Type 1 diabetes (T1D) is a chronic, debilitating autoimmune disease targeting pancreatic β cells. This process in T1D patients results in a decrease of the pancreatic β cell mass, which causes insufficient insulin production (1). The ensuing dysmetabolism leads to serious complications, including cardiovascular disease, diabetic neuropathy, and nephropathy (1). Although the exact etiology of T1D remains unknown, it is prevalent among young children and remains without a practical cure despite being one of the most common endocrine disorders. Successful transplantation of the pancreas or that of pancreatic islets can prevent or reduce some of the complications of T1D. However, organ shortage severely limits organ transplantation in these patients.

Pluripotent stem cells (PSCs) hold tremendous promise in addressing many of these shortcomings because their availability is unlimited and they are considered to be immune privileged (2–4). Both iPS and ES cells are capable of forming any cell type, if provided with appropriate developmental cues (4). Unlike ES cells that require the controversial destruction of embryos, iPS cells are derived from pre-existing somatic cells and enable the possibility of designing patient-tailored therapies (4). These characteristics of iPS cells provide the unique opportunity to engineer autologous IPCs that can be used to replace pancreatic β cells destroyed in T1D (5). Moreover, these cells have enormous value in drug screening as well as in modeling the pathogenesis of T1D.

The process of differentiating ES or iPS cells into pancreatic endocrine cells in vitro traces the developmental stages observed during embryogenesis (6). We and others (7–12) have begun to differentiate iPS cells into IPCs. Overall, it has proved challenging to generate mature and functional IPCs that possess hallmark features of adult pancreatic β cells, such as glucose responsiveness and rapid correction of hyperglycemia (8–12). More recently, two reports overcame these shortcomings and devised strategies to differentiate human ES and iPS cells into glucose-responsive IPCs that strongly resembled adult pancreatic β cells (13, 14). Unfortunately, the rate of glucose correction after transplantation of the cells into diabetic mice was 6–7 weeks. Here we show that we can correct hyperglycemia in less than 4 weeks.

We independently overcame the hurdles in the generation of IPCs from human iPS cells by replacing 2D culture platform systems used in prior protocols with a 3D differentiation culture system. The number of IPCs derived was superior to what had been originally described in the literature for traditional 2D
culture systems (8–12) and comparable with what has recently been achieved using suspension-based 3D cultures (13, 14). The reason for this improvement is that, during embryogenesis, the developing cells are arranged in 3D clusters, which support cell–cell signaling (15, 16). 3D differentiation of human iPS cells has notable precedent in the literature, having been used to derive functionally and morphologically superior tissues, such as cerebral organoids (15) and liver buds (17). Here, we established 3D cultures using Matrigel, which is an extracellular matrix containing rich bioactive substrates, to exploit scaffold-embedded signaling cues (18, 19). By combining a novel 3D bioscaffold-based culture platform with well-selected and optimized signaling cues, we envisioned that we could drastically improve the efficiency of generating glucose-responsive IPCs.

iPS cells derived from some T1D patients have been shown to have a lower efficiency in generating pancreatic progenitor cells expressing Pdx1 (20). It is not yet known why this is the case, especially because other T1D iPS cell lines have been used to make IPCs efficiently. If this resistance to differentiation is common to a significant number of T1D cell lines, autologous iPS cell therapy for T1D will be a challenge. As a possible way to overcome this, we show here that transient demethylation treatment during the differentiation of a T1D iPS cell line that, in our experience, poorly differentiates into IPCs, can significantly improve the yield of functional IPCs.

Results

**Differentiation of T1D and nondiabetic (ND) iPS cells into definitive endodermal cells**

We and others have published preliminary data on the differentiation of iPS cells from healthy individuals (7–14); however, the differentiation of iPS cells into IPC has remained elusive. Here, we incorporated additional critical signaling cues that instruct iPS cells to become pancreatic β cells to further improve the yield of IPCs (Fig. 1A). The differentiation process drives the cells through five stages lasting a total of 27 days. The first stage of differentiation toward IPCs is the conversion of iPS cells into definitive endodermal cells (DE cells) (6) after treatment with Activin A and Wnt3a signaling (10) (Stage 1). DE cells are characterized by the expression of CXCR4 and simultaneous expression of the transcription factor Sox17. The resulting cells are next exposed to FGF-7, which acts to convert the DE cells into the posterior foregut (10) (Stage 2). Following this step, the cells are treated with retinoic acid and SANT-1, an inhibitor of the Shh pathway (10), to posteriorize the gut tube and promote pancreatic differentiation as opposed to hepatic specification (21). Reinforcing suppression of hepatic differentiation, Noggin is added to this media mixture as well (10). It functions to inhibit BMP signaling (22). The aim of this phase is to generate Pdx1+ pancreatic precursor cells (Stage 3). The cells are then instructed to become pancreatic endocrine precursor cells by carrying over certain cues from Stage 3, such as Noggin and low-dose retinoic acid signaling, and supplementing them with inhibitors of TGF-β signaling (23) and suppression of Notch signaling (13, 14), which actively inhibit endocrine differentiation by the process of lateral inhibition (24). Suppression of TGF-β signaling is accomplished by the treatment with ALK5 inhibitor II (25). Repression of Notch signaling is indirectly accomplished by blockade of its ligand, γ-secretase, using DAPT (26). In addition, this stage of differentiation introduces several drivers of pancreatic β cell development, such as thyroid hormone (T3) (13, 14) and the incretin GLP-1 (27) (Stage 4). Finally, in the last stage of differentiation, specific inducers of insulin are added in addition to agents that promote the maturation and development of pancreatic β cells, such as nicotinamide (28–30), IGF-1 (27, 31), GLP-1, and T3.

Using this protocol, ND and T1D iPS cells were first differentiated into DE cells in parallel, and the efficacy of differentiation was assessed on day 5 by determining the expression of CXCR4, Sox17, and platelet-derived growth factor receptor (PDGFR)-α. Co-expression of CXCR4 and Sox17 typifies lineage commitment to the endoderm. Undifferentiated iPS cells were utilized as negative controls and did not express any of the aforementioned markers (Fig. 1B). Both T1D-1 and ND differentiated cultures contained >90% CXCR4+ Sox17+ endodermal cells (Fig. 1B). Additionally, these cells were mostly PDGFR-α− (Fig. 1B), which suggests that they are true endodermal cells and are not arrested in the transitory, immature mesendodermal state (32). Thus, we concluded that we were able to achieve a near pure population of DE cells from both ND and T1D iPS cells at comparable yields. For further differentiation into IPCs, we dissociated the 2D differentiating DE cells by scraping the monolayers into chunks of cells, and then depositing these cell clusters into Matrigel blocks (1:1 diluted with DMEM/F-12). The cells coalesced into discrete spheroids that embedded into the Matrigel within 24 h. At this point both cell types were transferred to plates containing Matrigel, allowing differentiation of the cells on a 3D platform.

**T1D iPS cells predominantly derive hollow cysts that do not express insulin**

Early in the 3D differentiation procedure, we recognized that the DE cells from both T1D and ND IPC cultures coalesced into compact cell clusters. However, in the final stage of the differentiation, we observed the formation of clusters with two distinct morphological phenotypes: hollow cysts that appeared like cysts and compact spheroids (Fig. 1C). Strikingly, we observed that the T1D IPC cultures consisted almost entirely of hollow cysts, whereas the ND iPS cells gave rise to a ratio of 50:50 of hollow cysts to compact spheroids (Fig. 1E). The cysts emerged gradually with the differentiation process, becoming more prominent toward the end of the differentiation. The whole differentiation period lasted 27 days. To further characterize these hollow cysts and compact spheroids, they were stained for insulin. The hollow cysts collapsed upon fixation in paraformaldehyde (Fig. 1D), which is an observation that is consistent with what has been described in the literature with ex vivo culture of mouse embryonic pancreatic progenitor cells (33). When we stained these structures for insulin, the compact spheroids, but not the hollow cysts, stained positive for insulin (Fig. 1D).

Remarkably, the morphology of the compact spheroids resembled that of pancreatic islets (Fig. 1D). This result suggested that to optimize the yield of IPCs, the number of the compact spheroids needed to increase.
The differentiation of T1D iPS cells into IPCs has lower efficiency compared with that of ND iPS cells

As determined by flow cytometry, the 25–50.5% yield of IPCs derived from ND iPS cells (across two different iPS cell lines) is comparable to the frequency of β cells found in primary human islets (34). This yield of IPCs from ND iPS cells is far superior when compared with previous reports, where the yield of IPCs was only 10–15% (7–12), and is comparable to what was achieved more recently by two groups in 2014 using suspension-based 3D cultures (13, 14). We con-
included that 3D differentiation is superior for iPS cell differentiation compared with 2D differentiation. However, despite the effectiveness of our protocol, the yield of IPCs from a T1D-1 iPS cell line we possessed was only 15.9%, which was significantly lower than those of ND iPS cells (Figs. 2A and 4B). Thus, these data allow us to conclude that from a T1D-1 iPS cell line we possessed was only 15.9%, which was significantly lower than those of ND iPS cells (Figs. 2A and 4B). Thus, these data allow us to conclude that

Figure 1. Solid spheroids but not hollow cysts found predominantly in T1D differentiating cultures express insulin. A, this differentiation schema shows in a clockwise manner the five stages of differentiation that iPS cells undergo to become IPCs. B, T1D-1 and ND iPS cells were differentiated in parallel under Stage 1 to generate CXCR4+/SOX17+/PDGFRα−/DE cells. Over 90% of the resultant cells derived from both T1D and ND iPS cells co-expressed CXCR4 and Sox17, and almost all of these cells were PDGFRα−, suggesting that they are endodermal but not mesendodermal. C, to confirm that those low doses of 5-Aza-DC would still demethylate cells, we performed a dot blot for 5-methylcytosine on genomic DNA isolated from untreated T1D-1 iPS cells or iPS cells that were treated with 1 nm or 10 nm 5-Aza-DC (n = 3). Untreated iPS cells possessed significant 5-methylcytosine content (leftmost column), evidenced by the dark spot where the DNA was blotted. As can be observed by lightening of the spots at the 1 nm and 10 nm doses, 5-Aza-DC appeared to effectively demethylate the cells (1 min represents the exposure time for the blot).

Figure 2. IPCs derived from T1D iPS cells poorly express Pdx1 and insulin. A, to investigate why the T1D cells poorly differentiated into IPCs, mRNA levels of various pancreatic genes were quantified in ND IPCs and T1D-1 IPCs. Insulin expression in Stage 5 was accompanied by a striking decrease in Glucagon expression in the ND differentiating cultures. However, the T1D-1 culture expressed significantly lower levels of Insulin and Glucagon compared with ND IPCs. Pdx1 expression in the T1D-1 IPCs was significantly lower than in the ND IPCs (n = 5). These data were generated by normalizing threshold cycle (CT) values to an iPS cell line. The internal control used in this experiment was the TATA binding protein, which was used as a housekeeping gene. Data are represented as mean ± S.E.; *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, 50.5% of the ND IPCs are positive for insulin compared with only 15.9% of T1D-1 IPCs. The flow analysis was repeated four times for each cell line. C, to confirm that those low doses of 5-Aza-DC would still demethylate cells, we performed a dot blot for 5-methylcytosine on genomic DNA isolated from untreated T1D-1 iPS cells or iPS cells that were treated with 1 nm or 10 nm 5-Aza-DC (n = 3). Untreated iPS cells possessed significant 5-methylcytosine content (leftmost column), evidenced by the dark spot where the DNA was blotted. As can be observed by lightening of the spots at the 1 nm and 10 nm doses, 5-Aza-DC appeared to effectively demethylate the cells (1 min represents the exposure time for the blot).
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the differentiation of T1D cell lines into insulin-expressing cells is diminished.

T1D iPS cell-derived differentiating cells poorly express Pdx1

To further investigate why the differentiation of T1D iPS cells was impaired, we next corroborated the immunofluorescence and flow cytometry data by gene expression studies. Gene expression for several genes was studied in Stages 4 and 5 of both T1D and ND differentiation cultures. As can be seen in Fig. 2B, the expression of the Insulin transcript in the ND cells increases from Stage 4 to Stage 5, and this is accompanied by a decline in the expression of Glucagon, suggesting commitment of the cells toward insulin-expressing cells in the last stage of the differentiation. However, in the T1D IPC differentiating cultures, we observed significantly lower expression of Insulin at both Stages 4 and 5, confirming our previous results. We also observed significantly poorer expression of Glucagon in the T1D IPC cultures compared with ND IPC cultures. The expression of other genes, such as Somatostatin and Glucokinase was not significantly different between the T1D-1 and ND cultures, and Ghrelin showed a very small but significant difference (Fig. 2B).

To determine whether the inefficiency in differentiation manifests earlier than the last stage of differentiation, we determined the expression of Pdx1 in T1D and ND IPC differentiating cultures. Pdx1 is the master regulator gene in the pancreas and its expression appears midway through the differentiation process (10). In ND cultures, Pdx1 is expressed in high levels in Stage 4 (Fig. 2B), which precedes the expression of Insulin in Stage 5, consistent with embryonic development of the pancreas (35). Pdx1 levels continue to increase in Stage 5 in the ND culture. However, at both Stages 4 and 5, the T1D-1 cultures expressed significantly lower levels of Pdx1 than did the ND culture (Fig. 2B). Pdx1 is indispensable for the development of pancreatic β cells (6). Pdx1 knock-out mice fail to form a pancreas (36), which is evidence for how critical this gene is in the development of the pancreas. This likely explains why the expression of downstream genes, such as Insulin, is also impaired in the T1D differentiating cultures.

Effective differentiation of T1D iPS cells requires precise temporal modulation of demethylation treatment

So far we demonstrated that the differentiation of T1D iPS into IPCs was impaired for reasons that are still unclear. Considering the importance of epigenetics in cell differentiation and the poor expression of Pdx1 in T1D differentiating cultures, we hypothesized that epigenetic barriers were likely responsible for the poor yield of IPCs derived from T1D iPS cells. We reasoned that the dysmetabolism in T1D might be responsible for the epigenetic changes which could lead to the poor differentiation of T1D iPS cells. To address this problem, we utilized 5-Aza-2’-deoxycytidine (5-Aza-DC), a potent demethylating agent that inhibits the DNA methyltransferase (Dnmt), thereby inhibiting methyl group deposition on cytosine residues of DNA, including on gene promoters (37). This allows for the binding of the transcriptional machinery and promotes gene expression. We further argued that the use of a demethylating agent might induce a more labile, permissive state of the stem cells, allowing for greater cell responses to differentiation stimuli, and ultimately enhance the yield of IPCs from T1D iPS cells.

We established a dose-screen experiment to identify the optimal dose of 5-Aza-DC for treatment that would preserve cell viability while effectively demethylating the DNA of the cells. We utilized 1 nm and 10 nm 5-Aza-DC for our pilot differentiation. To determine the effectiveness of the proposed doses in demethylating iPS cells, we performed a dot blot assay (38) for 5-methylcytosine on genomic DNA isolated from iPS cells that were treated with 1 nm or 10 nm 5-Aza-DC for 18 h. Untreated iPS cells were used as negative controls. At the 1 nm and 10 nm 5-Aza-DC treatments, we observed loss of methylation in iPS cells as evidenced by the lighter spots on the film, corresponding to lower levels of 5-methylcytosine in these cells (Fig. 2C).

Having confirmed that the doses above resulted in decreased 5-methylcytosine content in the iPS cells, we next sought to optimize the time point for the demethylation. We considered two possible time points for the demethylation treatment: 1) at the start of the differentiation into DE cells, or 2) after the generation of DE cells, before the cells progress into the stage in which Pdx1+ cells are generated. We observed that the treatment of iPS cells with 10 nm 5-Aza-DC on day 0 of differentiation (before initiating the generation of DE cells) resulted in cells on day 5 that were arrested in the immature CXCR4+PDGFR-α+Sox17+ mesendodermal state (Fig. 3A). In contrast, demethylation of the cells on day 4 of differentiation, which is the day when DE cells are harvested, yielded a pure population of CXCR4+ Sox17+ PDGFR-α−DE cells, similar to what we were able to generate without any demethylation agent. This pilot experiment allowed us to conclude that the 5-Aza-DC treatment was ideal after the generation of DE cells.

Demethylation of T1D DE cells rescues the expression of Pdx1 and leads to the generation of islet-like compact cell clusters

After identifying the appropriate doses and time point at which demethylation would be implemented, we initiated a full differentiation of T1D iPS cells into IPCs, demethylating the DE cells on day 4 for 18 h before transferring them onto Matrigel. We observed a striking impact of the 5-Aza-DC treatment in the T1D cultures. Typically, the T1D iPS cells we possessed gave rise to a disorganized mix of cysts and spheroids, with a dominant presence of hollow cysts. At both doses tested, 5-Aza-DC treatment instead promoted the formation of compact cell clusters (Fig. 3B). Juxtaposition of the clusters derived from the two treatment conditions showed that regardless of the dose, demethylation generated cell clusters that uniquely resembled human islets (Fig. 3B). We stained these cell clusters with dithizone, which is an organic compound that complexes with Zn2+ ions found in insulin hexamers of β cell insulin granules, and thus suggests the presence of insulin (39). Dithizone staining revealed the strong red color of the compact clusters found in the 1 nm and 10 nm 5-Aza-DC treated cultures, which was reminiscent of islets (Fig. 3C). This was in contrast to what was observed in the IPCs derived from untreated iPS cells,
which stained brown in a manner similar to undifferentiated iPS cells (Fig. 3C).

Next, we assessed whether the demethylation rescued the expression of Pdx1 in the differentiating T1D IPC cultures. We chose to address this question by determining the expression of Pdx1 in the differentiating cells using flow cytometry. Undifferentiated iPS cells were used as negative controls in these experiments (Fig. 3D), and the mouse insulinoma β TC3 or human cadaveric β cells served as positive controls (Fig. 3D).

As described above, without the demethylation step, the yield of insulin-expressing cells from T1D iPS cells was lower than ND iPS cells (Fig. 2A), which is consistent with the 12% yield of Pdx1+ pancreatic progenitor cells observed in regular differentiations at the end of Stage 4 (Fig. 3D). However, after demethylation, we observed robust expression of Pdx1 (~95%)
at the end of Stage 4 (Fig. 3D). Curiously, demethylation of differentiating ND cells did not significantly improve the yield of Pdx1⁺ cells. 71.1% of the non-demethylated cells expressed Pdx1 compared with 76.1% after demethylation (Fig. 3D). In T1D differentiating cultures, a significant proportion of cells co-expressed Pdx1 and the pancreatic β cell–specific transcription factor Nkx6.1, which is critical for maintaining the identity and function of pancreatic β cells (40, 41) (Fig. 3E). Altogether, these data suggested that the transient demethylation treatment allowed the expression of Pdx1 in differentiating T1D cultures.

**Demethylation of T1D DE cells significantly improves the differentiation of DE cells into pancreatic cells**

These data then led us to wonder if 5-Aza-DC treatment enhanced the expression of downstream targets of Pdx1, such as insulin (42), and made the cells more receptive to differentiation cues while averting commitment toward alternative lineages. For this experiment, we were able to obtain a separate T1D iPS cell line, which also showed resistance to differentiating into IPCs. As can be seen in Fig. 4A, demethylation improved the yield of IPCs from 13.7 to 56.7% in T1D-1 and from 1 to 59.4% in T1D-2, which was very similar to human islets (58.4%). T1D-1 iPS cells were assessed for insulin expression as a negative control. Notably, the yield of IPCs from ND iPS cells was not changed even after 5-Aza-DC treatment (Fig. 4B).

A key sign of the efficiency of the differentiation is the relative ratio of β cells versus α cells. Specifically, we sought to determine the proportion of IPCs relative to those that stain for glucagon. Most reports published so far generate multihor- monal cultures that express both insulin and glucagon (7–12). As can be seen in Fig. 4C, the proportion of glucagon-expressing cells was quite high when the iPS cells were not demethylated, whereas they were depleted after the demethylation step (Fig. 4C). Nondemethylated cultures yielded up to 13% insulin-expressing cells and 50% glucagon-producing cells. However, with demethylation at a dose of 10 nM 5-Aza-DC, 56% of the cells expressed insulin, with a much smaller portion of the cells expressing glucagon (Fig. 4). This result also demonstrates that, at higher concentrations of the demethylating agent, the yield of IPCs from ND iPS cells was not changed even after 5-Aza-DC treatment (Fig. 4B).
nearly all the glucagon-secreting cells are gone. Because the highest yield resulted from treatment of the cells with 10 nM 5-Aza-DC, we chose to use this dose for all subsequent experiments. This experiment has been repeated multiple times and as many as 65% insulin-positive cells were measured in some cultures. Pooling of data from several differentiations reveals

Figure 5. T1D IPCs possess insulin granules and are glucose-responsive. A, iPS and ES cells typically give rise to a multimodal pool of precursor cells that only acquire maturity and monohormonal expression of insulin after transplantation in mice. After utilizing 5-Aza-DC to generate islet-like compact clusters, we sought to characterize the expression of insulin and glucagon within these cell clusters (n = 4). Consistent with the flow cytometry data, 5-Aza-DC appears to promote the generation of unihormonal insulin-expressing cells while averting the formation of glucagon-producing cells. Human islets possess both insulin-expressing and glucagon-expressing cells. Scale bar = 50 μm. B, IPCs stain positive for cytoplasmic C-peptide, confirming de novo production of insulin, and express Nkx6.1 in the nucleus. Tricolor merge on top left and two-color combinations are shown in the other panels. Scale bar = 10 μm. C, human islets (left panel) possess insulin granules of various maturities that are differentiated by the color and shape of the core granule. Mature angular insulin granule (blue box), maturing round insulin granule (green box), and early round gray insulin granule (red box) are identified by the electron microscopy (4000X). However, all granules possess a characteristic “halo” surrounding them, which is very specific to the insulin granule. Similar to islets, T1D-1 IPCs protocol (right panel) possesses insulin granules of various maturities, confirming their authenticity and similarity to human islets (n = 3 experiments). D, comparison of the number of granules per cell in islets and IPCs reveals a nonsignificant difference between the two cell types. Data are represented as mean ± S.E. n = 57 IPCs and 28 islet pancreatic β cells counted. E and F, after baseline equilibration in 2.8 mM glucose solution (low glucose or LG), exposure to 28 mM glucose (high glucose or HG) resulted in significant increase in insulin secretion by both IPCs (E) and islets (F). G, the fold increase in insulin secretion is higher for IPCs than for islets. n = 4 (two experiments of duplicates); p < 0.001. The variation in cell numbers across the cell batches was normalized by total protein content per well.

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that 5-Aza-DC treatment consistently enhances the yield of IPCs 4-fold (Fig. 4D).

T1D IPCs derived from demethylated DE cells express pancreatic β cell–specific markers and possess insulin granules at similar levels to cadaveric β cells

Until very recently, mainstream published protocols for the generation of IPCs from iPS or ES cells generally gave rise to a multihormonal pool of cells, of which very few cells express only insulin (7–12). We showed so far that our newly established protocol allowed for the selective generation of insulin-expressing cells from T1D iPS cells while generating very few glucagon-expressing cells. Immunofluorescence analysis of the differentiated cell clusters showed that the cells were mostly insulin-secreting cells only with a very small percentage of glucagon-secreting cells (Fig. 5A). Thus, demethylation appears to promote the generation of monohormonal insulin-expressing cells while averting the formation of glucagon-producing cells.

Additionally, we stained for the nuclear transcription factor Nkx6.1, which is critical for the maintenance of pancreatic β cell function and identity (40, 41). The cells were co-stained for the insulin precursor C-peptide, which is synonymous with de novo production of insulin (27, 43). As evidenced in Fig. 5B, T1D IPCs derived via demethylation show robust expression of C-peptide in the cytoplasm as well as strong nuclear expression of Nkx6.1 (Fig. 5B). This confirms the flow cytometry results for Nkx6.1 (Fig. 3E) and affirms our conviction that we have established a protocol that efficiently generates IPCs.

Next, we analyzed the ultrastructure of these cells by transmission electron microscopy (TEM). We observed a unique pancreatic β cell–like morphology of the granules contained in the T1D IPCs (Fig. 5C, bottom panel). Unlike our first protocol that was published recently (7), these granules are identical to those present in cadaveric β cells (Fig. 5C, top panel). Insulin granules undergo various stages of maturation that are differentiated by the shape and darkness of the core (13, 14). The most mature insulin granules are angular because of the hexamer complexation of insulin with zinc, which creates a crystalline shape (44). However, insulin granules universally possess a characteristic “halo” (which is an artifact of the gluteraldehyde fixation) that is not found on any other hormone granule (44). This feature is thus a unique and specific indication of β cell–like phenotype.

As can be seen in the lower panel of Fig. 5C, IPCs resemble pancreatic β cells in their possession of the three different insulin granule subtypes, all of which have the characteristic halo surrounding them. Additionally, the number of granules found in the IPCs was not statistically different from the number of granules in primary human islets (Fig. 5D). Thus, we have generated human IPCs from T1D iPS cells that strongly resemble human β cells in their ultrastructure in addition to their expression of insulin and other pancreatic β cell–specific markers.

T1D IPCs are functional and are glucose-responsive

Until now, we argued that demethylation promoted the selective generation of insulin-producing cells which are bound in tight cell clusters that strongly resemble islets. Perhaps the most important criterion for defining the authenticity of the generated IPCs is to observe whether they secrete insulin when stimulated with high glucose (45). This is important because these cells should respond to glucose spikes in vivo caused by food intake. Besides, pluripotent stem cell–generated IPCs have failed to respond to glucose stimulation until very recently. Two recent reports described for the first time the generation of glucose-responsive cells from human ES cells (13, 14). Maehr et al. reported the generation of pluripotent stem cells from patients with type 1 diabetes and analyzed phenotype of the IPCs (46). However, as of yet, the generation of functional, glucose-responsive IPCs from human iPS cells of T1D patients has not been demonstrated.

To address the glucose responsiveness of these IPCs, we subjected T1D IPC cell clusters to a glucose-stimulated insulin secretion (GSIS) assay, which involves exposing cells sequentially to low glucose (2.8 mM) and subsequently to high glucose (28 mM). Insulin content in the supernatants was measured by ELISA. Although the absolute content of insulin produced, when normalized by total protein, was significantly lower for IPCs (2 µIU/ml) than islets (250 µIU/ml) (Fig. 5, E and F), the -fold increase in insulin production by IPCs is higher than that for islets (Fig. 5G). As evidenced in Fig. 5G, these IPCs are glucose-responsive in a manner that has never been observed before in IPCs derived from T1D iPS cells. This result demonstrates the generation of functional IPCs from human iPS cells derived from T1D patients.

Human iPS cell– derived IPCs rapidly correct hyperglycemia in diabetic mice

To determine whether the T1D IPCs are functional in vivo, immunodeficient Rag2−/−γc−/− mice were made diabetic by multiple low doses of streptozotocin. Mice were injected subcutaneously with 1.2–1.4 × 10⁶ of 5-Aza-DC–treated T1D-1 iPS cell–derived IPCs in the shoulder region. Remarkably, the hyperglycemia plateaued in less than 2 weeks after transplantation and started to fall rapidly. Within 4 weeks, mice either were normoglycemic or achieved near normoglycemia. None of the eight mice died or developed teratomas (Fig. 6A). All mice normalized blood glucose levels. Additionally, we subjected mice that showed stable correction of hyperglycemia to a glucose tolerance test (n = 4), in which they received a supraphysiologic glucose bolus i.p. The management of this glucose spike was then assessed over a period of time to determine the kinetics of blood glucose regulation. Remarkably, in contrast to nondiabetic control mice, which failed to correct hyperglycemia and were ultimately sacrificed, IPC-transplanted mice completely recovered to normoglycemia in 4 h (Fig. 6B). This is evidence for how the IPCs endowed these formerly diabetic mice with the ability to tolerate and manage glucose spikes. However, we noted that the correction of hyperglycemia was significantly delayed compared with nondiabetic control mice, suggesting that there is a need for further improvement. Perhaps increasing the cell numbers might speed up the recovery from the glucose bolus levels. Still, the complete return to normoglycemia in IPC-transplanted mice is striking evidence of the remarkable ability of these cells to manage supraphysiologic glucose spikes. Excision of the transplanted cells after 8 weeks of subcutaneous transplantation revealed an organoid.
Our data demonstrate that we have established a robust protocol for the generation of IPCs from either healthy or T1D iPS cells. These findings align with recent reports (13, 14) that utilize different 3D differentiation methods that altogether have facilitated an enormous advance from prior protocols that generally yielded only 10–15% insulin-positive cells from human ES cells or iPS cells derived from nondiabetic patients (7–12). Our protocol is simple and reproducible and does not require exorbitant amounts of expensive reagents and growth factors. Here, we utilized a highly optimized system to generate a virtually pure population of CXCR4+ Sox17+ DE cells that did not express PDGFR-α, a mesodermal and mesendodermal marker (32). These cells were then driven through four more developmental stages in a 3D platform to yield >50% insulin-positive IPCs. These cells were organized in compact cell clusters that resemble islets and expressed insulin as determined by a glucose stimulation assay, flow cytometry, quantitative RT-PCR, and immunofluorescence. Although we detected some somatostatin-expressing cells and insulin-producing cells, glucagon was not detectable.

However, the success of this protocol in yielding IPCs from ND iPS cells was clearly not recapitulated in two T1D iPS cells we possessed. Although the early differentiation of T1D and ND iPS cells into DE cells (Stage 1) was equivalent, downstream 3D differentiation of the iPS cells showed striking disparities between the two cell types. This was first observed in morphological differences between T1D and ND IPC differentiating cultures, whereby the T1D IPC culture yielded 85% insulin-negative hollow cysts and 15% insulin-expressing compact clusters, but the ND culture gave rise to a 50:50 mixture of the two cluster types (Fig. 1E). Interestingly, these hollow cysts have been observed by another group that differentiated human ES cells into islet-like cluster dishes (47).

Our observation of the dichotomy in cluster types is consistent with data that were presented in a manuscript that reported the use of growth factor–depleted Matrigel to expand mouse embryonic pancreatic progenitor cells (33). Depending on their medium, that group was able to derive two discrete sets of cell clusters. In one form of media, pancreatic progenitors underwent differentiation in vitro into complex organoids consisting of mature pancreatic endocrine, acinar, and ductal cells. However, a different set of media sustained pancreatic progenitors that assembled into hollow spheres, which consisted of cells that had not differentiated into mature pancreatic cells.

Correlating these findings with our own data, this alludes to a bivalent state for the clusters in our differentiating cultures, which consist of compact spheroids that contain mature IPCs and express insulin, as well as hollow, vacuolar cysts that contain immature progenitor cells that do not yet express insulin. Significantly, the dominant presence of hollow cysts in the T1D IPC cultures suggested that most of the cells were immature and that their normal development into IPCs was impaired.

Indeed, as determined through several parameters, we have shown that the generation of insulin-expressing cells from two T1D iPS cell lines in our possession is impaired. Tracing the disparity in the differentiations back to earlier in the pancreatic
The differentiation program revealed that the expression of the \( Pdx1 \) transcript was extremely poor in T1D iPS cell differentiating cultures, suggesting that the differentiation of T1D DE cells into \( Pdx1^+ \) pancreatic progenitor cells was impaired. Naturally, this would translate into significantly impaired yield of insulin-expressing IPCs at the end of the differentiation. Thus, we conclude from these data that the differentiation of some T1D iPS cells into IPCs is impaired compared with ND iPS cells. This could be because of variability between different iPS cell lines. However, the Melton group also showed poor differentiation of one T1D cell line in their possession, suggesting that this phenomenon may be worth investigating in larger panels of T1D iPS cell lines. Indeed, our findings are consistent with their utilization of T1D iPS cells as a negative control for generating \( Pdx1^+ \) pancreatic progenitor cells (20). Whereas that study stopped differentiating cells at Stage 3, our studies advance several steps ahead of this report because we compared the differentiation of ND and T1D iPS cells all the way to Stage 5 using a robust 3D differentiation protocol. Additionally, whereas that prior report briefly introduced its finding, here we have devised a protocol that overcomes this hurdle and allows for robust differentiation of resistant human T1D iPS cells into functional, therapeutic IPCs that respond to high glucose stimulation.

After obtaining the gene expression data showing the impaired expression of the \( Pdx1 \) transcript in T1D differentiating cultures, we reasoned that there were epigenetic barriers that hindered the expression of critical genes for pancreatic \( \beta \) cell specification, such as \( Pdx1 \). Thus, we hypothesized that using epigenetic modifiers such as 5-Aza-DC would allow for the expression of \( Pdx1 \) and downstream genes, which altogether would ultimately improve the differentiation outcome and result in a high yield of insulin-positive IPCs from resistant T1D iPS cells.

Following this hypothesis, we thus treated the T1D culture with 5-Aza-DC after the generation of DE cells, which is the time point after which impaired differentiation is observed. Treatment of iPS cells with 5-Aza-DC yielded >95% \( Pdx1^+ \) pancreatic progenitor cells from T1D iPS cells at the end of Stage 4, which eventually gave rise to >50% insulin-expressing IPCs organized into compact, islet-like clusters. This yield is comparable to what we observed in human islets and in ND IPCs differentiating cultures. When we determined the proportion of insulin-expressing cells versus glucagon-expressing cells in the cultures, we found that 5-Aza-DC improved the yield of IPCs in a concentration-dependent manner while averting the generation of glucagon-expressing cells. This suggested that demethylation was making the differentiating cells more receptive to pancreatic differentiation cues. This specific but still unexplored mechanistic role of 5-Aza-DC in enhancing the differentiation of T1D iPS cells into IPCs strongly suggests a metabolic, inflammatory, or other cause of epigenetic variability in those cells versus ND cells at the level of methylases, methyltransferases, or methylation patterns. This gap in the mechanistic understanding of our findings demands further investigation. Additionally, we arrived at our choice of demethylation agent by serendipity and without specific evidence that it would up-regulate the expression of \( Pdx1 \). We also appreciate that 5-Aza DC may not be the ideal demethylating agent because it...
could lead to DNA damage. Altogether, these facts embolden the need to investigate other demethylation agents in the future.

Further characterization of these cells showed that IPCs possessed insulin granules and had the capability for GSIS. However, we found that glucose- and potassium chloride–responsive insulin secretion by the IPCs still remained 1/100th to 1/125th of human islets (Fig. 5, E and F). If not an issue of insulin content, our findings could be explained by a secretory defect in the IPCs that should be identified and corrected. This raises questions about the need for the in vivo microenvironment before the cells fully mature. Whether this significant difference between IPCs and native islets is because of decreased insulin content or impaired secretion of insulin is still unclear. Indeed, a limitation of this study is that we have not fully developed an understanding of the insulin content and biosynthesis machinery of iPS cell–derived IPCs compared with islets from a true quantitative standpoint. Our use of TEM in Fig. 5 is much more robust in allowing us to make qualitative conclusions. The number of granules is not a suitable surrogate for cellular insulin content and so we would need to address this question further in future studies.

Nonetheless, confirmation that our cells are true β cells came from the transplantation data, which show very rapid correction of hyperglycemia in diabetic mice. In comparison to most other data published by others, our cells abrogate the rise in hyperglycemia and rapidly induce normoglycemia in 4 weeks. In a clinical situation, this would be highly desirable. Additionally, we showed through a glucose tolerance test that IPC–transplanted mice were able to effectively manage a supraphysiologic glucose load, returning to baseline normoglycemia after 4 h, whereas nontransplanted diabetic counterparts remained hyperglycemic. Importantly, however, we observed a striking delay in the correction of hyperglycemia in IPC-transplanted mice compared with nondiabetic, healthy mice. Although we did not collect data on the kinetics of human insulin secretion in these mice during the procedure, we speculate that this markedly slowed return to normoglycemia is because of impaired insulin secretion in IPCs compared with native islets, which is a phenomenon that further bolsters the need for deeper analysis.

One other remarkable finding in our study is that it appears that the location of the transplanted cells in the recipient mice does not matter. In a previous report, we transplanted the cells under the kidney capsule, where the cells induced neovascularization and corrected hyperglycemia (7). Because the kidney capsule can maintain the cells in one spot, we were able to harvest a much larger organoid after 3 months. Here, we probed another site, namely the subcutaneous space on the shoulder flank as published by others (38), which is why the cells are embedded in adipocytes. The rationale for doing this is that the skin is highly vascularized and the procedure is simpler than the major surgery that is involved in subcapsular kidney transplantation. We do not attribute differences in the speed by which normalization of the glucose levels occurred or in the degree by which correction happened to the injection site but instead to the maturity of the cells. Clearly, the cells generated through our latest protocol were significantly more mature than in our prior report, as demonstrated by the greater number of granules and the superior speed with which they corrected hyperglycemia. Interestingly, the IPCs were surrounded by adipocytes, which is consistent with the observation that insulin promotes adipocyte growth (48). Lastly, none of the mice developed teratomas. Thus, our data show rapid correction of hyperglycemia in diabetic mice using IPCs derived from T1D human iPS cells. Notably, we explored if removal of the graft resulted in hyperglycemia. Although it did lead to a significant rise in blood glucose levels (data not shown), overt hyperglycemia (>250 mg/dl) was not seen. This is likely because graft excision was not guaranteed, considering the original subcutaneous injection of the cells. This is a drawback of this method as opposed to injecting the cells under the kidney capsule.

Our findings are highly significant because iPS cell–based therapy for T1D will, in all likelihood, involve the patient’s own somatic cell–derived iPS cells (4, 5). With most prior reports using human ES cells or iPS cells derived from health subjects (7–12), we expect more studies to better understand the influence of T1D on the differentiation of iPS cells derived from individuals suffering from T1D. Indeed, our studies underscore the need to address inadequacies in the differentiation of some T1D iPS cells lines into IPCs before such therapy is hastily translated into the clinic. Here, we demonstrate a highly efficient protocol for using demethylation as a strategy to induce directed derivation of IPCs from resistant T1D patient–derived iPS cells. The success of a model in which iPS cells will one day be generated from T1D patients and used to generate IPCs will be highly dependent on the results of our current studies, which we hope one day to advance into the clinical realm.

**Experimental procedures**

**Differentiation of iPS cells into IPCs**

To initiate the differentiation of iPS cells into definitive endoderm (DE) cells, they were first maintained in the STEMdiff Definitive Endoderm Kit (catalogue no. 05110, STEMCELL Technologies, Vancouver, BC) for 5 days, while cultured on feeder cells as colonies. On day 5, expression of DE cell markers, such as CXCR4 and Sox17, was assessed on these cells to ensure that differentiation was proceeding properly. After confirmation that the culture contained >90% CXCR4+ Sox17+ cells, the rest of the DE cells were harvested and 3D differentiation was initiated with Media 2. If demethylation of the cells was performed, this was done according to details provided in the supplemental information. On the day of 3D differentiation (day 5), a 1:1 (v/v) mixture of liquid Matrigel hESC-Qualified Matrix (catalogue no. 354277, Corning Inc., Tewksbury MA) was mixed with cold DMEM/F-12 in a chilled conical tube. Then, in a 24-well plate, 100 μl of the 1:1 mixture was deposited in the center of each well and the plate was incubated at 37 °C for 5 min. When a rigid dome had formed in the center of each well, 400 μl of the 1:1 mixture was added on top of this dome and the plate was shaken to allow the Matrigel mixture to spread evenly, resulting in a 500-μl layer of Matrigel in each well. The plate was replaced at 37 °C for 3 h to allow the Matrigel to solidify sufficiently.
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2.5 h into the incubation, the DE cells were harvested and suspended in warm Media 2, which contains Y27632, a Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor intended to promote cell survival. This cell suspension (2.5–5 × 10^4/well) was distributed on top of the Matrigel, with each well thus containing 500 μl of the Matrigel mixture and 500 μl of the cell suspension. Generally, within the first 24 h, most of the DE cell clusters embed into the Matrigel scaffold, forming sphere-like clusters at varying depths in the Matrigel layer. The plate was left undisturbed for 24 h, at which point, the first media change in 3D culture was performed as following protocol (supplemental Table S1).

**Glucose-stimulated insulin secretion assay**

We performed static glucose-stimulated insulin secretion (GSIS) assays to determine the glucose responsiveness of IPC clusters (days 27–30 of culture) as compared with human islets (supplied by the Integrated Islet Distribution Program (IIDP)). We followed IIDP standard operating procedures to perform the GSIS assays (detailed in the supplemental information), and utilized the human Ultrasensitive Insulin ELISA (catalogue no. 80-INSHUU-E01.1, ALPCO Diagnostics, Salem, NH) according to manufacturer’s instructions for quantitation of insulin levels in the supernatant of these samples. The amount of insulin produced was normalized by the total protein in each sample, which was calculated via the Bradford assay of the lysate generated from the cell clusters.

**Mice and transplantation**

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa and the Iowa City Veterans Affairs Medical Center, and procedures were conducted in accordance with NIH guidelines. Other details of materials and methods are provided in the supplemental information.

**Author contributions**—G. S. M., E.-M. K., and N. Z. designed the experiments. G. S. M. and N. Z. wrote the manuscript. G. S. M. and E.-M. K. performed most experiments with the help of N. Z. All authors discussed the results and commented on the manuscript.

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**Note added in proof**—In the version of this article that was published as a Paper in Press on March 30, 2017, Figs. 1D and 3B were presented such that neighboring images appeared to be derived from each other. Additionally, the immunofluorescent image of human islet in Fig. 1D appeared as a composite. These presentational errors have been corrected and do not affect the results or conclusions of this work.

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