WFS1

Egli and colleagues provided the first example for the use of hiPSCs to create insulin-producing cells from patients with Wolfram Syndrome (WS) (119). hiPSCs were generated from individuals with diabetes caused by mutations in the *WFS1* gene and healthy-donor controls. Differentiation of these cells towards β-like cells revealed increased levels of ER stress molecules and decreased insulin content in WFS1-deficient β-like cells. Overall, insulin processing and secretion in response to various

TABLE 1 | Summary of reports modeling maturity-onset diabetes of the young (MODY) mutation effects.

Gene studied	Pancreatic defects reported in humans	Effects recapitulated in mice	Genome editing approach	Differentiation protocol	Type of human pluripotent stem cell	*In vitro* phenotypes	Ref.
***HNF4A* (MODY1)**	*HNF4A* heterozygous mutations affect both liver and pancreas development. MODY1 patients present neonatal hyperinsulinemia and impairment in β cell function. They present normal insulin sensitivity but decreased insulin secretion.	Rodent models do not accurately recapitulate the MODY1 phenotype in humans. The available *Hnf4a* general knockout murine model is embryonic lethal, while heterozygous mice present normal glucose tolerance and do not show any diabetic features.	NA	(62)	hiPSCs were derived from MODY1 mutation carriers. Their family members, without the mutation, were used as controls.	The *HNF4A* mutation studied did not prevent the formation of insulin+ cells *in vitro*. Also, no defects in β-like cells differentiated from *HNF4A* mutant hiPSCs were found.	(105)
			NA	Adapted from (62).	Control hiPSC lines (CSES7 and IPSO lines) and MODY1 patient-derived hiPSCs.	Researchers report that cells from the MPC stage show increased expression of endocrine progenitor transcription factors, including *PAX6*, *NEUROD1* and *NEUROG3*.	(106)
			Site-directed mutagenesis.	(61)	hiPSCs were derived from non-diabetic and MODY1 patients.	Key developmental genes such as *HNF1B*, *PDX1*, *GATA4*, and *RFX6* are downregulated at the foregut progenitor stage, prior to MPC specification. Still, terminally differentiated β-like cells can be produced and express selective β cell markers and C-peptide. The functional capacity of these cells could not be appropriately elucidated due to limitations of the *in vitro* protocol used.	(107)
***GCK* (MODY2)**	Patients with *GCK* heterozygous mutations present progressive β-cell dysfunction, fasting hyperglycemia and reduced insulin secretion. These result in a mild diabetes phenotype that generally does not require anti-diabetes medication.	Homozygous mutant mice exhibit growth retardation and die soon after birth as consequence of severe hyperglycemia. Heterozygous mutant mice only present slightly elevated blood glucose levels from birth, with disturbed glucose tolerance and glucose-induced insulin secretion.	NA	NA	Non-edited MODY2 and PNDM patient-derived hiPSCs.	This work reports the generation of iPSCs from MODY2 patients. The researchers did not analyze differentiation into the pancreatic lineage.	(108)
HNF1A (MODY3)	Patients with *HNF1A* heterozygous mutations show β cell dysfunction and hyperglycemia due to insufficient insulin release in response to increased blood glucose levels.	Mouse models do not fully mimic the human disease phenotype. Mice with heterozygous mutations in *Hnf1a* are healthy and mice with homozygous null mutations present a diabetic phenotype.	CRISPR-CAS9 system.	(62), with minor modifications.	Genome-edited hESCs (MEL1 and H1) and human β-cell lines (EndoC-BH).	Differentiation from *HNF1A*$^{+/-}$ hESC show reduced number of INS+ cells. β-like cells present defects in mitochondrial function and the glycolysis process. Decreased expression of β cell transcription factors and genes associated with insulin synthesis. Reduced β cell proliferation and increased apoptosis.	(109)
			NA	(61), with some modifications.	hiPSCs were derived from MODY3 patients. hiPSCs derived from a healthy donor were used as control.	*HNF1A* MODY3 mutations caused decreased *GLUT2* expression, which was associated with reduced glucose uptake and ATP production. The mutant *HNF1A* β-like cells present decreased insulin secretion in response to high glucose.	(110)

(Continued)

TABLE 1 | Continued

Gene studied	Pancreatic defects reported in humans	Effects recapitulated in mice	Genome editing approach	Differentiation protocol	Type of human pluripotent stem cell	*In vitro* phenotypes	Ref.
***PDX1* (MODY4)**	*PDX1* heterozygous mutations are associated with insulin secretion deficiency. Common point heterozygous mutations in the *PDX1* transactivation domain impair human pancreatic β cell formation and function, and contribute to increased risk for diabetes. Pancreatic developmental anomalies related to *PDX1* mutations are reported only in neonatal diabetes cases.	Homozygous *Pdx1*-deficient mice fail to generate a pancreas, while heterozygous animals develop a pancreas but become diabetic in adulthood due to β cell apoptosis.	TALEN and CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Monoallelic *PDX1* mutations are associated with decreased PDX1 protein expression. These compromise endocrine differentiation and lead to reduction in the number of INS+ cells derived *in vitro*.	(111)
			CRISPR/Cas9.	Based on (62).	Genome-edited hiPSCs and patient-derived hiPSCs.	Heterozygous mutations impair *in vitro* β cell differentiation and function. Homozygous point mutations in the *PDX1* transactivation domain do not only impact pancreatic endocrine lineage development, but also impair glucose-responsive function of β cells through misregulation of several PDX1 target genes.	(112)
***HNF1B* (MODY5)**	Patients with *HNF1B* heterozygous mutations commonly exhibit pancreatic hypoplasia, β-cell dysfunction and insulin resistance.	*Hnf1b*$^{-/-}$ mice present pancreatic agenesis, exhibiting loss of expression of several pancreatic genes, including Pax6, which regulate β-cell function. In contrast with MODY5 patients, *Hnf1b*$^{+/-}$ mice do not develop diabetes.	NA	Adapted from (52).	MODY5 patient-derived hiPSCs.	Upregulation of multiple key pancreatic transcription factors at the DE and MPC stage, including *FOXA2*, *PDX1*, *GATA4* and *GATA6*. Interestingly, expression of HNF1B itself was induced in mutant hiPSC-derived MPCs. Reduction of PAX6 expression.	(113)

NA, not applicable.

secretagogues was comparable to healthy controls, but the former displayed increased activity of unfolded protein response (UPR) pathways.

More recently, Maxwell et al. used CRISPR/Cas9 to correct a diabetes-causing pathogenic variant in *WFS1* hiPSCs (120). Noteworthy, β-like cells differentiated from *WFS1*-corrected hiPSCs showed robust and dynamic insulin secretion in response to glucose, and reversed streptozocin-induced diabetes when transplanted into mice. Single-cell RNA-seq transcriptome profiling showed that indeed these cells displayed increased insulin levels and decreased expression of genes associated with endoplasmic reticulum stress. Taken together, these studies illustrate the potential of *in vitro* pancreatic differentiation from hPSCs to study how mechanisms related to cellular stress can affect diabetes onset.

PDX1

Homozygous null mutations in *PDX1* result in pancreatic agenesis both in mice and humans (13, 121–123). Human patients with *PDX1* heterozygous inactivating mutations exhibit MODY4 diabetes caused by defects in β cell function and/or the maintenance of β cell mass in adults (36). In rodents, it has been reported that *Pdx1*$^{+/-}$ mice can develop a functional pancreas (121, 122) but become diabetic in adulthood due to β cell apoptosis (124).

Another pioneer study to model monogenic diabetes was reported by Huangfu and colleagues, who used TALEN and CRISPR-Cas-mediated gene editing combined with hPSC-directed differentiation. These researchers provide a systematic analysis of the role for PDX1 and seven additional pancreatic transcription factors (RFX6, PTF1A, GLIS3, MNX1, NGN3, HES1 and ARX) in pancreatic cell commitment (111). Noteworthy, they created mono- or biallelic frameshift mutations in all these genes and used untargeted isogenic cell lines as controls. This analysis not only defined the specific developmental steps affected by these mutations in a model of human pancreas differentiation, but also revealed new mechanisms. **Tables 1**, **2** show a summary of their results for the genes previously associated with MODY and/or PNDM. An interesting finding of this work was that monoallelic frameshift translation mutations disrupting the PDX1 protein sequence cause a reduction (up to 65%) in the number of insulin+ cells derived *in vitro*. These findings suggest a haploinsufficient requirement for *PDX1* in pancreatic endocrine development. Importantly, this phenotype correlates with the observation that patients with heterozygous mutations in *PDX1* present with diabetes from an early age (125). These results further validate that decreased amounts of PDX1 could lead to β cell dysfunction, a decrease in β cell mass during fetal development and/or the maintenance of β cell mass in adults (124, 126, 127).

TABLE 2 | Summary of reports modeling monogenic mutations associated with permanent neonatal diabetes mellitus (PNDM) or pancreatic agenesis.

Gene studied	Pancreatic defects reported in humans	Effects recapitulated in mice	Genome editing approach	Differentiation protocol	Type of human pluripotent stem cell	*In vitro* phenotypes	Ref.
GATA6	*GATA6* heterozygous inactivating mutations result in pancreatic agenesis.	*Gata6* heterozygous mice are fertile and phenotypically normal. *Gata6* null mice are embryonic lethal. Biallelic loss of *Gata6* and its paralog *Gata4* result in a phenotype similar to human PNDM *GATA6*-mutated patients.	CRISPR/Cas9-mediated genome editing.	Adapted from (52).	Patient-derived hiPSCs and genome-edited hESCs. Isogenic, mutation-corrected, hiPSCs were used as controls.	*GATA6* homozygous mutations lead to impaired DE differentiation. Rescue of DE defects in these cells by re-expression of other GATA family members allows β-like cell production with a lower efficiency. hPSCs with *GATA6* heterozygous mutations show defects in DE differentiation. β-like cells produced in both cases are defective in the GSIS and in insulin processing.	(114)
			CRISPR/Cas9-mediated genome editing.	(61, 62, 111), with some modifications.	Genome-edited hESCs (H1 and HUES8).	Differentiation of *GATA6*$^{-/-}$ hPSCs revealed impaired DE commitment and pancreatic endocrine differentiation. No defects in DE differentiation from *GATA6*$^{+/-}$ hPSCs, but a lower number of *PDX1*+ *NKX6.1*+ pancreatic progenitors and β-like cells was produced.	(115)
			TALENs	(55), adapted from (52).	hiPSCs derived from pancreatic agenesis patients with *GATA6* heterozygous mutations. Genome-edited hESCs (H9) and hiPSCs. Non-mutated hESCs and hiPSCs were used as isogenic controls.	*GATA6* heterozygous hPSCs present a modest decrease in the generation of DE, which differentiate less efficiently into MPCs and EPs. *GATA6*-null hPSCs fail to enter the DE lineage.	(116)
			CRISPR-CAS9-mediated genome editing.	Adapted from (61, 62, 54).	hiPSCs derived from a patient with pancreatic agenesis. Isogenic, mutation-corrected hiPSCs were used as control.	hiPSCs with *GATA6* heterozygous mutations present reduced efficiency for generation of pancreatic progenitor cells *in vitro*. Correction of these mutations allowed identifying a non-coding SNP that additionally contributes to the phenotype observed.	(117)
PDX1	Homozygous mutations in *PDX1* result in pancreatic agenesis. PDX1 heterozygous patients exhibit diabetes caused by defects in β cell function and/or the maintenance of β cell mass in adults.	Homozygous mutations in *Pdx1*cause pancreatic agenesis, while heterozygous animals develop a pancreas but become diabetic in adulthood due to β cell apoptosis.	TALEN ad CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Differentiation of *PDX1*$^{+/-}$ mutant hESCs present a 65% reduction of INS+ cells at the β-like cell stage, which are mainly polyhormonal cells using the protocol described in this study.	(111)

(Continued)

TABLE 2 | Continued

Gene studied	Pancreatic defects reported in humans	Effects recapitulated in mice	Genome editing approach	Differentiation protocol	Type of human pluripotent stem cell	*In vitro* phenotypes	Ref.
RFX6	Patients carrying biallelic *RFX6* inactivating mutations present a reduction in the pancreas size and obstruction of the small intestine. These patients present defects in the formation of pancreatic progenitors and their further differentiation into functional endocrine cells.	Similar to humans, *Rfx6*-null mice show variable degrees of pancreatic hypoplasia and premature death.	TALEN ad CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Differentiation of *RFX6*$^{-/-}$ mutant hESCs show a reduction in the number of *PDX1*+ pancreatic progenitor cells. Severe reduction in β-like cells and complete absence of α cells.	(111)
			CRISPR/Cas9-mediated genome editing.	Adapted from (62)	hiPSCs were derived from patients with MRS and from their healthy, heterozygous father. hESCs (H9) was used as control.	hiPSCs with *RFX6* homozygous mutations show normal DE and PFG differentiation, but fail to robustly activate *PDX1*. MPCs and endocrine-competent progenitors differentiate less efficiently from these cells.	(118)
PTF1A	Homozygous inactivating mutations in *PTF1A* cause pancreatic and cerebellar agenesis.	*Ptf1a*-null mice present a complete absence of exocrine pancreatic tissue, but all islet endocrine cell types are present until the late stages of embryogenesis.	TALEN ad CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Differentiation of *PTF1A*$^{-/-}$ mutant hESCs do not present defects in pancreatic endocrine differentiation using the protocol described.	(111)
GLIS3	Biallelic mutations of *GLIS3* underlie a rare clinical syndrome, characterized by neonatal diabetes and congenital hypothyroidism.	Global *Glis3*$^{-/-}$ mice die of severe neonatal diabetes shortly after birth. Minor differences in gene dosage of Glis3 produce substantive changes in the expression levels of Ngn3 and Ins1, leading to a variable phenotype among the multiple *Glis3*-KO mouse lines.	TALEN ad CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Differentiation of *GLIS3*$^{-/-}$ mutant hESCs do not present defects in pancreatic endocrine differentiation using the protocol described.	(111)
			CRISPR/Cas9-mediated genome editing.	(64)	Genome-edited hESCs.	Differentiation of *GLIS3*$^{-/-}$ mutant hESCs show impaired expression of pancreatic endocrine-associated genes, including *PDX1*, *NEUROD1*, *NKX6.1*, and *MAFA*, and present increased β-like cell death. A chemical screen identified a drug candidate that rescues mutant GLIS3-associated β-cell death both *in vitro* and *in vivo*.	(64)

(Continued)

TABLE 2 | Continued

Gene studied	Pancreatic defects reported in humans	Effects recapitulated in mice	Genome editing approach	Differentiation protocol	Type of human pluripotent stem cell	*In vitro* phenotypes	Ref.
MNX1	Homozygous mutations in *MNX1* are associated with the occurrence of diabetes in infancy without evidence of exocrine pancreatic dysfunction. Reduced number of pancreatic endocrine cells, including β cells.	*Mnx1*-deficient mice show pancreatic dorsal-lobe agenesis and smaller pancreatic islets, while *Mnx1* gain-of-function in the pancreas leads to aberrant pancreatic development.	TALEN ad CRISPR/Cas9.	Adapted from (52, 54).	Genome-edited hESCs (HUES8).	Differentiation of $MNX1^{-/-}$ mutant hESCs do not present defects in pancreatic endocrine differentiation using the protocol described.	(111)

DE, definitive endoderm; MPC, multipotent pancreatic progenitor cells; PFG, posterior foregut; GSIS, glucose-stimulated insulin secretion; MRS, Mitchell-Riley syndrome; KO, knock out; ER, endoplasmic reticulum.

More recently, Lickert and colleagues generated hiPSCs from two patients with heterozygous missense mutations in the *PDX1* coding region ($PDX1^{P33T/+}$ and $PDX1^{C18R/+}$) leading to single amino acid exchanges in its transactivation domain (112). By comparing with a control hiPSC line derived from a healthy donor, the authors showed that MPC differentiation was not affected in patient-derived hiPSCs. However, the *PDX1* heterozygous point mutations impaired the differentiation of β-like cells and affected their response to glucose. A more severe effect was observed when artificially introducing the same point mutations in homozygosis (*i.e.* $PDX1^{P33T/P33T}$ and $PDX1^{C18R/C18R}$) in isogenic cell lines derived from the original control cell. Interestingly, this resulted in impaired *NKX6.1* induction in MPCs just in one of the cell lines ($PDX1^{P33T/P33T}$). Nevertheless, when differentiated towards insulin producing cells, both homozygous cell lines yielded a decreased number of β-like cells with impaired glucose response. The authors also generated additional isogenic lines carrying different heterozygous mutations in the *PDX1* transactivation domain, to generate a frame-shift mutation ($PDX1^{+/-}$). This created a more severe phenotype to the one observed in the patient-derived hiPSCs, leading to similar outcomes as obtained from the homozygous isogenic $PDX1^{P33T/P33T}$ point mutated cells. Further transcriptomic analyses of MPCs differentiated from these cell lines ascribed the observed effects to downregulation of key PDX1-bound genes including *MEG3* and *NNA*, which are involved in pancreas development and insulin secretion.

Taken together, these results illustrate how predisposition to develop diabetes can be provoked at the stage of pancreatic endocrine lineage development by genetic mutations on a gene that plays a key role at this timepoint. These anomalies could impair the glucose-responsive function of β-like cells through misregulation of genes involved in β cell development, maturation, and function. These results also emphasize that the choice between patient-derived hiPSCs or healthy donor hiPSCs with mutations artificially introduced, as well as the choice of the control cell line used, can affect experimental outcomes and their interpretations. In this context, patient-derived hiPSCs could carry additional mutations in non-coding regulatory regions and/or other genes which might further impair the *in vitro* differentiation outcomes. This effects have been elegantly exposed in a recent work by Gadue and colleagues (117), which will be discussed in more detail below, in the *GATA6* section of this review. In contrast, the use of healthy donor hiPSCs with mutations artificially introduced has the advantage of enabling the use of isogenic cell lines (*i.e.* non-mutated hiPSCs) to exclude additional effects of the genetic background.

RFX6

Lack of Rfx6 in mice blocks differentiation of all islet cell types, with the exception of pancreatic-polypeptide-producing cells, while *RFX6* mutations in humans result in PNDM (93, 100, 128). Modeling of the RFX6 requirement for human endocrine pancreas development has been addressed by Zhu et al. Their findings, in agreement with current knowledge, show a reduction of endocrine cell commitment from pancreatic progenitor cells derived from $RFX6^{-/-}$ mutant hPSCs (111).

In a more recent study, Trott et al. used hiPSCs derived from individuals with Mitchell–Riley syndrome (MRS) to specifically associate the role of *RFX6* mutations and the lack of pancreatic endocrine cells in a human model of pancreas development (118). X-ray microtomography of one of these patients confirmed the spectrum of congenital defects typical of MRS (loss of the pancreas body and tail), and exome sequencing identified a homozygous non-sense mutation in *RFX6*. hiPSCs derived from this patient and differentiated along the pancreatic cell lineage revealed that these cells efficiently differentiate into posterior foregut cells but exhibited a reduction in the pancreatic endoderm differentiation, which was accompanied by expression of genes associated with mesoderm differentiation. These findings indicate that RFX6 is crucial for maintaining the transcriptional program that specifies early pancreatic endoderm in humans.

NEUROG3

While loss of Ngn3 function has been associated with complete lack of pancreatic endocrine cells in mice (129), the phenotype in humans is variable [recently reviewed in (21)]. In this sense, while some patients with homozygous or compound heterozygous *NEUROG3* mutations show glycemic control into adulthood, indicating a functional endocrine pancreas, others present neonatal diabetes (96, 130, 131). A recent study suggests that each mutation could have unique effects on the structure and function of NGN3 (132). To further understand this divergence, the requirement of NGN3 for the generation of insulin-producing cells during human development has been addressed using hPSC differentiation. Zhu et al. reported that *in vitro* endocrine pancreatic differentiation of hPSCs with biallelic mutations in *NEUROG3* formed some insulin-producing cells (111), whereas another study reported a total lack of endocrine cells differentiated from *NEUROG3*$^{-/-}$ hPSCs (133). The latter work described that as little as 10% *NEUROG3* expression is sufficient for the formation of pancreatic endocrine cells, supporting that NGN3 is essential for endocrine pancreas development in humans. The divergence between differentiation protocols used in each laboratory and the influence of genetic background could explain the varied phenotypes observed between these two studies. Interestingly, a new adult mouse islet resident pancreatic endocrine progenitor cell population has been recently reported (134). These cells express the surface marker *Procr*, are *Neurog3* negative and, when isolated and co-cultured with endothelial cells, are able to give rise to islet-like clusters containing all endocrine cell types. Apparently, differentiation of this adult progenitor cell population into endocrine cells does not involve *Neurog3* expression, raising the intriguing question of whether such a population exists in humans and, if so, whether *in vitro* pancreatic differentiation from hPSCs is able to follow this "alternative" path for endocrine cell production. Such possibility could explain the divergence between different reports concerning the requirement of NGN3 in endocrine cell production. Taken together, these studies illustrate the complexity, as well as the potential, associated with hPSC differentiation for modeling the impact of genetic mutations on human development.

GLIS3

It has been reported that global *Glis3*$^{-/-}$ mice die of severe neonatal diabetes shortly after birth (135). Minor differences in gene dosage of *Glis3* produce substantive changes in the expression levels of *Neurog3* and *Ins1*, leading to a variable phenotype among the multiple *Glis3*-KO mouse lines (136). In agreement with these phenotypes, human biallelic mutations in *GLIS3* underlie a rare clinical syndrome, characterized by neonatal diabetes and congenital hypothyroidism (92).

The first report of the *in vitro* modeling for the requirement of GLIS3 in human pancreas development was provided by Zhu et al. These researchers did not find defects in pancreatic endocrine differentiation using *GLIS3*$^{-/-}$ mutant hESCs, when using a first generation *in vitro* pancreatic differentiation protocol that allows producing poly-hormonal cells (111).

More recently, Amin et al. developed an improved differentiation protocol that allowed the production of monohormonal β-like cells with enhanced functionality (64). Noteworthy, this protocol allowed the generation of robust *GLIS3* expression at the PDX1+/NKX6.1+ pancreatic progenitor cell stage, in contrast with previously reported protocols (52, 111). Using this improved protocol, they were able to demonstrate that differentiation of *GLIS3*$^{-/-}$ mutant hESCs presented impaired expression of pancreatic endocrine-associated genes, including *PDX1*, *NEUROD1*, *NKX6.1*, and *MAFA*. These cells also showed increased β-like cell death. These findings contrast with those reported by Zhu et al. (111). The difference could be explained by the improvements in the differentiation protocol, which allow a closer recapitulation of the differentiation steps to produce β-like cells. Furthermore, providing an illustrative example of the utility of the *in vitro* β cell differentiation protocols, these researchers performed a chemical screen that allowed the identification of a novel drug candidate that rescued mutant *GLIS3*-associated β-cell death both *in vitro* and *in vivo* (64).

HNF1A

Hnf1a has been shown to regulate the expression pattern of islet-specific genes involved in key functions of this tissue (137). In the mouse, while homozygous knockout (*Hnf1a*$^{-/-}$) results in insulin secretory defects and higher blood glucose concentrations, heterozygous knockout (*Hnf1a*$^{+/-}$) do not display this phenotype (138). This is in sharp contrast with the MODY3 pathology in humans, in which heterozygous mutations result in diabetes (139). In an attempt to elucidate the mechanisms by which dysfunctional HNF1A affects pancreatic development and/or β cell function, Gadue and colleagues have modeled MODY3 using CRISPR-Cas9 genome-edited hESCs and EndoC-BH human cell lines (109). Loss of HNF1A function was accomplished by deletion and premature termination in one or both *HNF1A* alleles, resulting in heterozygous and homozygous KO mutations. Their results suggest that HNF1A plays an essential role in endocrine cell development, as its loss leads to abnormal expression of genes related to β cell function and diabetes. Noteworthy, complete loss of HNF1A did not impair the production of pancreatic progenitors, but this factor was necessary for proper endocrine cell development as revealed by decreased expression of *PAX4*, and impaired insulin expression and secretion. Interestingly, HNF1A loss of function (deletion in one or both alleles of *HNF1A*) led to increased expression of α cell markers, including glucagon. The authors suggest that the increase found in α cells derived from this model system appears to be human-specific, since *Hnf1a* knockout mice do not display this phenotype.

Another key finding of this work was the identification of a previously unannotated human-specific long intergenic non-coding RNA (*lncRNA*). The *LINC01139*, designated *LINKA*, was shown to act as a downstream target of HNF1A. *In vitro* endocrine pancreatic differentiation of *LINKA*-deficient hESCs showed no effect on the production of pancreatic progenitors, but revealed a limited bias towards the production of α cells. Furthermore, β-like cells produced from *LINKA*-deficient hESCs showed a decrease in maximal respiration capacity to a similar extent as seen in the *HNF1A* heterozygous cells. Taken together, their findings point to a role for *LINKA* in the regulation of a

subset of HNF1A target genes with implications in cellular respiration. The *in vivo* relevance of *LINKA* for diabetes onset remains to be explored. Of note, a significant variability was observed in the expression changes among the hESC lines used in this study. These could be partially explained by the impact of the genetic background, which could lead to differences in the efficiency of differentiation protocol when applied to each cell line.

A more recent report was provided by Teo and colleagues (110). These researchers used MODY3 patient-derived hiPSCs to study the impact of a recently reported patient-specific heterozygous HNF1A$^{+/H126D}$ mutation (140). The authors used hiPSCs reprogrammed from a healthy donor and H9 hESCs as two independent wild type controls. Molecular dynamics simulations predicted that the H126D mutation could compromise DNA binding and gene target transcription. Indeed, RNA-seq and ChIP-seq analyses performed on MODY3 hiPSC-derived endocrine progenitors revealed that the expression of several HNF1A gene targets was affected by the mutation. An in-depth analysis of the effects on the β-like cells derived from HNF1A$^{+/H126D}$ hiPSCs demonstrated that the *HNF1A* mutation causes a GLUT2 deficiency, that is associated with reduced glucose uptake and ATP production. Their findings reveal the importance of HNF1A in regulating GLUT2 and several genes involved in the MODY3 pathology that may partly account for the lack of insulin secretion clinically observed in these patients. This report extends the findings reported by Cardenas-Diaz et al. (109) by revealing additional mechanisms triggered by the *HNF1A* mutations on the rest of the stimulus-secretion coupling pathway and on HNF1A transcriptional targets in human β-like cells. Noteworthy, Teo and colleagues performed RNA-seq and ChIP-seq at the endocrine progenitor cell stage. They did not found a differential regulation of the *LINC01139* (*LINKA*) at this stage, and unfortunately the expression of this lncRNA in β-like cells derived from HNF1A$^{+/H126D}$ hiPSCs is not reported. It remains to be elucidated whether *LINC01139* is also downregulated in the latter model. Potential discrepancies on the regulation of this lncRNA could be accounted by the different approaches followed in each work to evaluate the effects of HNF1A haploinsufficiency. On one hand Cardenas-Diaz et al. artificially introduced KO mutations by generating a genomic deletion leading to premature termination in one or both *HNF1A* alleles, and non-mutated isogenic cell lines were used as controls. This approach has the advantage of using an isogenic control cell line, which neutralizes contributions from the genomic background. However, the mutations introduced generate a strong HNF1A loss of function that might not appropriately recapitulate the mechanisms that take place in MODY3 patients. On the other hand, Teo and colleagues used MODY3 patient-derived hiPSCs carrying a mutation that causes an amino acid substitution (HNF1A$^{+/H126D}$) and used hiPSCs derived from a healthy donor and H9 hESCs as wild type controls. This approach has the advantage of using hiPSCs derived from patient cells, accounting for a closer model to the MODY3 disease. However, the use of non-isogenic hPSCs as controls does not allow accounting for potential effects derived from the different genomic backgrounds. As presented in more detail in the next section, these might introduce an additional bias in the differentiation outcome. In summary, further studies are required to elucidate whether deregulation of *LINC01139* plays a relevant role in MODY3 diabetes.

GATA 6

Mono allelic mutations in *GATA6* have been linked with pancreas agenesis in humans (141) while the knockout of the same gene has little effect on pancreatic development in the mouse. Indeed, only knockout of both *Gata4* and *Gata6* results in pancreatic agenesis (142, 143). Thus, GATA6 seems to have a different or at least a more extensive function in human development. To confirm this observation, Shi et al. used CRISPR/Cas9 to create hPSCs carrying frameshift mutations in *GATA6*, alone or in combination with *GATA4* mutations (115). Their results show that GATA6$^{+/-}$ haploinsufficiency alters pancreatic progenitor cell differentiation leading to a reduced number of glucose-responsive β-like cells. Given that heterozygous inactivating mutations in *GATA6* have been linked with pancreas agenesis, these findings suggest that the severity of the phenotype could vary according to additional genetic, epigenetic, and/or environmental factors that were not accounted by the differentiation process. Interestingly, the authors also describe dosage-sensitive requirements for GATA6 and GATA4 in the formation of both definitive endoderm and pancreatic progenitor cells, confirming the complex interplays between these factors observed in genetic studies in the mouse.

In another study, Tiyaboonchai et al. used hiPSCs derived from a patient with pancreatic agenesis associated with a heterozygous *GATA6* frameshift mutation, which leads to production of a truncated protein. These researchers also used CRISPR/Cas9 genome editing to introduce this mutation on both alleles of the same hiPSC line (114). Noteworthy, hiPSC lines with homozygous mutations failed to differentiate into endoderm. Re-expression of *GATA6* or other *GATA* family members restored this defect. The use of endodermal progenitor cell lines established from the hiPSC allelic series, which expressed *GATA6* at lower levels but *GATA4* and *GATA3* at higher levels, allowed bypassing the endoderm defect and focusing on pancreatic β cell differentiation. The authors found that all mutant lines were able to differentiate into pancreatic β-like cells, but the response to glucose in these cells was functionally defective. Also, they showed that the clear decrease in pancreas specification and β-like cell generation was associated with limited endogenous retinoic acid signaling during *in vitro* pancreas induction using the *GATA6* mutant cell lines.

Additional information was provided by Chia et al. who combined both gene-edited and patient-derived hPSCs to study the function of GATA6 (116). These authors found that *GATA6* heterozygous hPSCs show a limited reduction in endoderm formation, while *GATA6*-null hPSCs can only form mesoderm-like cells. Thus, GATA6 seems to be upstream of the

endoderm program in humans. Consistent with this hypothesis, genome-wide studies showed that GATA6 binds and cooperates with EOMES/SMAD2/3 to regulate the expression of master endoderm genes. In addition, the early deficit of GATA6$^{+/-}$ in definitive endoderm was accompanied by a significant reduction in PDX1+ pancreatic progenitors and C-peptide+ β-like cells. These findings show that, in humans, the formation of definitive endoderm and acquisition of pancreatic fate are exquisitely sensitive to *GATA6* gene dosage.

Taken together, the above-mentioned reports revealed different levels of requirement of GATA6 for pancreatic differentiation between protocols, labs and cell lines (**Table 2**). In this context, a very recent report by Gadue and colleagues provides an illustrative example which might help to understand this apparent divergence. These researchers generated a hiPSC line derived from a pancreatic agenesis patient, harboring a heterozygous 4 bp duplication in exon 2 of *GATA6* leading to a premature STOP codon, a genetically matched control line, and an identically artificially-mutated ESC line. Using these cell lines the authors identified a minor allele frequency of a SNP located downstream of *GATA6* which was associated with the level of expression of this gene (117). In their *in vitro* model, the expression of the GATA6 protein remained depressed in pancreatic progenitor cells even after correction of the coding mutation. Screening the regulatory regions of the *GATA6* gene in the patient cells and an additional pancreas agenesis hiPSC line revealed the above-mentioned SNP. Noteworthy, introducing this non-coding disease modifier SNP by CRISPR/Cas9 in control hESCs confirmed that it depressed *GATA6* expression in pancreas precursors. Thus, the phenotypic diversity found in *GATA6* heterozygous patients and the outcome of *in vitro* studies could be explained in part by this genetic variant.

The findings reported by Gadue and colleagues suggest that caution has to be taken when interpreting the results of monogenic diabetes modeling using patient-derived hiPSCs. Additional genomic variants might contribute to the *in vitro* differentiation outcomes, making it difficult to compare the results obtained by different groups. Nevertheless, some of the studies mentioned above did use the same hPSC line, including the original H9 line derived by JA Thomson and colleagues (144). In such cases, it is worth to underline that each group used different protocols of differentiation. Specific additives could compensate for the decrease in *GATA6* expression. For example, retinoic acid seems to support GATA6 function in pancreatic specification. Addition/increase of this morphogen could modulate the effect of *GATA6* haploinsufficiency. Taken together, these results illustrate the challenges and, at the same time, highlight the unique interest of investigating the function of key transcription factors in pancreatic development using hPSCs.

HNF4A

In mouse, it has been shown that full inactivation Hnf4a is embryonically lethal, while heterozygote knockout mice are normoglycemic and do not present diabetes features (145–147). In contrast, MODY1 patients carrying heterozygous mutations in *HNF4A* present diabetes due to impaired β cell function (148). Patient-derived hiPSCs have been recently used to address the potential mechanisms involved in this phenotype. Ræder and colleagues reported the use of hiPSCs derived from patients carrying a non-sense *HNF4A* mutation associated with MODY1 to study its effect on pancreas and β cell differentiation (105). Noteworthy, the mutation studied in this work (p. Ile271fs) generates a truncated HNF4A product from one of the alleles. The authors show that insulin-positive cells could be generated *in vitro* from these cells, suggesting that this human *HNF4A* mutation neither blocked the expression of the insulin gene nor the production of insulin-producing cells *in vitro*. However, they acknowledge that the insulin-producing cells derived are immature as a result of the β cell differentiation protocol *per se*, leaving open the possibility that HNF4A could have more subtle effects on the functionality of fully mature β cells.

In another study, Braverman-Gross et al. generated hiPSCs from MODY1 patients harboring a different non-sense mutation in the *HNF4A* gene and evaluated its differentiation along the pancreatic lineage (106). In this case, the mutation studied affects all *HNF4A* transcripts and impairs the protein dimerization and transactivation domains. Pancreatic progenitors differentiated from these cells exhibited an upregulation of other key pancreatic transcription factors, including *PAX6*, *NEUROD1*, and *NEUROG3*. The authors suggest that such gene expression increase could be a compensatory mechanism utilized by MODY1 cells to overcome the reduction in *HNF4A* expression. Interestingly, they also note that the differential expression of HNF4A target genes in posterior foregut progenitors derived from mutant cells is affected by the number of HNF4A DNA binding sites, its transcription start site distance, and the number of other transcription factor binding sites. Unfortunately, the authors of this work did not extend the differentiation protocol to evaluate proportion and functionality of β-like cells derived from these hiPSC samples.

MODY1 disease modeling was also more recently accomplished by Teo and colleagues using hiPSCs derived from patients with frameshift mutations that introduce a premature stop codon in *HNF4A*, leading to an unstable mRNA and overall lowered HNF4A levels (107). This mutation is the same one (p. Ile271fs) studied by Ræder and colleagues. Control hiPSC lines were derived from a non-diabetic patient family member. The resulting cell lines were differentiated into liver and pancreatic endocrine cells. Phenotypic analyses showed that *HNF4A* haploinsufficiency affects foregut endoderm gene expression signatures, contributing to long-term consequences on hepatic and pancreatic cell fates. While key developmental genes were perturbed by *HNF4A* haploinsufficiency at the pancreatic progenitor stage (including *HNF1B*, *PDX1*, *GATA4*, and *RFX6*), these mutant hiPSCs were still able to procure β-like cells expressing specific markers, including insulin and C-peptide. However, the β-like cells derived with the assayed *in vitro* protocol were not fully mature. More critical effects of *HNF4A* mutations taking place during the β cell maturation process or on already mature β cells could not be properly evaluated with the protocol described in this work.

Taken together, the results reported so far from hiPSC models used to study the effects of different *HNF4A* mutations suggest that the effects of such mutations might be more relevant at the functional level of the β cells produced. The generation of fully functional β-like cells from *in vitro* differentiation protocols still remains a challenge. Thus, evaluating the functionality of the β-like cells produced from control or patient-derived hiPSC cannot be appropriately assessed with the current differentiation protocols. On the other hand, it should be noted that while two of these studies evaluated the effects of the same mutation, the results described by Braverman-Gross et al. analyzed a different *HNF4A* mutation. These mutations lead to HNF4A loss-of-function through different mechanisms, thus potentially explaining the different outcomes obtained in each of the reports. Last, but not least, it should be kept in mind when using patient-derived hiPSCs that additional mutations in other genes or in *HNF4A* regulatory regions could also modulate the outcome of the *in vitro* differentiation experiments, as illustrated above for *GATA6*. To conclude, additional studies are necessary to address how *HNF4A* mutations cause MODY in humans, especially using the next generation of pancreatic differentiation protocols that improve the production of fully mature β-like cells.

HNF1B

Teo and colleagues established a well-controlled patient-derived hiPSC pancreatic differentiation model to elucidate the molecular mechanisms underlying MODY5 pancreatic hypoplasia (113). Differentiation of MODY5-hiPSCs into pancreatic progenitors showed that the HNF1B$^{S148L/+}$ mutation causes the up-regulation of several key endocrine pancreas-enriched transcription factors including *PDX1*. Pancreatic differentiation using these cells did not block *PDX1*, *PTF1A*, *GATA4*, and *GATA6* expression, suggesting that MODY5-mediated pancreatic hypoplasia in this case is mechanistically independent from the effect associated with these transcription factors. On the other hand, the point mutation in *HNF1B* caused an indirect reduction in the expression of the insulin gene activator *PAX6*, suggesting that loss of one copy of *HNF1B* in humans impairs β cell development and function. Although these findings are consistent with the potential occurrence of maturity-onset diabetes, they fail to uncover the mechanism by which *HNF1B* haploinsufficiency results in pancreatic hypoplasia.

To further address this question, we recently used an alternative hiPSC pancreatic differentiation model to elucidate the molecular mechanisms underlying HNF1B-associated diabetes (Khairi et al, manuscript submitted). To evaluate the transcriptional differences in the *HNF1B* haploinsufficient cells, we used bulk RNA-seq at several stages of the pancreatic differentiation protocol (from DE to β-like cells), immunofluorescence staining, and scRNA-seq at the MPC stage. Our analyses show that absence of HNF1B blocks the specification of the pancreatic fate from the foregut progenitor stage. In contrast, *HNF1B* haploinsufficiency allows differentiation of MPCs and the generation of functional β-like cells although at a lower frequency than the control isogenic cell line. We further report that *HNF1B* haploinsufficiency impairs cell proliferation in foregut progenitors and MPCs. Our results show that HNF1B plays a key role in the production and expansion of pancreatic progenitors and suggest that this factor could regulate the expression of several Hippo pathway components in MPCs. Thus, the level of HNF1B, combined with environmental stimuli, could define the number of pancreatic progenitor cells generated during development and therefore contribute to the susceptibility to diabetes during childhood/adulthood.

PTF1A

It has been described that homozygous inactivating mutations in *PTF1A* cause pancreatic and cerebellar agenesis (98). In agreement, *Ptf1a* null mice present a complete absence of exocrine pancreatic tissue, but all islet endocrine cell types are present until the late stages of embryogenesis (149). Zhu et al. reported the *in vitro* modeling of the PTF1A requirement for human pancreas development. Using *PTF1A*$^{-/-}$ hESCs and a first generation *in vitro* pancreatic differentiation protocol, these researchers did not find defects in pancreatic endocrine differentiation (111). This finding is in agreement with previous reports showing that *Ptf1a* is not required for the specification of Ngn3+ endocrine progenitors or the differentiation of mature β cells in mice (150).

The study of *PTF1A* regulation provides another example of how human *in vitro* pancreatic differentiation can guide the discovery of a developmental regulatory mechanism, in this case consisting in the identification of recessive mutations in a distal non-coding region (151). Identification of genetic mutations resulting in pancreatic agenesis can be challenging as these can be located in regulatory regions far away from known regulators. Accordingly, genome sequencing of a cohort of patients presenting pancreatic agenesis revealed several mutations in a distal non-coding region located >1 Mb upstream the *PTF1A* gene. Enhancer profiling in MPCs, derived *in vitro* from hPSCs, confirmed the functional importance of this regulatory sequence in humans (151). The mutation sites coincided with a FOXA2 binding site profiled by ChIP-seq in *in vitro* MPCs. Further mechanistic experiments performed *in vitro* confirmed that the targeted region acts as an enhancer in human MPCs, and that patient mutations affect PDX1 and FOXA2 binding. These findings allowed us to propose that the mutated enhancer region is in charge of triggering the early *PTF1A* expression in the gut region where the pancreas is specified. This study illustrates how human genetic and *in vitro* differentiation of hPSCs can be combined to define mechanisms driving developmental diseases.

INS

Balboa et al. generated a model based on hiPSCs from patients carrying *INS* mutations and engineered isogenic CRISPR-Cas9 mutation-corrected lines. These cells were differentiated to β-like cells (152). Using this model, the authors show that the *INS* mutations lead to accumulation of proinsulin misfolding, increased signs of ER-stress, and reduced proliferation in *INS*-mutant β-like cells compared with corrected controls. Following transplantation into mice, *INS*-mutant grafts presented reduced insulin secretion and further increased ER-stress, associated with

decreased *PDX1* expression and β cell size, as well as mitochondrial alterations. The authors conclude that neonatal diabetes-associated *INS*-mutations lead to defective β cell mass expansion, contributing to neonatal diabetes development.

In another recent study, Egli and colleagues generated hiPSCs from fibroblasts of a patient with PNDM and undetectable insulin at birth due to a homozygous mutation in the translation start site of the insulin gene (153). Their results show that the differentiation of *INS* mutant cells resulted in hormone-negative hiPSCs, and the correction of this mutation by CRISPR-Cas9 restored insulin production and secretion to levels comparable to those of wild type endocrine cells. The authors also demonstrate that the insulin-producing cells of corrected patient hiPSCs protect mice from diabetes, providing a proof-of-principle study for the use of replacement therapy as a treatment for monogenic diabetes.

STAT3

Saarimäki-Vire et al. used hiPSCs derived from a patient with PNDM and pancreatic hypoplasia to investigate the effects of an activating *STAT3* mutation on pancreatic development (154). Noteworthy, the mutation studied has been identified as the cause of PNDM in association with early onset autoimmunity. These authors demonstrate that the mutation in *STAT3* leads to premature endocrine differentiation through binding and direct induction of *NEUROG3* by the increased nuclear shuttling of the mutated protein. They also showed that correction of the *STAT3* mutation using CRISPR/Cas9 completely reversed the disease phenotype. These results demonstrate that, in addition to the early onset autoimmunity, the same mutation leads to a primary developmental defect in pancreatic organogenesis.

CONCLUSION AND FUTURE DIRECTIONS

The field of hPSCs has allowed important advances in our understanding of the molecular mechanisms underlying the different forms of monogenic diabetes. Indeed, the establishment of hPSC-based *in vitro* platforms offers a unique opportunity to study pancreas development and to investigate the pathophysiology underlying monogenic diabetes. This basic knowledge paves the way to the development of new treatments, not only for diabetes induced by genetic mutations, but also more broadly for personalized medicine therapies in the context of type I and type II diabetes. Nonetheless, several challenges require attention. Current *in vitro* β-like cell differentiation protocols have been markedly improved and may be sufficient to recapitulate several of the MODY phenotypes in the hPSC-based model. However, one of their greatest limitations remains the lack of metabolic maturation of the β-like cells derived. A solution to turn the differentiated cells into fully mature and functional β cells has been their transplantation in mouse to allow for the latest steps of cell differentiation to take place *in vivo*. Alternative methods involve culture in 3D and cell self-aggregation into islet-like clusters to produce β-like cells with improved functionality. The emergence of scRNA-seq is expected to lead to the identification of new markers involved in pancreatic β cell maturation, thus allowing improved benchmarking of the *in vitro* differentiation protocol outcomes. Also, scRNA-seq applied to human embryonic pancreatic tissue might provide additional insights into the developmental cues that differ among mice and humans. This will provide additional input to improve the *in vitro* differentiation protocols by modulating yet unknown signaling cues.

The other growing challenge is the divergence of results between different groups studying the same mutation/genes but using either different hPSC lines and/or different protocols. Indeed, genetic background and culture conditions can have a strong effect on phenotype, thus leading to different experimental outcomes. New hiPSC lines derived from monogenic diabetic patients continue to be reported. A very recent study described the generation of hiPSCs derived from MODY2 patients, but in this case its differentiation into the pancreatic lineage was not evaluated so far (108). Thus, there is a need to develop standard hiPSC lines which could be shared between laboratories. More importantly, the use of "universal" culture conditions to grow and to differentiate hPSC lines would be incredibly useful to allow the comparison of data generated and to precisely establish the importance of genetic background on the phenotype observed *in vitro*. Such standardization implies that culture conditions are fully described and shared between laboratories. The use of isogenic control hPSC lines is also essential and is helping to overcome the limitations related to the variability between lines, especially when compared with the use of family controls, which is inherent to differences in genetic background. Numerous studies have successfully used CRISPR/Cas9 tools to generate isogenic hPSC lines by introducing patient-specific mutations, editing genes in control-hPSC lines to investigate the implication of a single genetic variant on β cell differentiation and function. Here, we revisited the latest advances in the application of *in vitro* pancreatic cell differentiation from hPSCs to model several types of monogenic diabetes. Much work remains to be done to improve the modeling of monogenic diabetes but, as it stems from this review, *in vitro* pancreatic differentiation from hPSCs is definitely gaining momentum.

AUTHOR CONTRIBUTIONS

All authors reviewed the literature, wrote, and edited the paper. JIB prepared the table. LV and SAR-S conceptualized the review topic and contents, and approved the paper. All authors contributed to the article and approved the submitted version.

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Use of Induced Pluripotent Stem Cells to Build Isogenic Systems and Investigate Type 1 Diabetes

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Type 1 diabetes (T1D) is a disease that arises due to complex immunogenetic mechanisms. Key cell-cell interactions involved in the pathogenesis of T1D are activation of autoreactive T cells by dendritic cells (DC), migration of T cells across endothelial cells (EC) lining capillary walls into the islets of Langerhans, interaction of T cells with macrophages in the islets, and killing of β-cells by autoreactive $CD8^+$ T cells. Overall, pathogenic cell-cell interactions are likely regulated by the individual's collection of genetic T1D-risk variants. To accurately model the role of genetics, it is essential to build systems to interrogate single candidate genes in isolation during the interactions of cells that are essential for disease development. However, obtaining single-donor matched cells relevant to T1D is a challenge. Sourcing these genetic variants from human induced pluripotent stem cells (iPSC) avoids this limitation. Herein, we have differentiated iPSC from one donor into DC, macrophages, EC, and β-cells. Additionally, we also engineered T cell avatars from the same donor to provide an *in vitro* platform to study genetic influences on these critical cellular interactions. This proof of concept demonstrates the ability to derive an isogenic system from a single donor to study these relevant cell-cell interactions. Our system constitutes an interdisciplinary approach with a controlled environment that provides a proof-of-concept for future studies to determine the role of disease alleles (e.g. *IFIH1, PTPN22, SH2B3, TYK2)* in regulating cell-cell interactions and cell-specific contributions to the pathogenesis of T1D.

Keywords: type 1 diabetes mellitus, isogenic, human, beta cells, immunity, autoimmunity, induced pluripotent stem cells

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INTRODUCTION

Type 1 diabetes (T1D) is a debilitating autoimmune disease that is caused by T cell-mediated destruction of β-cells in the islets of Langerhans in the pancreas. This results in lifelong dependence on exogenous insulin and can lead to many complications that degrade quality of life for patients living with T1D. With the advent of genome-wide association studies (GWAS) a big picture view of the genetic contributions of T1D has come into focus. While human leukocyte antigen (HLA) loci, encoding the major histocompatibility (MHC) proteins, make up the bulk of genetic risk for T1D, over 100 non-HLA loci have been identified with polymorphisms that increase or decrease risk for T1D (1–12). However, studying the phenotypic outcome of these polymorphisms has proven difficult due to the heterogeneous nature of the human population and the inaccessibility of pancreatic tissue from individuals with signs of islet autoimmunity (i.e. islet autoantibodies) and individuals with clinical T1D.

Over the past half-century, immune and non-immune cells have been implicated in T1D (13–15). Key interactions involved in the pathogenesis of T1D in humans (**Figure 1**) include; A) the initial interactions between dendritic cells (DC) and β-cells where DC uptake β-cell antigens from dead, dying, or stressed β-cells, B) the activation of autoreactive $CD4^+$ and $CD8^+$ T cells by the β-cell antigen-loaded DC, C) the interaction between autoreactive $CD8^+$ T cells and the endothelial cells (EC) lining the capillaries that supply the islets of Langerhans as autoreactive $CD8^+$ T cells undergo transendothelial migration to access the islets, D) the targeting of β-cells for cell-mediated lysis by autoreactive $CD8^+$ T cells, and E) the *in situ* amplification of T cell responses by DC and macrophages in the islets. Genetics influence T1D pathogenesis and deleterious cellular function; however, genes implicated in T1D do not function in isolation. Numerous implicated genes cluster into pathways likely to create situations where different combinations of candidate genes can contribute equally to increased T1D risk (16). The development of a simple and flexible human model is essential to deconvolute genetic risk and make progress in understanding human T1D. We propose the use of models constructed from single-donor iPSC-derived cells. The utility of iPSC to investigate the relationship between rare and common gene variants and gene expression in pluripotent cells was recently highlighted (17).

Human iPSC exhibit similar gene expression patterns to human embryonic stem cells (ESC) and have the capacity to differentiate to all cell types of the human body. Generating iPSC and differentiating them to the cell types of interest (**Figure 1**) from a single donor preserves the genetic background and integrity of disease alleles, potentially making for an excellent isogenic model system to study T1D pathogenesis. In support of these isogenic systems, advances in stem cell technologies have

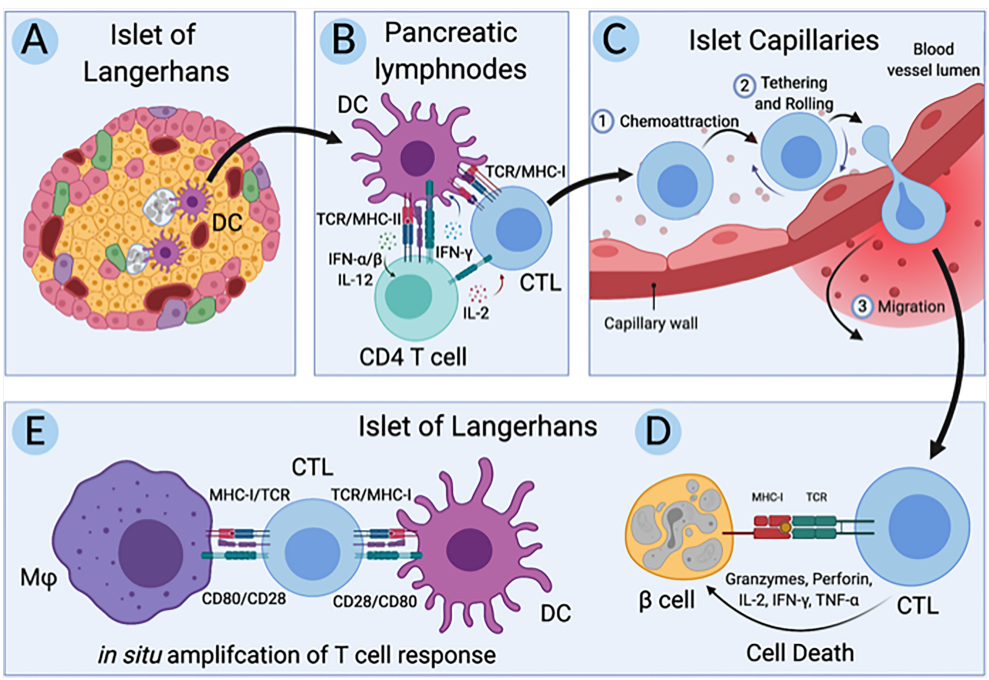

FIGURE 1 | Key cellular interactions involved in pathogenesis of T1D in humans. **(A)** First dendritic cells (DC) uptake antigen from dead, dying, and stressed β-cells in the islets of Langerhans in individuals at risk for type 1 diabetes (T1D). **(B)** Second, these β-cell-antigen-loaded DC migrate to the pancreatic lymph nodes where they present β-cell antigen to autoreactive $CD4^+$ and $CD8^+$ T cells, initiating an adaptive immune response. **(C)** Third, these autoreactive $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs), migrate back to the pancreas and undergo transendothelial migration across the endothelium lining capillaries that supply the islets of Langerhans. **(D)** Fourth, these CTLs directly target and lyse β-cells while **(E)** resident and infiltrating macrophages and DC amplify the CTL response *in situ*. Created with BioRender.com.

resulted in protocols allowing for differentiation of iPSC to functional replicas of cells essential for T1D, including the hard to access β-cells. Additionally, methods for engineering primary human T cells have advanced our understanding of immunity and autoimmunity (18–20). Here we selected three candidate iPSC lines to build the isogenic system, 1-018, 1-023, and 2395, that had previously been differentiated to stem cell derived beta-like cells (sBC), as this is the most time intensive and thus the rate limiting step for construction of these isogenic models (21–23). Based on HLA requirements and necessity of successful differentiation into pancreatic β-cells, endothelial cells, macrophages, and dendritic cells, line 2395 was selected. In addition, T cells were purified from this same donor. Employing the most recent and advanced methods for culturing and differentiating iPSC that have similar functionality to cells *in vivo*, we show that these iPSC-derived cells can be used for modeling and interrogating cell-cell interactions that are relevant to the pathogenesis of T1D (**Figure 1**). Utilizing these cellular approaches will allow investigators to examine relationships of genotype with cell function and cell-cell interactions.

MATERIALS AND METHODS

Reprogramming Somatic Cells to iPSC

The generation and characterization of the iPSC lines 1-018 and 1-023 was previously described (21–23). Derivation of iPSC from donor 2395 was as previously described (24). Briefly, peripheral blood was collected from donor 2395 under informed consent by the University of Florida Diabetes Institute Study Bank with Institutional Review Board approval (IRB201400703). $CD34^+$ peripheral stem cells were isolated from peripheral blood and expanded using the Complete Kit for Human Whole Blood $CD34^+$ Cells (Stem Cell Technologies, Vancouver, Canada) following the manufacturer's protocol. Expanded $CD34^+$ peripheral stem cells were transduced with the Sendai viral vector, SeVdp(KOSM)302L, which encodes the four reprogramming factors, OCT4, SOX2, KLF4, and c-MYC (20, 25, 26). Following transduction, cells were cultured on hESC qualified Matrigel- (Corning, Corning, NY) coated dishes in ReproTeSR medium (Stem Cell Technologies) and medium was changed daily until iPSC colonies were observed. iPSC colonies were manually isolated then expanded on Matrigel-coated dishes in mTeSR1 medium (StemCell Technologies, Vancouver, Canada).

Following reprogramming, donor 2395 iPSC were assessed for pluripotency using the StemDiff Trilineage Differentiation Kit (StemCell Technologies) following the manufacturer's protocol. iPSC were subjected to karyotyping to verify a normal karyotype by Cell Line Genetics (Madison, WI, USA). Undifferentiated donor 2395 iPSC were stained with anti-NANOG-AF647, anti-SSEA-4-PE, and anti-Oct-4-AF488 (BioLegend, San Diego, CA) using the Fixation/Permeabilization Solution kit from BD Biosciences (San Jose, CA) and analyzed on an Accuri C6 flow cytometer (BD Biosciences). Information for all antibodies used during the included studies are contained in **Supplementary Table 1**.

Differentiation of iPSC to Stem Cell-Derived Beta-Like Cells

The iPSC lines used in this study, 1-018, 1-023, and 2395, were initially selected because they had previously been differentiated to stem cell derived beta-like cells (sBC). The iPSC lines, 1-018 and 1-023, were differentiated to sBC as previously described (22). Donor 2395 iPSC were maintained on Matrigel-coated 6 well plates in mTeSR+ medium (StemCell Technologies) and differentiated to sBC as previously described (27, 28). Differentiation to sBC was carried out in suspension-based, low attachment suspension culture plates as described (29) or in an ABLE bioreactor magnetic stirring system (Reprocell, Beltsville, MD, USA) as follows. For differentiations, 70 - 80% confluent iPSC cultures were washed with PBS and incubated in TrypLE Express Enzyme (Gibco, Watham, MD, USA) for 8 min at 37°C followed by quenching with mTeSR+. Bioreactors were seeded at 0.5×10^6 cells/mL in mTeSR+ medium supplemented with 10 μM ROCK inhibitor. Bioreactors were placed on a magnetic stirring system set at 60 RPM in a cell culture incubator at 5% CO_2 to induce sphere formation for 48-72 hours. To begin the differentiation process, spheres were collected in a 50 mL Falcon tube, allowed to settle by gravity, washed once with RPMI (Gibco) + 0.2% FBS, and re-suspended in day 0 medium (RPMI containing 0.2% FBS, 1:5,000 ITS (Gibco), 100 ng/mL Activin-A (R&D Systems), and 3 μM CHIR (StemCell Technologies)). The differentiation medium was changed daily by letting spheres settle by gravity for 3-10 min. Approximately 80% of spent supernatant was removed by aspiration, fresh medium was added, and bioreactors were placed back on stirrer system. Differentiation media are as follows: day 1-2, RPMI containing 0.2% FBS, 1:2,000 ITS, and 100 ng/mL Activin A; day 3-4, RPMI containing 2% FBS, 1:1,000 ITS, and 50 ng/mL KGF (Peprotech); day 5, DMEM with 4.5 g/L D-glucose (Gibco) containing 1:100 SM1 (StemCell Technologies), 1:100 NEAA (Gibco), 1 mM Sodium Pyruvate (Gibco), 1:100 GlutaMAX (Gibco), 3 nM TTNPB, (R&D Systems), 250 nM Sant-1 (R&D Systems), 250 nM LDN (StemCell Technologies), 30 nM PMA (Sigma Aldrich), 50 μg/mL 2-phospho-L-ascorbic acid trisodium salt (VitC; Sigma); day 6, DMEM with 4.5 g/L D-glucose containing 1:100 SM1, 1:100 NEAA, 1 mM Sodium Pyruvate, 1:100 GlutaMAX, 3 nM TTNPB and 50 μg/mL VitC; day 7, DMEM containing 1:100 SM1, 1:100 NEAA, 1 mM Sodium Pyruvate, 1:100 GlutaMAX, 3 nM TTNPB and 50 μg/mL VitC; day 8-9, DMEM containing 1:100 SM1, 1:100 NEAA, 1 mM Sodium Pyruvate, 1:100 GlutaMAX, 200 ng/ml EGF and 50 ng/mL KGF; days 10-16, DMEM containing 2% fraction V BSA, 1:100 NEAA, 1 mM Sodium Pyruvate, 1:100 GlutaMAX, 1:100 ITS, 10 μg/ml Heparin (Sigma), 2 mM N-Acetyl-L-cysteine (Cysteine; Sigma), 10 μM Zinc sulfate heptahydrate (Zinc; Sigma), 1x BME, 10 μM Alk5i II RepSox (R&D Systems), 2 μM 3,3',5-Triiodo-L-thyronine sodium salt (T3; Sigma), 0.5 μM LDN, 1 μM Gamma Secretase Inhibitor XX (XXi) (AsisChem, Waltham, MA, USA) and 1:250 1 M NaOH to adjust pH to ~7.4;

day 17+, CMRL (Gibco) containing 1% BSA, 1:100 NEAA, 1 mM Sodium Pyruvate, 1:100 GlutaMAX, 10 µg/mL Heparin, 2 mM Cysteine, 10 µM Zinc, 1x BME, 10 µM Alk5i II RepSox, 1 µM T3, 50 µg/mL VitC, and 1:250 NaOH to adjust pH to ~7.4 (also referred to as maturation medium). All media contained 1x PenStrep (Gibco). Medium was changed every other day starting day 11. Validation and function of sBC, including staining for glucagon and insulin as well as glucose-stimulated insulin secretion were performed as previously described (22, 28, 29).

HLA Typing of iPSC

DNA was isolated from iPSC lines, 1-018, 1-023, and 2395, and subjected to genotyping at 974,650 unique loci using a custom SNP array. The Axiom Precision Medicine Research Array (ThermoFisher Scientific) was modified to include content from the ImmunoChip v2.0 (30) as well as previously reported credible T1D risk variants (10), and additional content to create the UFDIchip. UFDIchips were processed on an Affymetrix Gene Titan instrument with external sample handling on a BioMek FX dual arm robotic workstation. Data processing and quality control pipelines included standard quality control procedures at the SNP, sample, and plate levels using Axiom™ Analysis Suite 3.0 (ThermoFisher Scientific) set to the default stringency thresholds as recommended, as well as for genetic versus reported sex. The analysis pipeline also includes race imputation using EthSeq (31), relatedness using KING (32), and imputation to 40M SNPs using the Human Reference Consortium (version 1.1) with the Michigan Imputation Server (33). The array also includes dense coverage of the highly polymorphic HLA region allowing accurate imputation of HLA haplotypes to 4-digit resolution (34). SNP data are available upon request. HLA alleles arranged into putative extended haplotypes using the Allele Frequency Net Database (http://allelefrequencies.net/).

Differentiation of iPSC to Monocytes, Monocyte-Derived Macrophages, and Monocyte-Derived Dendritic Cells

Donor 2395 iPSC were differentiated to monocytes, monocyte-derived dendritic cells (moDC), and monocyte-derived macrophages (MDM), as described (35). Briefly, iPSC were differentiated to hematopoietic progenitors using the STEMdiff Hematopoietic Kit (StemCell Technologies) over 12 days. On day 12, hematopoietic progenitors were harvested and the hemogenic endothelium left behind in the 12-well plate was cultured in X-VIVO15 (Lonza, Basel, Switzerland) supplemented with β-mercaptoethanol (Gibco), Glutamax (Gibco), 25 ng/mL recombinant human IL-3 (rhIL-3, PeproTech, Rocky Hill, NJ), and 100 ng/mL recombinant human M-CSF (rhM-CSF, PeproTech). The hemogenic endothelium transitioned into monocyte factories and produced non-adherent $CD14^+$ monocytes that can be harvested repeatedly starting around day 21. Thereafter, $CD14^+$ cells are harvested every 2-7 days. Monocyte factories were replaced on Day 94. iPSC-monocytes were differentiated to iPSC-MDM as previously described (24, 35). Briefly, iPSC-monocytes were plated in high-glucose DMEM supplemented with 10% FBS (Genesee Scientific, San Diego, CA) or 10% human serum (Atlanta Biologicals, Flowery Branch, GA), 1000 U/mL Penicillin/streptomycin (Genessee Scientific), Glutamax, and 10 ng/ml of rhM-CSF for 7 days before use in experiments. iPSC-monocytes from Donor 2395 were differentiated to iPSC-moDC as previously described (36). Briefly, the $CD14^+$ iPSC-monocytes were plated in GMP DC medium (CellGenix, Freiburg im Breisgau, Germany) supplemented with Glutamax, 50 ng/mL recombinant human IL-4 (PeproTech), and 50 ng/mL recombinant human GM-CSF (PeproTech) and cultured for 6 days. On day 7, iPSC-moDC were plated for experiments.

Differentiation of iPSC to Endothelial Cells

Donor 2395 iPSC were differentiated to iPSC-derived endothelial cells (iPSC-EC) as previously described (37). This newly published protocol was adapted from a previously published protocol (38). Briefly, donor 2395 iPSC colonies were dissociated to single cells with Accutase (StemCell Technologies) and plated on Matrigel-coated plates at a density of 5×10^5 cells per cm^2 in mTESR1 medium (StemCell Technologies) supplemented with 10 ng/mL of the RHO/ROCK pathway inhibitor, Y-27632 (StemCell Technologies) on day 0. On day 1, the medium was changed to STEMdiff Mesoderm Induction medium (StemCell Technologies) and the medium was changed daily. On day 3, medium was replaced with STEMdiff APEL medium (StemCell Technologies) supplemented with 25 ng/mL BMP-4 (PeproTech) and 50 ng/mL VEGF (Peprotech). On day 5, the medium was replaced with fresh STEMdiff APEL medium supplemented with 25 ng/mL BMP-4 and 50 ng/mL VEGF. On day 7, $CD34^+$ cells were enriched with the EasySep Human Cord Blood CD34 Positive Selection Kit II (StemCell Technologies) without using the RosetteSep™ Human Cord Blood CD34 Pre-Enrichment Cocktail. The enriched iPSC-derived $CD34^+$ cells were plated at a density of 3.5×10^5 cells per cm^2 onto fibronectin (Millipore) coated dishes (5 ng/cm^2) and cultured in Endothelial Growth Medium 2 (EGM2, Lonza) supplemented with 50 ng/mL VEGF with medium changes every other day until they reached confluency. After the 1st passage, cells were cultured in regular EGM2 without extra VEGF and split at a 1:3 ratio onto fibronectin-coated plates (5 ng/cm^2) as they approached confluency. iPSC-EC were cryopreserved in 0.5mL of CryoStor CS10 Freeze Media (BioLife Solutions, Bothell, WA) at 1×10^6 cells/vial according to the manufacturer's protocol. Phase contrast photomicrographs of 2395 iPSC-EC were collected using an EVOS FL Cell Imaging System (Fisher Scientific).

Generation of $CD8^+$ T Cell Avatars From Donor 2395

Antigen-specific $CD8^+$ T Cell avatars were produced and validated as previously published (20). Briefly, $CD8^+$ T cells were isolated from peripheral blood of donor 2395 *via* negative selection using the RosetteSep Human $CD8^+$ T Cell Enrichment Cocktail (StemCell Technologies) according to the manufacturer's protocol. Naïve $CD8^+$ T cells ($CD45RA^+CD45RO^-$) were isolated by FACS with a

FACSAria III Cell Sorter (BD Biosciences). Naïve $CD8^+$ T cells were activated for two days with Human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, Waltham, MA). After two days, cells were transduced with lentiviral vectors, either pCCL.IGRPopt.eGFP, encoding a TCR recognizing an HLA-A*02-01-restricted epitope derived from the T1D-relevant autoantigen, glucose-6-phosphatase 2 (G6PC2) commonly referred to as islet-specific G6CP-related protein (IGRP) (39, 40) or LV.Mart1.TCR.RK, encoding a TCR recognizing an HLA-A*02-01-restricted epitope derived from the non-T1D-relevant auto-antigen, MART-1 (18, 19). Following transduction, the IGRP and the MART-1 CD8+ T cell avatars (IGRP avatars and MART-1 avatars) were expanded for 7 more days then cryopreserved.

Co-Culturing Donor 2395 iPSC-moDC and iPSC-MDM With Donor 2395 $CD8^+$ T Cells

To verify the ability of iPSC-moDC and iPSC-MDM to induce antigen-specific T cell expansion, iPSC-moDC and MDM were treated with 10 ng/mL of LPS (Sigma-Aldrich, St. Louis, MO) and 10 ng/mL of IFNγ (Peprotech) for 48 hours. After iPSC-moDC and MDM were activated for 48 hours, they were loaded for 2 hours with both CEFX Ultra SuperStim Pool MHC-I and MHC-II Subsets (JPT innovative peptide solutions, Berlin, Germany), for a total of 80 MHC Class I restricted epitopes and 68 MHC Class II restricted epitopes derived from a broad range of common human viruses, as well as *Clostridium tetani* and *Toxoplasma gondii*. After 2 hours, iPSC-moDC and MDM were washed with T cell expansion medium (RPMI supplemented with 10% HyClone FBS (GE Healthcare, Chicago, IL, USA), 1000 U/mL Penicillin/streptomycin, 1mM Sodium Pyruvate (Corning), 10mM HEPES (Corning), nonessential amino acids (Corning), Glutamax, and β-mercaptoethanol) and the medium was replaced with T cell expansion medium. Freshly isolated total T cells from donor 2395 were stained with CellTrace CFSE (ThermoFisher), added to the CEFX peptide-loaded iPSC-moDC or MDM and co-cultured for 5 days. After 5 days, expansion of total T cells was analyzed *via* flow cytometry on an Accuri C6 Flow Cytometer.

In Vitro Hydrodynamic Flow Chamber Adhesion Assay

The *in vitro* hydrodynamic flow chamber adhesion assay was performed as previously described (15, 41). Briefly, iPSC-EC were grown in monolayers to 95-100% confluence on a 0.2 mm Luer μ-slide (channel slide) pre-coated with fibronectin. The iPSC-EC monolayers were then loaded with either vehicle solution, $IGRP_{265-273}$ (the antigenic peptide recognized by the IGRP avatars), or Melan-A_{26-35} (the antigenic peptide recognized by the MART-1 avatars) at a final concentration of 10 μg/ml and incubated overnight. The next day, the iPSC-EC-containing channel slide(s) were loaded on the stage of a confocal microscope heated to 37°C. Ten thousand MART-1 or IGRP avatars labeled with 200 nM CellTracker Green (Molecular Probes, Eugene, OR) were added to the channel slide and allowed to settle for 30 minutes. HBSS buffer with $MgCl_2$ and $CaCl_2$ was then flowed across the channel slide by a syringe-driven pump at a series of increasing flow rates ranging from 1 to 80 dyne/cm^2. After passing the buffer through the channel slide for 1 minute for each shear flow rate, bright field and fluorescent images of the slide were acquired by confocal microscopy. T cells that remained attached after each flow rate were then counted using ImageJ software (42).

Chromium Release Assay

The chromium release assay to assess antigen-specific targeting of sBC or the immortalized human β-cell line, BetaLox5 cells (βL5), by donor 2395 avatars was performed as previously described (20, 43). Briefly, sBC clusters from donor 2395, 1-018, or 1-023 were dispersed in enzyme-free Cell Dissociation Buffer (Gibco) for 10 minutes at 37°C with gentle pipetting. Dispersed sBC were plated at 40,000 cells/well or βL5 cells were plated at 10,000 cells/well in 96-well flat-bottom plates. Target cells were loaded with Melan-A_{27-35}. Target cells were then labeled with ^{51}Cr at 1 μCi/well for 3 hours and then washed 3 times with culture medium. Donor 2395 MART-1 avatars were added at effector to target (E:T) ratios of 0:1, 5:1, and 10:1 and co-cultured for 16 hours. Afterwards, the supernatants were collected, cells were lysed in 2% SDS, and cell lysate was collected. ^{51}Cr release was measured with a gamma counter and specific lysis was calculated as follows:

$$\%Specific\ Lysis = Experimental\frac{(\#Release)}{(\#Release)+(\#Lysate)} - Spontaneous\frac{(\#Release)}{(\#Release)+(\#Lysate)}$$

where spontaneous release is the 0:1 E:T ratio and experimental release is measured in the 5:1 and 10:1 E:T ratios.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism version 8.4.3 (San Diego, CA) using unpaired Student's t test, one-way ANOVA, or two-way ANOVA, as indicated in the figure legends.

RESULTS

Reprogramming Donor 2395 $CD34^+$ Peripheral Stem Cells to iPSC

Our proposed isogenic system for T1D (**Figure 1**) requires constant supplies of β-cells, DCs, T cells, endothelial cells, and macrophages from the same donor. The major goal of this effort was to determine if iPSC lines had potential to differentiate into all of these cell types allowing construction of a fully isogenic system. For the work described here all of these cell types, except for T cells, were to be differentiated from donor iPSC lines. Before working with these iPSC lines, they were all verified to express pluripotency markers and have a normal karyotype. iPSC from lines 1-023 and 1-018 were validated and published previously (21–23). iPSC generated from donor 2395 showed similar morphology to normal hESCs (**Figure 2A**) and expressed the pluripotency markers, Oct4, SSEA-4, and NANOG (**Figure 2B**). 2395 iPSC were pluripotent, they differentiated into $Nestin^+$ ectoderm, $FoxA2^+Sox17^+$ endoderm, and $NCAM^+Brachyury^+$ mesoderm (**Figure 2C**).

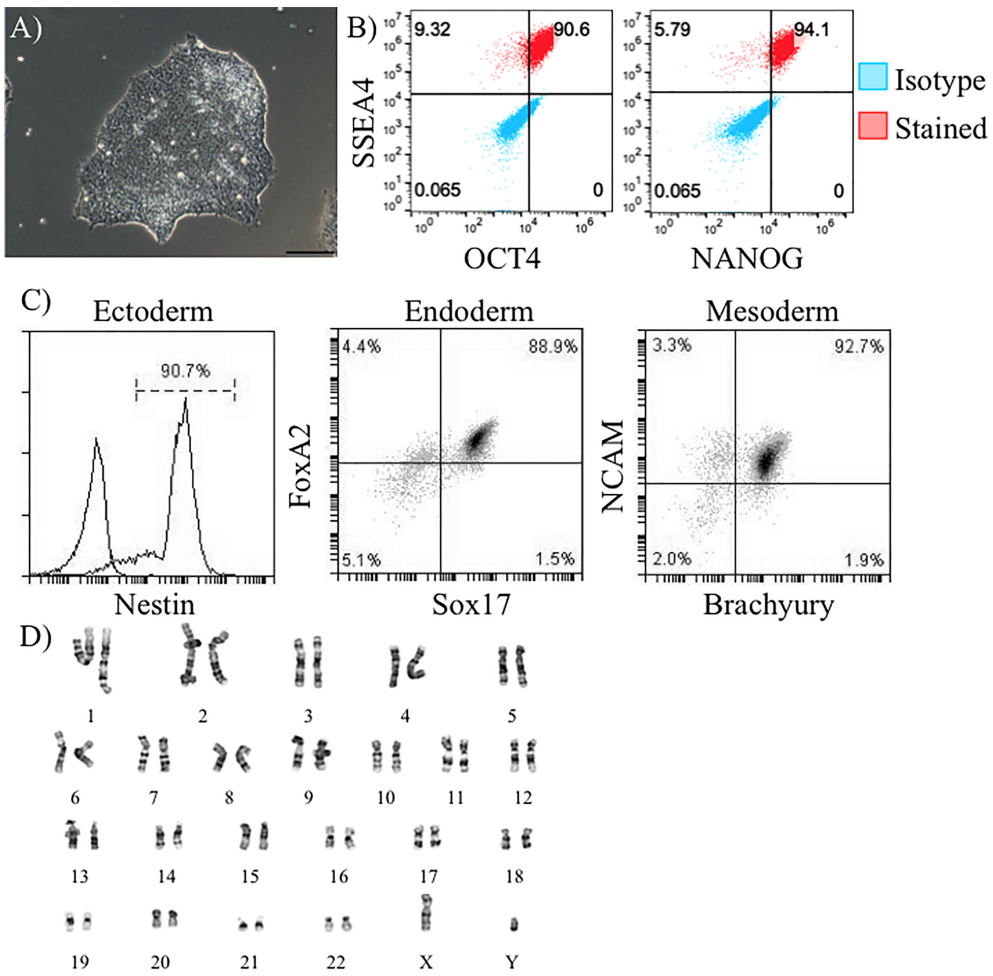

FIGURE 2 | Verification of pluripotency of donor 2395 iPSC. **(A)** A phase contrast image of an iPSC colony from donor 2395 showing densely packed cells with a defined border characteristic of iPSC. **(B)** Donor 2395 iPSC are positive for Oct4, SSEA4, and NANOG expression. **(C)** Donor 2395 iPSC express Nestin when differentiated to ectoderm, express FoxA2 and Sox17 when differentiated to endoderm, and express NCAM and Brachyury when differentiated to mesoderm. **(D)** Karyotypic analysis of donor 2395 iPSC reveals a normal karyotype.

Karyotyping analysis of 2395 iPSC showed a normal chromosome count of 46,XY (**Figure 2D**).

Donor 2395 iPSC Differentiate Into Insulin Producing Beta-Like Cells That Respond to Glucose

β-cells participate in the pathogenesis of T1D by shedding antigen (**Figure 1A**) to initiate the autoimmune response and are also targeted for destruction by the immune system in T1D (**Figure 1D**). Any fully isogenic system to study these steps in T1D (**Figure 1**) will require a renewable source of islets or β-cells. Stem cell-derived β-cells (sBC) can fulfill this need. With published protocols for differentiation of β-cells available, we proceeded to differentiate iPSC from the three lines (1-018, 1-023, and 2395) into sBC. Lines 1-018 and 1-023 have previously been differentiated into validated sBC in islet like clusters with expression of c-peptide and secretion of insulin (22, 44). iPSC from line 2395 were differentiated to sBC by first differentiating to definitive endoderm (DE). Cultures were only moved forward if they reached >80% $SOX17^+$ at the DE stage (**Figure 3A**). At the β-like stage, cells reached 28.1 ± 3.0% $insulin^+glucagon^-$ (**Figures 3B, C**; n = 3 separate differentiations). Fluorescent micrographs (**Figure 3D**) demonstrate an islet-like cluster of sBC from 2395 with cells expressing NXK6.1, PDX-1, and c-peptide. To verify that the sBC were functional, those differentiated from 1-018, 1-023, and 2395 were subjected to static glucose-stimulated insulin secretory assays and compared to primary human islets. Basal insulin release at 3mM glucose in human islets [2.3 ± 1.8 μg insulin/10 ng DNA (n=6)] was higher than that observed from all three sBC clusters [1-023 (0.051 ± 0.043 μg insulin/10 ng DNA (n=3)], 2395 [0.04 ± 0.01 μg insulin/10 ng DNA (n=6)], and 1-018 [0.007 ± 0.005 μg insulin/10 ng DNA (n=3)]. Clusters from all three lines secreted insulin, albeit less than the average stimulation index of primary islets (**Figure 3E**).

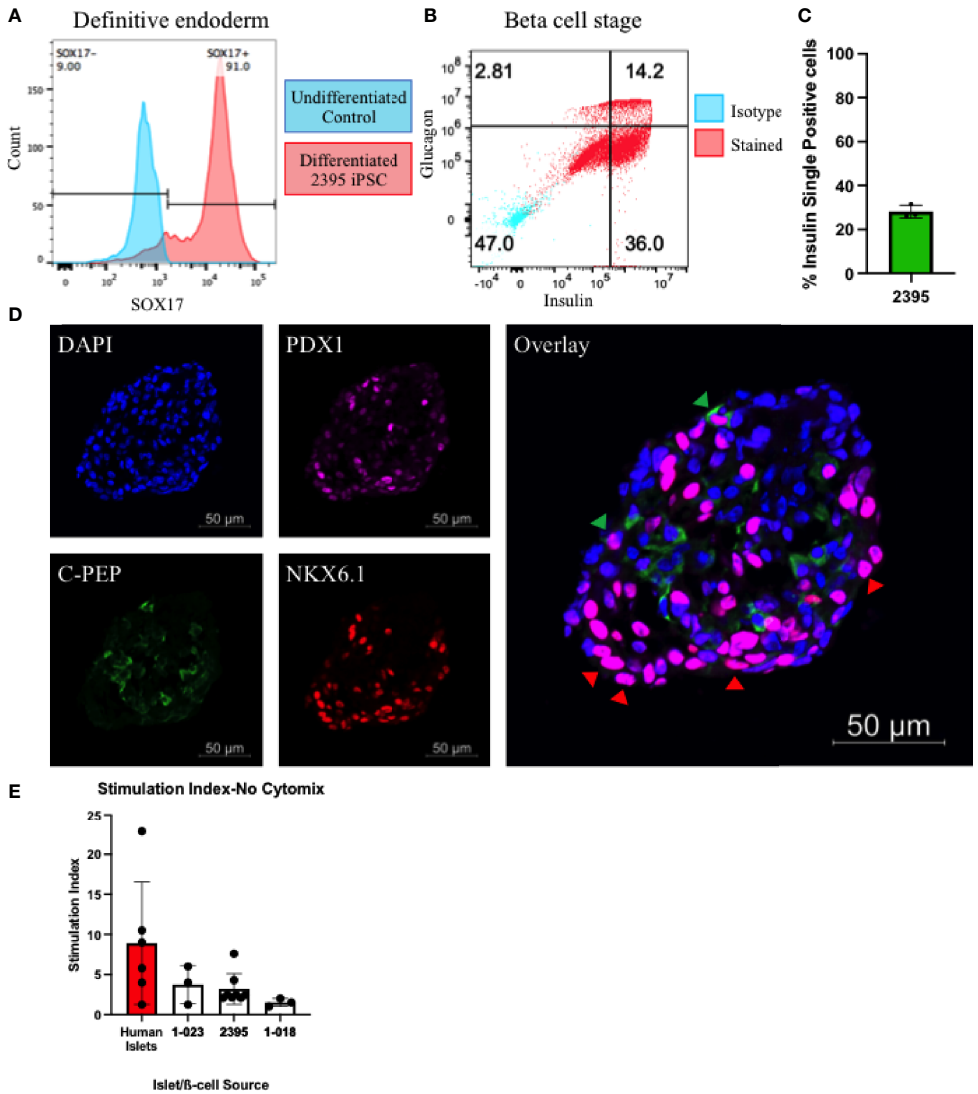

FIGURE 3 | Donor 2395 iPSC differentiate to stem cell derived beta-like cells (sBC). **(A)** Donor 2395 iPSC upregulate SOX17 when differentiated to definitive endoderm. **(B)** When donor 2395 definitive endoderm is differentiated to the β-cell stage, many polyhormonal (insulin$^+$glucagon$^+$) and monohormonal (insulin$^+$) cells arise. **(C)** The percent of insulin$^+$glucagon$^-$ cells from 3 repeats of 2395 sBC differentiations. **(D)** An sBC from donor 2395 was fixed, labeled with DAPI, stained with antibodies against PDX1, C-peptide, and NKX6.1 revealing some C-peptide/NKX6.1 double positive β-cells (green arrow heads) as well as many NKX6.1/PDX1 double positive cells (red arrow heads). **(E)** Donor 2395 sBC as well as iPS-β-cells from lines 1-018 and 1-023 exhibit glucose-stimulated insulin secretion. Stimulation index = insulin secretion in 16.7mM glucose/insulin secretion in 3mM glucose.

While there were no significant differences, 1-023 and 2395 outperformed clusters from 1-018 on average. In summary these lines differentiated into glucose-responsive sBC allowing a first step towards building *in vitro* systems for studying the interactions of isogenic immune cells with β-cells.

HLA Typing of iPSC 1-018, 1-023, and 2395

Class II HLA alleles confer the strongest genetic risk for pathogenesis of T1D, highlighting the importance of CD4$^+$ T cells. CD8$^+$ T cells are the predominant cell in human insulitis emphasizing that HLA Class I alleles play a major role in T1D development. HLA Class I regulates many of the key steps thought to be vital for T1D initiation such as T cell activation and targeted deletion of pancreatic β-cells (**Figure 1**). For these reasons it is essential that iPSC harbor useful HLA alleles for the study of T1D. HLA alleles were determined for all three iPSC lines (**Table 1**). The studies here will focus on the interactions of HLA-A*0201 restricted CD8$^+$ T cell avatars (20) with iPSC-derived cells expressing HLA-A*0201 with cognate peptide. Lines 2395 and 1-023 carry HLA-A*0201 while 1-018 does not (**Table 1**). Therefore, we prioritized the former two iPSC lines for further studies.

TABLE 1 | iPSC Donor Demographics and HLA Alleles for lines 1-018, 1-023, and 2395.

Donor	Age at Study	Sex	T1D Status	Age at Onset	HLA-A	HLA-B	HLA-DQA1	HLA-DQB1	HLA-DRB1
1-018	32	Female	T1D	10	0101	1801	0301	0302	0402
					3101	7301	0301	0201	0405
1-023	23	Male	Control	Not applicable	0201	3501	0102	0502	1101
					6801	5101	0501	0301	1506
2395	52	Male	Control	Not applicable	2901	4403	0201	0202	0701
					0201	4002	0505	0301	0701

Alleles are arranged into putative extended haplotypes using the Allele Frequency Net Database (http://allelefrequencies.net/).

Donor 2395 iPSC Efficiently Differentiate to Monocytes, MDM, and MoDC

While the initiating events in T1D are not decisively characterized, initiating a productive T cell response requires at least two signals during the interaction of APC with T cells (**Figure 1B**). The first signal is binding of the TCR on the T cell to its cognate antigen/MHC complex on an APC and the second signal is the costimulatory signal in the form of co-receptors on the T cell binding their respective ligands on an APC (e.g. CD28 binding CD80/CD86, CD27 binding CD70, CD226 binding CD155, OX-40 binding OX-40L, etc.) (45, 46). APC such as DC and macrophages serve to initiate T cell responses in the lymph nodes and enhance T cell responses at sites of inflammation. In order to model interactions between macrophages and T cells or DC and T cells, we differentiated donor 2395 iPSC to monocytes, then MDM and moDC (**Figure 4**). Similar to primary classical peripheral blood monocytes, these iPSC-monocytes are $CD14^+CD64^+CD16^-$ (**Figure 4A**). Also similar to their primary counterparts, iPSC-MDM are $CD11b^+CD68^+$ (**Figure 4B**) and iPSC-moDC are $MHC\text{-}II^+CDllc^+$ (**Figure 4C**). Attempts were made to differentiate the iPSC line, 1-023, to monocytes however they never produced $CD34^+$ hematopoietic progenitors following hematopoietic differentiation and they also never produced $CD14^+$ monocytes following monocyte induction. Due to the inability of 1-023 iPSC to differentiate into Monocytes, MDM, and MoDC, we prioritized iPSC line 2395 for the remaining studies.

Donor 2395 iPSC-moDC and iPSC-MDM Elicit Donor 2395 $CD4^+$ and $CD8^+$ T Cell Expansion in an Antigen Specific Manner

To determine if iPSC-moDC and MDM are able to elicit an antigen specific $CD4^+$ and $CD8^+$ T cell response, iPSC-moDC or MDM from donor 2395 were activated with LPS/IFNγ, loaded with CEFX Ultra SuperStim Pool MHC-I and MHC-II subsets, and co-cultured with CFSE (ThermoFisher) stained total T cells from donor 2395. When 2395 total T cells were cultured with iPSC-MDM alone (**Figure 4D**; closed black circles), or LPS/IFNγ-activated iPSC-MDM (**Figure 4D**; open black circles) there was little T cell expansion observed. However, when 2395 total T cells were cultured with CEFX peptide-loaded MDM (**Figure 4D**; closed red squares), or LPS/IFNγ-treated, CEFX peptide-loaded iPSC-MDM (**Figure 4D**; open red squares), there was significantly more expansion than when they were cultured with iPSC-MDM without peptide loading. iPSC-moDC also induced T cell expansion in an antigen specific manner. When total T cells were cultured with peptide-loaded iPSC-moDC (**Figure 4E**; closed red diamonds), expansion was increased compared to T cells cultured with iPSC-moDC alone (**Figure 4E**; closed black triangles). However, when iPSC-moDC were activated with LPS/IFNγ first, there was no significant difference in T cell expansion when iPSC-moDC were peptide loaded (**Figure 4E**; open red diamond) compared to iPSC-moDC without peptide loading (**Figure 4E**; open black triangles).

Donor 2395 $CD8^+$ T Cell Avatars Bind to Donor 2395 EC in an Antigen Specific Manner

After T cells are activated, they must traffic to the islets of Langerhans. To exit the islet capillaries and enter the islets, T cells must migrate across the endothelial cell (EC) layer lining the insides of the capillaries in a process called extravasation [(47) and **Figure 1C**]. In this process, T cells first migrate to the site of inflammation *via* chemotaxis, they then must tether and roll across activated ECs before adhering, and finally transmigrating across the epithelium (47). To model the interactions of $CD8^+$ T cells with vascular endothelium, we assessed $CD8^+$ T cell avatars adhesion to an antigen-loaded iPSC-EC monolayer using cells from the same donor. As described recently (37), 2395 iPSC differentiate into iPSC-EC with morphology (**Supplementary Figure 1A**), cell surface marker profile (**Supplementary Figure 1B**), and functional capabilities similar to human aortic endothelial cells (HAEC). In this study 2395 iPSC efficiently differentiated into iPSC-EC with high a percentage of the cells being double positive for both CD31 and CD144 (84% ± 8, n=4). Here, an *in vitro* hydrodynamic flow chamber adhesion assay was performed with monolayers of iPSC-EC from donor 2395 and either IGRP avatars or MART-1 avatars from 2395. When the 2395 iPSC-EC monolayer was not presenting antigenic peptide, the 2395 IGRP avatars failed to adhere strongly and were displaced at higher laminar flow rates (**Figure 5A**; black). Similarly, when the 2395 iPSC-EC monolayer was pulsed with the peptide antigen recognized by the MART-1 TCR, Melan-$A_{26\text{-}35}$ (EAAGIGILTV), 2395 IGRP avatars detached at higher flow rates (**Figure 5A**; red). In contrast, 2395 IGRP avatars formed strong adhesions with the $IGRP_{265\text{-}273}$ peptide-pulsed iPSC-EC monolayer and remained attached at higher flow rates (**Figure 5A**; gray). We confirmed the system's utility with a

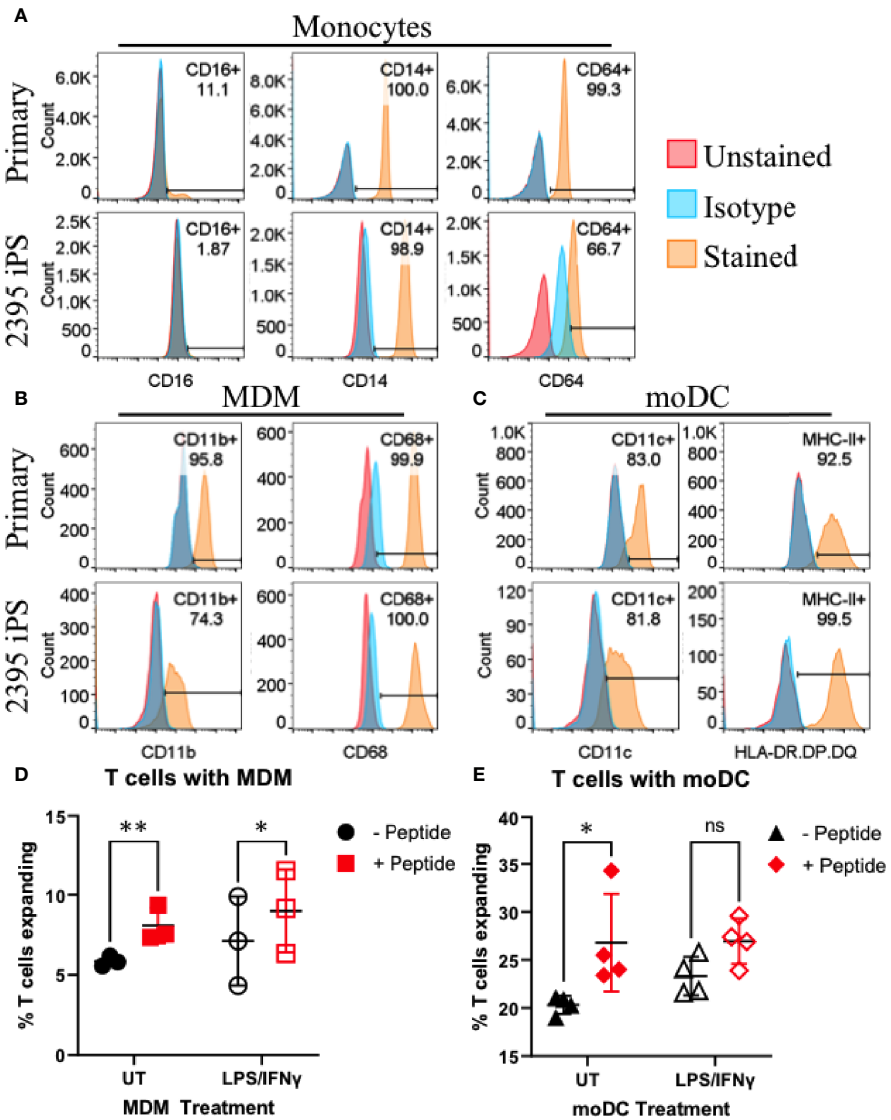

FIGURE 4 | Donor 2395 iPSC differentiate efficiently to monocytes, monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (moDC) and induce antigen specific $CD4^+$ and $CD8^+$ T cell expansion. **(A)** Donor 2395 iPSC-derived monocytes are $CD16^-$, $CD14^+$, and $CD64^+$ similar to primary classical monocytes isolated from blood. **(B)** Donor 2395 iPSC-derived MDM express CD11b and CD68 similar to primary MDM. **(C)** Donor 2395 iPSC-derived moDC express CD11c and MHC-II similar to primary moDC. **(D)** iPSC-MDM expand Total T cells in an antigen specific manner. When antigen (CEFX peptide pools) is present, there is a significantly increased expansion of T cells compared to when Total T cells are cultured with APC alone (2-way ANOVA **p = 0.0011 with 3 experimental repeats). **(E)** iPSC-moDC expand Total T cells in an antigen specific manner. When antigen is present, there is a significantly increased expansion of T cells compared to when Total T cells are cultured with APC alone (2-way ANOVA *p = 0.0104 with 4 experimental repeats). Significance shown on the graphs were from *post-hoc* analysis utilizing the Šidák method to correct for multiple comparisons. * indicates that $p < 0.05$, ** indicates that $p < 0.01$, and ns indicates not significant.

second TCR and antigen, 2395 MART-1 avatars and the peptide it recognizes, Melan-$A_{26\text{-}35}$. Using the laminar flow system without peptide the MART-1 avatars failed to form firm adhesions with the iPSC-EC monolayer (**Figure 5B**; black). Addition of $IGRP_{265\text{-}273}$ did not facilitate adhesion as this peptide is not recognized by the MART-1 avatars (**Figure 5A**; gray). In contrast, 2395 MART-1 avatars made firm adhesions with the Melan-$A_{26\text{-}35}$ peptide-pulsed iPSC-EC monolayer as observed by binding at higher flow rates (**Figure 5A**; red). As vascular adhesion interactions are critical for T cell trafficking into pancreatic islets for targeted destruction of β-cells in T1D (15, 48–50), this proof-of-concept study demonstrates the utility of an antigen-dependent system for assessing interactions of T cells with endothelial cells. The ability to model these interactions with human cells allows for intensive interrogation of genetics regulating vascular inflammation or therapeutics meant to disrupt lymphocyte adhesion with vascular endothelial cells (**Figure 1C**).

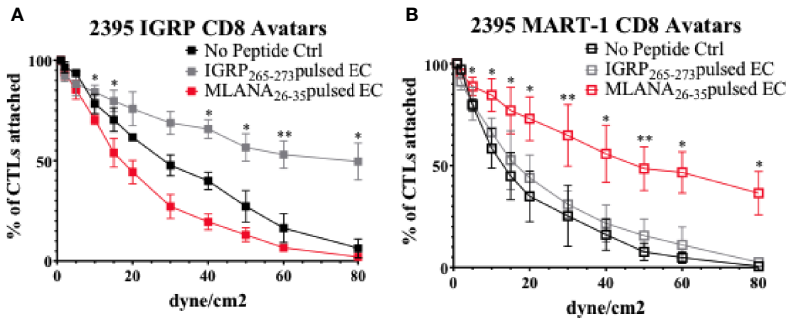

FIGURE 5 | Donor 2395 CD8$^+$ T cell avatars exhibit antigen specific binding to donor 2395 iPS-EC. **(A)** When donor 2395 IGRP CD8$^+$ T cell avatars are flowed across a donor 2395 iPSC-derived endothelial cell (iPS-EC) monolayer loaded with IGRP peptide, the IGRP CD8$^+$ T cell avatars adhere more than MART-1 CD8$^+$ T cell avatars or when the iPS-EC is not loaded with peptide. **(B)** When donor 2395 MART-1 CD8$^+$ T cell avatars are flowed across a donor 2395 iPSC-derived endothelial cell (iPS-EC) monolayer loaded with Melan-A_{26-35}, the MART-1 CD8$^+$ T cell avatars adhere more than IGRP CD8$^+$ T cell avatars or when the iPS-EC is not loaded with peptide. All *in vitro* hydrodynamic flow assay experiments were repeated 3 times. Significance shown on the graphs were from *post-hoc* analysis utilizing the Fisher's LSD test. * indicates that $p < 0.05$ and ** indicates that $p < 0.01$.

Donor 2395 CD8$^+$ T Cell Avatars Efficiently Target Donor 2395 sBC in an Antigen Specific Manner

To determine if sBC are able to be targeted by CD8$^+$ T cells in an antigen specific manner, a cell mediated lymphocytocixity (CML) assay was run with dispersed clusters of sBC differentiated from 1-023, 2395, or 1-018 as targets. The HLA-A*0201$^+$ human βL5 cell line and dispersed HLA-A*0201$^+$ human islet cells were used as positive controls (**Figure 6**). These groups were co-cultured with donor 2395 T cell avatars expressing a T cell receptor that recognizes a peptide from IGRP in the context of HLA-A*0201 (39, 40). The 2395 IGRP-avatars were able to target and kill the HLA-A*0201 expressing βL5 and primary human islets (**Figure 6A**). In addition, cells differentiated from 1-023 and 2395 were lysed by the avatars (**Figure 6A**) as they carry HLA-A*0201 (**Table 1**). Cells from 1-018 were not killed by the avatars (**Figure 6A**) as these cells do not have the correct HLA restriction (**Table 1**). We also assessed the ability of 2395 MART-1 T cell avatars that recognize a non-β-cell peptide derived from Melan-A_{26-35} (20, 51) to target and destroy cells that were pulsed with this Melan-A peptide or left untreated. MART-1-avatars were able to target and lyse both βL5 and 2395 sBC when the peptide was present in a fashion that was regulated by the number of MART-1 avatars added (**Figure 6B**). However, when the βL5 or 2395 sBC were not pulsed with Melan-A_{26-35}, neither population of cells was lysed. These results indicate that sBC can be utilized to study the interactions of β-cells with antigen specific T cells. In addition, as 2395 T cell avatars kill 2395 sBC in an antigen-specific fashion (**Figure 6B**) this isogenic system is a viable method to study interactions of autoreactive T cells with β-cells.

DISCUSSION AND CONCLUSION

While T1D develops in genetically at-risk individuals, our current understanding of the pathogenesis of T1D in humans continues to evolve due to the complexities of genetic risk in combination with environmental factors (52). Recent GWAS efforts have dramatically expanded the number of loci that are associated with both increased or decreased risk for T1D (1–12). The mechanisms linking the majority of these polymorphisms, alleles, and allotypes to the development of T1D are not clear. Disease heterogeneity in T1D may result from the dissimilar combinations of genes inherited by at risk individuals that instigate variations in onset age and disease endotypes. Indeed, specific combinations of risk HLA haplotypes have been linked to earlier age of onset as well as changes in the immune cell components of the insulitic lesion (53). Due to the impact of total combined genetic risk, it is necessary to sufficiently power mechanistic studies with large sample sizes to determine the responsibility of a single polymorphism to T1D and the cell types influenced by the greater than 100 polymorphisms that are linked to T1D (1–12). With the development of isogenic systems, such as that described here, methodologies have advanced that allow for interrogation of the effect of a single polymorphism in the absence of other genetic differences. Models derived from primary cells can lack reproducibility because certain cell types (i.e. β-cells) are not renewable; utilizing iPSC circumvents this issue. Herein we have shown that key cell types involved in the pathogenesis of T1D (**Figure 1**) can be derived from a single donor and used to construct an isogenic system to interrogate relevant cell-cell interactions. This renewable system provides a powerful platform to interrogate how specific genetic polymorphisms influence both cell function and cell-cell interactions in isolation of other genetic differences.

Thus far, we have observed that 2395 iPSC are pluripotentusing *via* trilineage differentiation, demonstrating that 2395 iPSC are capable of forming all 3 germ layers *in vitro* (**Figure 2C**). Further, 2395 iPSC express the pluripotency markers, OCT4 and NANOG (**Figure 2B**), and differentiate into functional cells from the endoderm (**Figures 3**, **5**, **6**) and mesoderm (**Figure 4**). However, prior to use of 2395 cells an *in vivo* model it will be essential to determine teratoma formation and tumorigenic potential. In this

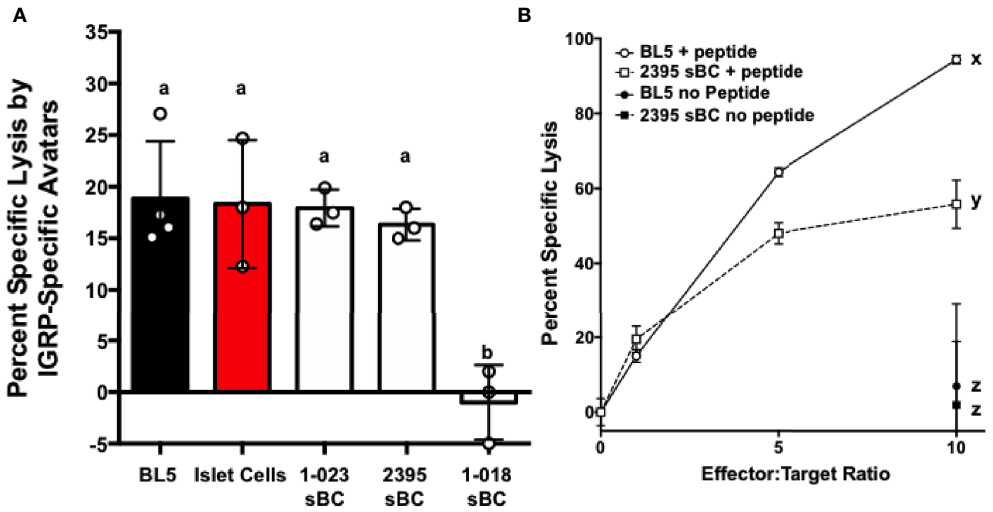

FIGURE 6 | sBC are efficiently targeted by donor 2395 CD8$^+$ T cell avatars in an antigen specific and HLA restricted manner. **(A)** Donor 2395 IGPR T cell avatars were co-cultured with βL5 cells, primary islet cells from a HLA-A*0201 positive donor, 1-023 sBC, or 2395 sBC the cells were effectively lysed at an effector to target (E:T) ratio of 10:1. As 1-018 sBC do not harbor HLA-A*0201 these cells were not lysed by these T cell avatars as they require the IGRP peptide to be presented by HLA-A*0201. **(B)** Donor 2395 MART-1-avatars were co-cultured with either βL5 cells or donor 2395 sBC at increasing E:T ratios (0:1, 1:1, 5:1, and 10:1). When pulsed with Melan-$A_{26\text{-}35}$, lysis of both βL5 and 2395 sBC increased with escalating E:T ratios. In the absence of Melan-$A_{26\text{-}35}$, no lysis was measured. *Letters denote significance: groups/lines with different letters were statistically significant ($P<0.05$), whereas those with the same letter were not statistically different. These data are from at least 3 independent studies performed in triplicate.

isogenic system derived from donor 2395, we have shown that both macrophages and DC can expand total T cells (**Figure 4**), that CD8$^+$ T cell avatars bind to EC in an antigen specific manner (**Figure 5**), and that CD8$^+$ T cell avatars can target and lyse sBC that is dependent on the trimolecular complex (**Figure 6**). The current system affords power for interrogating these simple cell-cell interactions as well as more complex cell-cell interactions such as macrophage, T cell, and β-cell interactions. While the system has great utility, it does however have limitations.

One of the current requirements of the current system is the need to recall specific donors to obtain T cells. This necessitates proper IRB approval and flexibility of research participants and investigators to schedule blood draws. Currently there are protocols for obtaining T cells from iPSC however these produce predominantly CD8$^+$ T cells and very few CD4$^+$ T cells (54, 55). The addition of iPSC-derived naïve autoreactive CD8$^+$ T cells to this system can be implemented to provide yet another way for interrogation of cell-cell interactions and eliminate the need to recall donors to obtain CD8$^+$ T cells. iPSC can be transduced with lentiviral vectors (56, 57), therefore a system to develop antigen specific iPSC-T cells could be employed wherein the iPSC are engineered to express a specific TCR to recognize self or nominal antigens.

Another shortcoming of this system and iPSC in general is that not all iPSC lines efficiently differentiate into all necessary cell types. For example, 1-018 sBC were not as functional as sBC from lines 1-023 or 2395 (**Figure 3E**). In addition, while 1-023 differentiated well into sBC, this iPSC line did not produce monocytes. Lack of monocytes prevented downstream differentiation to macrophages and dendritic cells. Other groups have observed that lineage differentiation capacity is dependent on the iPSC line necessitating protocol optimization for lines that fail to differentiate effectively (58). As such, methodological modifications specific for 1-023 may resolve the observed dearth of monocytes from this line. However, the necessity of individualized differentiation protocols could impact the throughput of any system. Another important consideration and potential difficulty results from the complexity of T1D genetics. This requires careful consideration of the cells utilized for differentiation and study. Due to the strong genetic influence of the HLA super locus on T1D and the need for specific HLA alleles to be present for antigen specific studies, genotyping and selecting iPSC with HLA alleles (**Table 1**) that match TCRs to be utilized is essential. Here we eliminated 1-018 from the system as this line did not have the necessary HLA-A*0201 allele to interact with the T cell avatars (**Table 1**). Indeed, 1-018 sBC were not targeted and lysed by the IGRP-Avatars (**Figure 6A**). The HLA Class I and Class II loci are the most polymorphic coding genes in the human genome (59), therefore matching HLA for the current study was prioritized. However, advances in gene editing technology may, in future studies, allow for extensive modification of HLA loci and obviate the requirement for excluding cells based on HLA allele types. Regardless of the more difficult requirements of the current system, there are many developing technologies that will improve future iterations.

One of the currently developing technologies is gene editing in iPSC. Gene editing can increase the flexibility of this system. There are numerous protocols for editing SNPs or knocking out genes in iPSC. While knockout is straightforward and there are

high-efficiency protocols available for iPSC, easy to implement and high-efficiency protocols for scarless editing of single bases, such as SNPs, are still being developed (60–66). Once these high-efficiency protocols are developed, editing SNPs in iPSC will allow us to interrogate how credible risk or resistance SNPs influence these cell-cell interactions. The ability to edit iPSC donor lines to harbor both the T1D-risk and T1D-resistance alleles at a single locus will potentially suppress variation by allowing edited donor lines to be compared to each other. These methods would have exceptional value to study the genetic influences in cells, organoids, or organs that are difficult to access or genetically modify in a homogenous fashion. This would greatly facilitate the study of rare genetic variants (e.g. *rs34536443* in *TYK2*, *rs2476601* in *PTPN22*, *rs35744605* in *IFIH1*, etc.) as editing iPSC lines to the rare minor alleles at T1D risk loci would allow for generation of numerous lines from different donors that could be studied in a pairwise fashion versus identifying and specifically recruiting donors with minor allele frequency (MAF) <0.05. For example, the frequency of individuals homozygous for the rare allele of *rs34536443* in *TYK2* was recently reported to be 213 per 100,000 with MAF = 0.04 (67, 68). The ability to edit a credible risk or resistance SNP will facilitate determination of the impact of these rare variants and likely bolster efforts seeking to resolve the actual SNP(s) in linkage disequilibrium (LD) that influence disease risk (e.g. the 233kb LD block on chromosome 16p13 that contains *CLEC16A*) (69–71). In addition, other technologies in development, such as ESC and iPSC-derived thymii that can output functional naïve T cells, are novel tools for studying human disease etiology. As thymic epithelial cells (iPSC-TEC) (72, 73) have the potential to regulate T cell production from progenitors, use of these evolving technologies in combination (gene editing and iPSC-TEC) would allow for studies to dissect how T1D-linked loci, or those linked to other diseases, regulate T cell development. These and other currently advancing technologies will greatly enhance isogenic systems, improving their capacity to model human diseases.

Isogenic systems derived from iPSC will greatly augment our ability to interrogate key cellular interactions involved in genetic regulation of T1D pathogenesis. While the system built here is focused on T1D, it can be utilized to study cell-cell interactions and genetic regulation in the context of other human diseases. In summary, we have presented an isogenic model that can currently be used to interrogate many T1D-relevant cell-cell interactions. As more technologies for gene editing and differentiating iPSC continue to be developed these tools can be implemented to build a more relevant model capable of answering more complex questions.

DATA AVAILABILITY STATEMENT

The SNP typing data will be available to academic investigators upon request.

AUTHOR CONTRIBUTIONS

LHA, SES, CEM, and MAW conceived the idea, designed the research plan, and participated in all aspects of writing the manuscript. KS, LS, SO, BN, MH, JT, NF, PS, IR, RC-G, DP, HR, AS, NT, TB, JC, and DE performed experiments, analyzed data, interpreted results of experiments, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.737276/full#supplementary-material

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