Reprogramming Adult Human Dermal Fibroblasts to Islet-Like Cells by Epigenetic Modification Coupled to Transcription Factor Modulation

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In this article, we describe novel conditions for culture, expansion, and transdifferentiation of primary human dermal fibroblasts (hDFs) to induce expression of transcription factors (TFs) and hormones characteristic of the islets of Langerhans. We show that histones associated with the insulin gene are hyperacetylated and that insulin gene DNA is less methylated in islet cells compared to cells that do not express insulin. Using two compounds that alter the epigenetic signature of cells, romidepsin (Romi), a histone deacetylase inhibitor, and 5-Azacytidine (5-AzC), a chemical analogue of cytidine that cannot be methylated, we show that hDFs exhibit a distinctive regulation of expression of TFs involved in islet development as well as of induction of glucagon and insulin. Overexpression of Pdx1, a TF important for islet differentiation, and silencing of musculoaponeurotic fibrosarcoma oncogene homolog B, a TF that is expressed in mature glucagon-producing cells, result in induction of insulin to a higher level compared to Romi and 5-AzC alone. The cells obtained from this protocol exhibit glucose-stimulated insulin secretion and lower blood glucose levels of diabetic mice. These data show that fully differentiated nonislet-derived cells could be made to transdifferentiate to islet-like cells and that combining epigenetic modulation with TF modulation leads to enhanced insulin expression.

Introduction

IN TYPE 1 DIABETES (T1D), THERE IS a loss of insulin-producing β cells and patients are dependent on daily insulin injections for their survival [1]. Several protocols to transplant islets from cadaveric donors have been developed [2,3], however, their use is limited mainly because of a shortage of donors [4,5]. Hence, generation of islet-like β -cells from other cell types may be used in place of bona fide islets and could result in significant improvement over current therapeutic approaches for patients with diabetes.

There have been advances in generating insulin-producing cells from other adult cell types. Ferber et al. [6–11] reported successful transdifferentiation of hepatocytes and keratinocytes into insulin-producing cells by ectopic expression of the transcription factor (TF) pancreatic and duodenal homeobox 1 (Pdx1), which is important for pancreatic development and β cell maturation. By combining Pdx1 with the TF Nkx6.1, they were able to induce insulin expression to a greater extent [7]. Other protocols using nonadult cells, including human embryonic stem cells, somatic stem cells, induced pluripotent stem cells (iPSCs), or mouse stem cells, were developed [12–24]. Additional factors were included in these protocols to increase insulin synthesis and glucose-responsive insulin secretion such as GLP1/exendin-4, Wnt3A, noggin, KAAD-cyc, B27, nicotinamide, activin A, retinoic acid, and growth factors.

Epigenetic modifications are thought to account for specification of gene expression in different tissues. In islets, as in all tissues, histone acetylation is associated with transcription activation [25] and has previously been indicated to play a role in regulating insulin and glucagon gene expression [26]. DNA methylation usually is associated with repression of transcription [27].

We studied DNA methylation in islet cells and in islet cells that had dedifferentiated in culture and no longer produced insulin and glucagon. Compared to islets cells, we observed that the insulin gene promoter is hypermethylated in dedifferentiated islet cells. Together with the histone underacetylation [26], we hypothesize that these two epigenetic changes may account, at least partially, for the repression in insulin and glucagon gene transcription.

Primary human dermal fibroblasts (hDFs) were recently shown capable of undergoing differentiation into a wide range of cell types, including neurons, blood progenitors, and adipocytes [28–34]. This process has been termed transdifferentiation. In this article, we describe a 5-day protocol to transdifferentiate primary hDFs into insulin- and glucagon-producing cells. We induce the expression of insulin through increasing acetylation and decreasing methylation by two epigenetic modifying compounds—romidepsin (Romi), a histone deacetylase inhibitor (HDACi) and 5-Azacytidine (5-AzC), a cytidine analog that cannot be methylated. To our

knowledge, only a single previous study reported [35] the use of a HDACi in an attempt to transdifferentiate adult cells, in this case, bone marrow cells to insulin-producing cells. We also studied the effect of silencing of musculoaponeurotic fibrosarcoma oncogene homolog B (MafB), a TF previously described to be specific for glucagon-producing α -cells in adults and inducing glucagon [36–38], in conjunction with overexpression of Pdx1, a pancreatic TF crucial for islet development, specific for adult insulin-producing cells [39,40] and an activator of insulin gene transcription. In combination with Romi and 5-AzC, we found that overexpression of Pdx1 resulted in higher insulin and glucagon transcription and silencing of MafB resulted in induction of insulin to higher levels as well as reduction of glucagon transcription.

Materials and Methods

Ethics statement

All animal work was conducted according to national and international guidelines.

Cells

Adult human primary dermal fibroblasts were obtained from ATCC. Human islets were obtained from cadaveric pancreases from the National Islet Cell Resource Center Basic Science Islet Distribution Program. Islets were dispersed with 0.05% Trypsin-EDTA (Cellgro; Mediatech) for 5 min at 37°C, and then infected for 72 h with an adenovirus-containing green fluorescent protein (GFP) under the control of the rat insulin promoter. β -cells were sorted with the BD FACSARIA II cell sorter. Dedifferentiated islets (islet-derived dedifferentiated cells that can be induced under serum deprivation to islet-like structures following proliferative expansion) were derived from fresh human islets as described previously [41]. Islet cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 2% fetal calf serum, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 2 mM glutamine.

Viruses

Adenoviruses expressing mouse Pdx1, mouse MafA, and mouse Nkx6.1 were obtained from the Beta Cell Biology Consortium. Adenovirus-expressing rat NeuroD1 was obtained from Dr. Harry Heimberg [42].

A lentivirus encoding the -1600bp of the rat glucagon promoter fused to the GFP reporter gene was generated by cloning it into pLenti4/V5-DEST. shMafB lentivirus was purchased from Sigma Aldrich (Mission RNAi system).

Materials

Romidepsin was generously provided by Celgene Corporation and the National Cancer Institute, NIH. 5-AzC was purchased from Sigma Aldrich.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with 100 μ g of cell chromatin extracts. Assay was carried out using the Active Motifs chromatin shearing kit. Chromatin was incubated with acetylated histone H3 and H4 antibodies (Millipore) as well as rabbit IgG (Abcam). Binding was analyzed by real-time

PCR with the following sets of PCR primers and 6FAM-labeled probes: hIns-275(F) TGTGAGCAGGGACAGGTC TG; hIns-275(R) TCCTCAGGACCAGCGGG; hINS-275(P) 6FAM-CCACCGGGCCCCCTGGTTAAGACTCTA; hGCG-207(F) CTT AAGTGATTTTCATGCGTGATTG; hGCG-207(R) TG GGA ATGGAGAGAGCAGCTT; hGCG-207(P) 6FAM-AAG TAGAAGGTGGATTTC; hPdx1: hPdx1-206 (F) ATTTTCGTAT GGGGAGATGTTTCG, hPdx1-206(R) CTCCGCCGCCACCC CAATTAAC, hPdx1206(P) 6FAM-CTACGTACCTATACAT AAACCGCCAA.

Insulin and glucagon measurements

Cells were incubated for 2 h at 37°C in the Krebs-Ringer bicarbonate buffer with Hepes (KRBH) containing 1.67 mM glucose. Indicated glucose or deoxyglucose concentrations were then added to the cells for 1 h at 37°C in KRBH. Glucagon in incubation buffers and cell extracts was measured by radioimmunoassay (Millipore), and insulin in incubation buffers and cell extracts was measured by enzyme-linked immunosorbent assay (Mercodia). Secretion was expressed as percentage of the total insulin content.

Immunofluorescence

Cells were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.15% Triton $\times$ 100 in a blocking buffer containing 4% normal donkey serum. Cells were incubated with rabbit anti-human glucagon (Dako North America), rabbit anti-human c-peptide (Linco Research), rabbit anti-MafA (Bethyl Laboratories), rabbit anti-MafB (Bethyl Laboratories), or rabbit anti-Pdx1 (Abcam) antibodies in a blocking buffer for 1 h at 37°C, washed with the buffer, and then incubated with fluorescent secondary antibodies (Life Technologies) in the blocking buffer for 1 h at room temperature.

Coverslips were washed, mounted in Mowiol[®] (Sigma-Aldrich) with DAPI (Sigma-Aldrich), and images captured on a Zeiss META NLO confocal microscope.

Fluorescence in situ hybridization (FISH)

Cells were fixed in 4% formaldehyde in 2 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate) for 15 min and followed by dehydration in ethanol.

Probes were purchased from Eurofins MWG Operon.

Ins:

[AminoC6+Alexa488]CCCGCCCAGCTCCACCTGCCCCAC CTGCAGGTCTCTGCCTCCCGGCG and
[AminoC6+Alexa488]GGTGTGAGCCGCACAGGTGTTGGT TCACAAAGGCTGCGGCTGGGTCAGG Gcg:
[AminoC6+Alexa546]GCCTGGGAAGCTGAGAATGATCT GGATTTCTCTCTGTGTCTTGAAGG and
[AminoC6+Alexa546]GACGTTTGGCAATGTTATTCCTGTT CCTCTTGGTATTCATCAACCACTGC

Approximately 20 ng of each of the biotinylated probes was added to each slide together with 10 μ g of salmon sperm DNA in a hybridization buffer (50% formamide/1 $\times$ SSC/10% dextran sulfate). Hybridization was performed for 16–20 h at 37°C. Posthybridization washing consisted of two 5-min immersions in 50% formamide and 2 $\times$ SSC, followed by 2 $\times$ SSC and 0.1 $\times$ SSC at 37°C. The slides were dipped briefly

in water, allowed to air dry, and Mowiol mounting fluid containing DAPI added. Fluorescence localization was analyzed by confocal microscopy (Carl Zeiss Jena).

Fluorescence-activated cell sorting (FACS)

Flow cytometry analysis was performed using a BD FACSaria II cell sorter (Becton Dickinson).

Real-time RT-PCR

Quantitative PCR was performed on DNA and cDNAs. All reactions were conducted in 96-well plates in 25 μ L. Each reaction contained cDNA from 100 ng total RNA, or DNA precipitated from 100 μ g of chromatin extracts, for ChIP experiments, and appropriate amount of Universal PCR Master Mix (Applied Biosystems) and Primers/Probe mix. All primers and probes are from Applied Biosystems Assay-on-Demand. The cycle threshold value >40 was considered undetectable and calculated as a Ct of 41.

Bisulfite DNA sequencing

Genomic DNA was isolated from 10^6 cells by using Non-Organic DNA Extraction Kit (Millipore). Briefly, cells were detached and collected by centrifugation. Cell membranes were disrupted by incubation with a hypotonic buffer, and nuclei were collected and enzymatically deproteinated. Genomic DNA was purified by ethanol precipitation. One milligram of genomic DNA was treated using the CpGenome DNA Modification Kit (Millipore), according to the manufacturer's instructions, to produce the bisulfite-treated DNA (BS-DNA). Final DNA pellets were resuspended in 25 μ L ddH₂O.

The insulin promoter was amplified using 1 μ L of BS-DNA, the following primers: forward TTGTTTTAGTTGTGAG TAGGGATAG and reverse AACTTTATAATCTCAAAAC CCATCTC (annealing temperature 55°C), and HotStarTaq Master Mix (Qiagen). A second round of PCR was performed to produce enough product, using 1 μ L of the first round PCR product (in a 50 μ L reaction), and the following primers: forw-BamHI AGGAGGATCC TTGTTTTAGTTGTGAG TAGGGATAG and rev-XhoI AGGACTCGAG AACTTTA TAATCTCAAAACCCATCTC (annealing temperature 55°C), which incorporate adhesive restriction sites. The PCR products were digested by BamHI and XhoI (New England Biolabs Ipswich) at 37°C for 2 h, purified by the QiaQuick PCR Purification Kit (Qiagen), and ligated with the pShuttle-V5 vector (predigested and purified). The ligation was used to transform competent *Escherichia coli* cells (One Shot[®] Omni-MAX[™] 2 T1^R; Invitrogen), and selected by Kana+ LB-agar plates overnight at 37°C. At least 20 colonies were picked up, and the inserts were amplified by PCR supplemented with 5% DMSO, with the following primers: pShuttleF TAAGA AGCTTGGTACCGAATTC and pShuttleR GCTCGCCG CAGC CGAACGACCG (annealing temperature 60°C). Clones with the correct size (about 450 bp) were analyzed by DNA sequencing with primers pShuttleF or pShuttleR (Eurofins MWG Operon).

Mice

About 1.5×10^6 of either hDFs that underwent the reprogramming protocol, or control hDFs were transplanted under the kidney capsule into NOD.Cg-Rag1^{tm1Wjl} Ins2^{Akita}

Il2rg^{tm1Wjl}/SzJ (NRG-Akita) female mice (Jackson Laboratory) at 11 weeks of age. Blood glucose levels were measured using the Ascensia Contour glucometer (Bayer) on a bi-weekly basis a week before surgery and up to 3 weeks postsurgery. The C-peptide was measured at 3 weeks postsurgery using the Mercodia rat ultrasensitive kit. A total of three mice per group are described here.

Data analysis

Data are presented as mean $\pm$ S.E (standard error) of at least three different experiments and analyzed by the Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Histone H3 and H4 were previously described to be acetylated in islets at the proximal promoter regions of the insulin, glucagon, and Pdx1 genes as well as exons 1 and 3 of the insulin gene [26,43–46]. This modification is often associated with activation of gene transcription [25]. As islet cells proliferate and adapt in culture, they stop expressing insulin and glucagon. In those dedifferentiated islet cells and in mesenchymal stem cells (MSCs), the insulin and glucagon were found to be less highly acetylated [26,47].

We examined whether other epigenetic modifications might account for the differences between islets and dedifferentiated islet cells. We studied DNA methylation at the insulin gene promoter and found it to be less highly methylated in islets compared to dedifferentiated islets (Table 1). Since gene methylation is often associated with repression [27], we hypothesized that the decreased methylation on the insulin gene in islet β cells compared to cells that do not express insulin would account for the differences in expression levels.

Building upon the above results, we used two compounds, Romi, a HDACi, and 5-AzC, a cytidine analogue that cannot be methylated, to change the epigenetic modifications of cells in culture. In preliminary experiments with dedifferentiated islet cells, where neither glucagon or insulin transcripts are detectable, 30 nM Romi upregulated glucagon mRNA levels, but not insulin levels and 1 μ M 5-AzC did not induce either gene. When the cells were treated with both Romi and 5-AzC, the expression of the insulin and glucagon genes was induced (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Based on these findings, we were encouraged to use these agents in other adult cell types.

Implementing transplantation protocols using islets or dedifferentiated islets is difficult because of the shortage of

TABLE 1. METHYLATION STATE OF THE INS PROMOTER IN β -CELLS AND DEDIFFERENTIATED ISLET CELLS

	% Methylation					
Promoter region	–234	–206	–180	–135	–102	–69
β -cells	61	61	43	52	13	65
Dedifferentiates islets	96	46	38	88	38	92

At least 20 bisulfite sequencing of the insulin proximal promoter in islet β -cells and dedifferentiated islets. Values are the percentage of DNA methylation within the different positions of CpG sites.

cadaveric donors. hDFs can be easily obtained from patients, expanded many fold in culture, and used for autologous transplantation. We obtained similar findings in hDFs on the insulin and glucagon genes as we did in dedifferentiated islet cells. In hDFs, Romi alone was able to induce only glucagon transcription and 5-AzC alone did not induce either glucagon or insulin (data not shown). In contrast, Romi plus 5-AzC induced an increase in both glucagon and insulin genes (Fig. 1). These increases in mRNA levels remained stable over time if cells were first treated with a low dose of Romi (3 nM) for 48 h, and then exposed to 30 nM Romi and 1 μ M 5-AzC every 48 h.

To better understand the reprogramming of hDFs into islet-like cells, we studied the effects of pancreas-specific TFs on their transdifferentiation. The use of pancreatic TFs was previously described to induce insulin gene transcription or even convert glucagon into insulin-expressing cells [48–50]. We chose to study five pancreas-specific TFs, namely, Pdx1, MafA, Nkx6.1, NeuroD1, and Pax4 by expressing them via adenoviral infection in hDFs. With the exception of Pdx1, none of these TFs, either individually or in combination, resulted in induction of insulin gene transcription (the data for Pdx1 is shown in Fig. 1). Although expression of some of the other TFs following treatment with Romi and 5-AzC resulted in an increase in expression of insulin compared to Romi and 5-AzC alone, only Pdx1 plus Romi and 5-AzC caused a robust increase in insulin mRNA. Similar results were obtained with hDFs obtained from two other individuals (data not shown). Pancreas-specific TFs as well as both Glut1 and Glut2 glucose transporters specific for glucagon- and insulin-producing cells, respectively, were also upregulated by Romi and 5-AzC, or by the TF Pdx1, or a combination of Romi and 5-AzC with Pdx1. Except for PPAR γ , none of the islet-specific markers we measured was detectable in hDFs. PPAR γ was upregulated by $\sim$ 20-fold by Romi and 5-AzC. We validated that the increases observed for MafA, MafB, and Pdx1 mRNAs were associated with increases in protein levels (Supplementary Fig. S2).

FIG. 1. Transcriptional effects of Romi, 5-Azacytidine (5-AzC), and transcription factor (TF) on islet-specific hormones, TFs, and transporters. Real-time PCR analysis of gene expression in human dermal fibroblasts (hDFs) undergoing infection by adenoviral pancreatic and duodenal homeobox 1 (Pdx1) for 72 h and/or addition of 30 nM Romi and 1 μ M 5-AzC for 48 h. Data are expressed relative to GAPDH mRNA. Values are the mean $\pm$ S.E. from three separate experiments, each analyzed in duplicate. *, statistical significance with $P < 0.05$ value using a Student's *t*-test.

FIG. 2. Following treatment of hDFs with Pdx1, Romi, and 5-AzC, the Gcg, Ins, and Pdx1 promoter regions are more acetylated. Chromatin immunoprecipitation for acetylated histones H3 and H4 in hDFs undergoing the reprogramming protocol. Values are the mean $\pm$ S.E. from three separate experiments, each analyzed in duplicate. *, statistical significance compared to hDF control levels with $P < 0.05$ value using a Student's *t*-test.

We therefore established a protocol to transdifferentiate hDFs into islet-like cells producing insulin and glucagon. Primary hDFs were infected with Pdx1 adenoviruses (MOI=50). Seventy-two hours later, 30 nM Romi and 1 μ M 5-AzC were added for 48 h. The analyses described below are for cells that underwent this reprogramming protocol after 5 days.

Comparable to what we observed in dedifferentiated islets, the insulin, glucagon, and Pdx1 promoter regions in hDFs are poorly acetylated (Fig. 2). Following addition of Romi and 5-AzC, histones H3 and H4 bound to the insulin, glucagon, and Pdx1 promoter regions are more highly acetylated as determined by ChIP (Fig. 2). Also, the insulin gene promoter is less methylated following the reprogramming protocol as estimated by bisulfite sequencing (Table 2). These two epigenetic modifications observed in hDFs following our reprogramming protocol may account for the changes in gene expression.

We confirmed the presence of c-peptide and glucagon by immunofluorescence staining. Over 80% of the cells stained positive for glucagon, while only around 2%–4% of the cells were positive for insulin. The TF Pdx1 was present in about 60% of the cells (Fig. 3a).

To study whether the cells are secreting insulin in response to glucose, we measured insulin in the medium after

TABLE 2. METHYLATION STATE OF THE INS PROMOTER IN hDFs AND hDFs AFTER THE REPROGRAMMING PROTOCOL

	% Methylation					
Promoter region	–234	–206	–180	–135	–102	–69
hDFs	100	100	91	89	58	91
Pdx1+Romi+5AzC	96	89	97	85	14	76

At least 20 bisulfite sequencing of the insulin proximal promoter in control hDFs and hDFs after the reprogramming protocols. Values are the percentage of DNA methylation within the different positions of CpG sites.

FIG. 3. Glucagon and insulin are produced and secreted from hDFs undergoing the reprogramming protocol. **(a)** *Left panel*—immunolabeling of glucagon (red) and c-peptide (green). *Right panel*—quantification of cells stained positive for each antibody tested. *, statistical significance with $P < 0.05$ value using a Student's *t*-test. **(b)** Enzyme-linked immunosorbent assay of the relative amount of insulin secreted versus the cellular insulin content in control hDFs and hDFs that have undergone the reprogramming protocol. **(c)** The ratio of glucagon secreted per cellular content of glucagon as measured by radioimmunoassay with different glucose concentrations in control cells and hDFs undergoing the reprogramming protocol. Data represent percent of hormones secreted versus the hormone content. Values are the mean $\pm$ S.E. from three separate experiments. *, statistical significance with $P < 0.05$ value using a Student's *t*-test.

incubation in low and high glucose concentrations (Fig. 3b). In a high glucose concentration (16.7 mM), cells that underwent the reprogramming protocol secrete twofold more insulin than these same cells in low glucose concentrations (1.67 mM). By contrast, high concentrations of deoxyglucose, a nonmetabolizable analogue of glucose, failed to induce insulin secretion from these cells. Glucagon secretion was significantly inhibited by high glucose concentrations, similar to islet cells. Deoxyglucose, however, did not reduce glucagon secretion (Fig. 3c). These data suggest coupling between insulin and glucagon storage within the cells and glucose sensing in the reprogrammed cells.

To better characterize the cells obtained from the reprogramming protocol, we stably infected primary hDFs with a lentivirus containing GFP under the control of the 1600 bp glucagon promoter. Following the reprogramming protocol, we sorted these cells by FACS (Fig. 4a). About 80% of the cells were GFP-positive. The GFP-positive cells were enriched with glucagon mRNA and did not express insulin mRNA (Fig. 4b). The GFP-negative population contained less glucagon mRNA, but was enriched for insulin mRNA. These results indicate that we obtained two cell populations: the majority of the cells express glucagon almost exclusively, while a small percentage of the cells express insulin.

To confirm that we obtained two distinct cell populations, we performed fluorescence in situ hybridization with probes for insulin and glucagon mRNAs. In concordance with the results obtained from the FACS sorting, there were no cells that coexpressed both glucagon and insulin, confirming the above results that there are two cell populations, one that expresses insulin and the other glucagon (Fig. 4c).

To further characterize the cell populations obtained, we performed immunostaining of the sorted cells. The GFP+ population was, as expected, enriched with glucagon-positive cells. The proportion of cells positive for Pdx1 was equally distributed between the GFP-positive and -negative populations. Interestingly, more GFP+ cells were also positive for MafA and MafB and all cells that stained positively for MafB were also positive for glucagon (Fig. 5a).

We hypothesized that by decreasing the MafB-positive cells we would decrease the proportion of glucagon-positive cells and potentially increase the proportion of insulin-positive cells. For that purpose, we stably infected hDFs with a lentivirus containing MafB shRNA before pursuing the reprogramming protocol. The level of MafB mRNA was decreased by 10-fold, but there was no effect on Pdx1 or MafA mRNA levels (Fig. 5b). We found that silencing of MafB resulted in increased insulin and decreased glucagon mRNA levels thereby improving our reprogramming protocol.

Finally, we transplanted NRG-Akita mice that are homozygous for the Rag1^{tm1Mom} and Il2rg^{tm1Mom} alleles and heterozygous for the Ins2^{Akita} allele, which are both immunodeficient and develop spontaneous hyperglycemia [51,52], with 1.5×10^6 hDF/mouse that underwent the reprogramming protocol as well as control hDF cells. We were able to detect the human c-peptide in the serum of mice transplanted with the cells that underwent the reprogramming protocol at 3 weeks postsurgery (18.4 ± 3.3 pmol/L), while no human c-peptide was detected in the control hDF transplanted mice. Blood glucose levels were also consistently lower in the mice transplanted with the reprogrammed fibroblasts: 477 ± 24 mg/dl glucose (mean value for days 7 to

FIG. 4. The cells originating from the reprogramming protocol consist of at least two populations, one that is enriched for glucagon and the other enriched for insulin. **(a)** Human primary dermal fibroblasts were stably infected with a lentivirus containing green fluorescent protein (GFP) under the control of the glucagon gene promoter. *Left panel*—control hDFs were infected with LacZ adenovirus, not Pdx1, and were not exposed to Romi and 5-AzC. *Right panel*—cells undergoing the reprogramming protocol. Cells were sorted by FACS into cells expressing GFP and GFP-negative cells. **(b)** Real-time PCR analysis of glucagon and insulin in hDFs undergoing the protocol. Data are expressed relative to GAPDH mRNA. Values are the mean $\pm$ S.E.M from three separate experiments, each analyzed in duplicate. *, statistical significance with $P < 0.05$ value using a Student's *t*-test. **(c)** Fluorescent in situ hybridization with insulin (green) and glucagon (red) probes in cells under the reprogramming protocol.

22) in mice transplanted with the reprogrammed hDFs versus 609 ± 40 mg/dl glucose (Fig. 6) in mice transplanted with control hDFs ($P < 0.01$). These results suggest that the reprogramming protocol described in this article generated cells that could potentially control glucose homeostasis in vivo.

Discussion

In this study, we showed that we can generate cells that secrete insulin in response to glucose from adult hDFs using two epigenetic modifying compounds. We further increased the efficiency of this process by adenoviral transduction of the Pdx1 TF in parallel to silencing the transcription of MafB.

We found that the majority of the cells that underwent the reprogramming protocol described in this article were glucagon-positive, while part of the remaining population was insulin-positive. Only a small number of cells coexpressed insulin and glucagon, similar to the adult endocrine pancreas. While it is possible that after 72 h in the reprogramming protocol the cells transition from a bi-hormonal state to a fully differentiated state [53], it is also possible that the single hormone-expressing cells we observed did not transition from a bi-hormonal stage, but were generated from a precursor cell that did not express any hormone. This is because it was previously described that other endocrine cells of the pancreas might originate from glucagon-expressing α -cells [53–55]. However, lineage-tagged transgenic mice showed that insulin and glucagon cells might originate independently in adults [56,57].

This study also demonstrates that human fibroblasts could be directly induced to become functional insulin- or glucagon-secreting cells without the need of going through an intermediate state to induce a pluripotent state by overexpressing pluripotent TFs. Pang et al. [58] as well as

Szabo et al. [28] have previously shown that fibroblasts can be differentiated into neuronal or blood progenitor cells. Other recent studies also support the idea that it is unnecessary to go through a pluripotent state to obtain a functional cell that secretes insulin in response to glucose [6,9,35].

DNA methylation and histone modifications constitute major mechanisms that are responsible for epigenetic regulation of gene expression during development and differentiation [59–61]. The use of HDACis and DNA methylation inhibitors has previously been proposed to be of use in cell reprogramming [35,62–65]. Trichostatin A, a HDACi, and 5-AzC were demonstrated to induce β -cell differentiation from rat multipotent nestin-positive bone marrow stem cells [63]. Similarly, it was shown that sodium butyrate and trichostatin A promote ductal differentiation at the expense of the acinar fate. Following that, cells with exocrine function are converted to endocrine cells, capable of producing hormones such as insulin and somatostatin [62,66]. Our study suggests that compounds that modify histone acetylation and DNA methylation can also be used in adult human fibroblasts to give similar expression of pancreatic hormones.

In agreement with our finding that methylation on the insulin promoter is inversely correlated with insulin expression, recent studies proposed that insulin gene expression is regulated by DNA methylation [67,68]. Kuroda et al. showed that while the degree of DNA methylation is low in the insulin promoter of β -cells, the same promoter is highly methylated in other cell types [68].

The TF Pdx1 has been shown to be essential for the normal proliferation and differentiation of embryonic pancreatic precursors [39,40,69]. Pdx1 is expressed early in all pancreatic progenitor cells and is later restricted to adult β -cells plus a fraction of δ -cells. Lineage tracing studies have illustrated

FIG. 5. Musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) and glucagon expression overlap and silencing of MafB results in more insulin and less glucagon expression. (a) Quantification from immunofluorescence staining of the sorted cells described in Figure 4a. Data shown as % of cells stained positive for each protein tested or a combination of glucagon and TF. (b) Quantitative real-time PCR of hDFs under the reprogramming protocol or silenced for MafB before the reprogramming protocol. Values are the mean $\pm$ S.E. from three separate experiments. *, statistical significance with $P < 0.05$ value using a Student's *t*-test.

that *Pdx1*-expressing cells at E8.5 are multipotent progenitors, giving rise to all pancreatic lineages [70–72]. Several studies have previously shown that ectopic expression of *Pdx1* can result in differentiation to a β -like cell phenotype. We show that overexpression of *Pdx1* in hDFs results in expression of glucagon, but not insulin. We observed a similar effect of *Pdx1* overexpression in MSCs [26]. However, addition of *Pdx1* before Romi and 5-AzC resulted in expression of both insulin and glucagon to a greater extent than Romi and 5-AzC alone.

The TF MafB is restricted to α -cells in adults. During pancreatic development, at E10.5, MafB is expressed in glucagon-positive cells and at E15.5, although all glucagon+ cells express MafB, a significant proportion of glucagon-negative cells express MafB. These early MafB+/Glucagon- cells express the panendocrine marker synaptophysin and some even express insulin [37,38], suggesting that MafB could play a role in the differentiation of β -cells. In addition, MafB knockout in mice was also shown to regulate the ex-

FIG. 6. Transplantation of reprogrammed hDFs heterozygous into Akita mice results in decreased blood glucose levels. *Cg-Rag1^{tm1Mom} Ins2^{Akita} Il2rg^{tm1Wjl}/SzJ* (NRG-Akita) female mice were transplanted with either three separate populations of hDFs, which were reprogrammed separately and transplanted into three mice (1.5×10^6 hDFs were infected with *Pdx1* adenoviruses and 72 h later, 30 nM Romi and 1 μ M 5-AzC were added for 48 h), or with control DFs. Blood glucose levels were measured before and postsurgery (surgery day=0). Values are the mean $\pm$ S.E. from three separate mice. *, statistical significance with $P < 0.05$ value, using a Student's *t*-test.

pression of genes specific for β -cell differentiation such as *Pdx1*, *Nkx6.1*, and *GLUT2* and to be crucial for β -cell development [36,73]. In this study, we noted that following the reprogramming protocol, MafB expression is increased. By lowering the expression of MafB, we obtain higher levels of insulin mRNA and less glucagon mRNA, indicating that fine tuning of MafB expression improves the transdifferentiation of hDFs toward a β -cell phenotype.

This study used adult human cells to transdifferentiate them into islet-like cells. Of note, hDFs are readily obtainable from individuals and the number of cells can be expanded in culture before undergoing the reprogramming protocol. The combination of using epigenetic modulating factors together with overexpression and silencing of key genes important for endocrine pancreas development resulted in the production of functional insulin-secreting cells that can secrete insulin and decrease glycemic levels in diabetic mice.

The method described in this article provides cells that could be obtained directly from a diabetic patient, reprogrammed, and transplanted back to the patient, hence, bypassing the need of a cadaveric donor and a potential rejection by the immune system. The presence of both functional insulin- and glucagon-secreting cells might improve the patient's glycemic control as is the case for exogenous bihormonal treatment for diabetes [74,75].

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Author Disclosure Statement

M.C.G. and L.S.K. have a patent application for the Method of Reprogramming Differentiated Somatic Cells to Islet-Like Cells.

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