**Klf6 protects β-cells against insulin resistance-induced dedifferentiation**

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**ABSTRACT**

**Objectives:** In the pathogenesis of type 2 diabetes, development of insulin resistance triggers an increase in pancreatic β-cell insulin secretion capacity and β-cell number. Failure of this compensatory mechanism is caused by a dedifferentiation of β-cells, which leads to insufficient insulin secretion and diabetic hyperglycemia. The β-cell factors that normally protect against dedifferentiation remain poorly defined. Here, through a systems biology approach, we identify the transcription factor Klf6 as a regulator of β-cell adaptation to metabolic stress.

**Methods:** We used a β-cell specific Klf6 knockout mouse model to investigate whether Klf6 may be a potential regulator of β-cell adaptation to a metabolic stress.

**Results:** We show that inactivation of Klf6 in β-cells blunts their proliferation induced by the insulin resistance of pregnancy, high-fat high-sucrose feeding, and insulin receptor antagonism. Transcriptomic analysis showed that Klf6 controls the expression of β-cell proliferation genes and, in the presence of insulin resistance, it prevents the down-expression of genes controlling mature β-cell identity and the induction of disallowed genes that impair insulin secretion. Its expression also limits the transdifferentiation of β-cells into α-cells.

**Conclusion:** Our study identifies a new transcription factor that protects β-cells against dedifferentiation, and which may be targeted to prevent diabetes development.

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**Keywords** Type 2 diabetes; Insulin resistance; β-Cell proliferation; Dedifferentiation; Transdifferentiation

1. INTRODUCTION

Pancreatic β-cells play a central role in glucose homeostasis by secreting insulin in response to increases in glycemia to stimulate glucose utilization by liver, fat, and muscle and to inhibit hepatic glucose production. In various physiological states, such as pregnancy, obesity, or aging, insulin target tissues become partially resistant to the action of this hormone and preserving euglycemia necessitates a compensatory increase in β-cell mass and insulin secretion capacity [1–3]. This functional adaptation is, however, limited and further worsening of insulin resistance may cause β-cell functional failure and reduced cellular mass, leading to development of diabetic hyperglycemia.

The mechanisms of β-cell adaptation to, or failure in insulin resistant states are not fully understood. Analysis of the pancreas of brain-dead organ donors showed that β-cell mass was increased in obesity but reduced in type 2 diabetes of long duration [4]. At the time of diabetes diagnosis, however, β-cell mass was found to be normal [5], implying that a defect in β-cell secretion capacity precedes loss of mass. The loss of β-cell function induced by insulin resistance is now recognized to be associated with, and probably caused by their dedifferentiation [6,7]. This process is characterized by the down-expression of genes controlling glucose-stimulated insulin secretion (GSIS) (Slc2a2, Gck, Kir6.2, Vdac1), genes encoding mature β-cell transcription factors (Pdx1, Nkx6.1, Pax6, Foxo1), and the expression of normally disallowed genes, such as Hk1, LdhA, and Slc16a2, which modify glucose metabolism and consequently further impair GSIS [6,7]. How β-cell dedifferentiation is triggered is not fully understood. Earlier experiments have shown that transplanting control islets into diabetic mice led to a rapid loss of Glut2 expression and of GSIS, whereas the transplantation of islets from diabetic animals into control mice led to the restoration of both parameters [8,9]. Thus, disturbances in the metabolic milieu of (pre)-diabetic mice may induce β-cell dedifferentiation and failure. Hyperglycemia, elevated plasma glucocorticoids, and free fatty acids [3,6,10–12] may contribute to induce these deregulations.

Another aspect of the development of β-cell dysfunctions in the presence of insulin resistance is their dependence on the genetic
architecture of each individual. It is indeed known that only a fraction of obese, insulin-resistant individuals progress to type 2 diabetes [13]. Similarly, mice with different genetic backgrounds display very heterogeneous deregulations of insulin secretion or insulin action when fed a high-fat, high-sucrose (HFHS) diet. To identify genes controlling β-cell functional adaptation, we recently used this mouse genetic diversity to perform a systems biology investigation of pancreatic islet adaptation to a metabolic stress. We characterized the physiological adaptation of mice from different inbred strains fed an HFHS diet for different periods of time and performed parallel islet transcriptomic analysis [14]. Weighted gene co-expression network analysis (WGCNA) [15] yielded islet gene modules that correlated with the measured phenotypes. Detailed analysis of one such module led to the identification of Elovi2, an enzyme that generates docosahexaenoic acid, an important regulator of GSIS which also confers protection against glucotoxicity-induced apoptosis [14,16].

Here, following a similar approach, we investigated the role of Krüppel-like factor 6 (Klf6), a zinc finger transcription factor [17], in β-cell adaptation to insulin resistance. The study of β-cell-specific Klf6 knockout mice showed that this transcription factor is required for β-cell proliferation during pregnancy, in response to high-fat diet feeding, and following pharmacological induction of insulin resistance. Furthermore, we show that Klf6 protects β-cells against insulin resistance-induced dedifferentiation and transdifferentiation into α-cells.

2. MATERIALS AND METHODS

2.1. Reagents

Mouse insulin and glucagon enzyme-linked immunosorbent assays (ELISAs) were purchased from Mercodia (Uppsala, Sweden) and radioimmunoassay (RIA) kits for insulin and glucagon were from Merck Millipore (MA, USA). Exendin-4 was purchased from Bachem (Bubendorf, Switzerland), and S961 and Exendin-4-Cy3 were kind gifts from Dr. Laue Schäffer and Dr. Jacob Hecksher-Sørensen (Novo Nordisk, Copenhagen, Denmark), respectively.

2.2. Antibodies

Rabbit anti-Ki67 was from Abcam (ab15580). Guinea pig antibodies to insulin and glucagon were purchased from Dako (A0564) and Linco (4031-01 F), respectively. Rabbit anti-GFP has been previously described [18]. Secondary antibodies were goat anti-rabbit immunoglobulin (IgG) antibodies coupled to Alexa-Fluor 488-labeled anti-guinea pig immunoglobulin antibodies (Invitrogen; A11073) and Alexa-Fluor 488-labeled anti-rabbit IgG antibodies (Invitrogen; A11008).

2.3. Mouse maintenance and diet

Male and female mice were housed in groups of 2–5 per cage on a 12-hr light/dark cycle with unlimited access to a standard rodent chow diet (Diet 3436, Provimi Kliba AG, Kaiseraugst, Switzerland) or an HFHS diet (Purified Diet 235HF, Safe Diets, Augy, France). Experiments were performed on 10- to 32-week-old mice with the respective age- and sex-matched control litters. The mouse experiments were approved by the Veterinary Office of Canton de Vaud, Switzerland.

2.4. Generation of β-cell-specific Klf6 knockout mice

Ins1Cre+ mice [19] were crossed with Klf6fl/fl mice [20] to generate Klf6fl/lox, Ins1Cre+ mice (Klf6KO) and Klf6fl/lox, Ins1Cre/- mice (Ctrl). Genomic DNA (gDNA) was extracted with the Quick gDNA MiniPrepTM kit according to manufacturer’s instructions (LucernaChem AG, Luzern, Switzerland), and genotyping was performed by PCR analysis using the following primers (see Figure 2) P1: 5’-TGGAGGAAATTTGGCAACAG-3’; P2: 5’-AACGATACCGGGTGCCACG-3’; P3: 5’-GCTCTTCGTCGGTCAATT-3’; P4: 5’-TGCTCTCAGTGGTGCCAG-3’. Cell lineage tracing experiments were performed using Rosa26tdTomato mice bred with Ins1Cre+ and with Klf6fl/lox, Ins1Cre+ to generate Klf6+/-; Rosa26tdTomato, Ins1Cre+/- and Klf6fl/lox, Rosa26tdTomato, Ins1Cre+/-.

2.5. Quantitative real-time PCR and gene expression analysis

Total RNA from islets was extracted using the RNeasy Plus micro kit (Qiagen, Hombrechtikon, Switzerland). cDNA was synthesized by reverse transcription of 1 μg of total RNA using random primers (Promega) and M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. Gene expression was measured in a final volume of 10 μl by quantitative real-time PCR using 5 μl of Power SYBR Green Master Mix (Applied Biosystems, Zug, Switzerland) and 2 μl of cDNA in a 7500 Fast Light Cycler System (Applied Biosystems, Zug, Switzerland). Results were normalized to the housekeeping gene mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Primers were obtained from Microsynth (Baligach, Switzerland). Specific mouse primers for each sequence are listed in Supplementary Table 5.

2.6. Glucose tolerance test and in vivo insulin secretion

For glucose tolerance tests, mice were fasted overnight and placed in individual cages. Blood glucose was measured from the tail vein using a Breeze2 glucometer (Bayer, Zürich, Switzerland) before and at the indicated times following an intraperitoneal (i.p.) injection of glucose (2 g/kg). The same protocol was applied for in vivo insulin secretion, in which blood sampling from the tail vein was used for plasma insulin level measurements by ELISA.

2.7. Pancreatic insulin and glucagon content

Excised pancreata were rapidly weighed and sonicated in ethanol-acid acid solution (75% EtOH; 0.55% HCl). After overnight storage at 4 ºC, samples were centrifuged at 4 °C for 5 min at 800 rpm. The supernatant was then centrifuged at 4 °C for 20 min at 4,000 rpm. The final supernatant was assessed for insulin and glucagon by radioimmunoassay (RIA). Results were normalized to initial pancreas weight.

2.8. Pancreatic islet isolation, insulin secretion, and proliferation assays

Islets from 10- to 12-week-old male mice were isolated by hand-picking following pancreata digestion by Liberase [21]. After overnight recovery in cell culture medium, insulin secretion measurements were performed on batches of 10 islets. Islets were first pre-incubated for 2 h in Krebs–Ringer Bicarbonate-HEPES buffer with bovine serum albumin (KRBH-BSA) (120 mM of NaCl, 4 mM of KH2PO4, 2 mM of KCl, 1.5 mM of CaCl2, 1.2 mM of MgCl2, 11 mM of glucose, 11 mM of NaHCO3, 5.5 mM of BSA, pH 7.4) supplemented with 2.8 mM of glucose. Medium was then replaced with KRBH-BSA supplemented with either 2.8 mM or 16.7 mM of glucose, and incubations were continued for 1 h. Secreted insulin and islet insulin contents were measured by radioimmunoassay for islet insulin content measurements, islets were sonicated in ethanol-acid acid. For proliferation measurements, 10 islets were placed on extracellular matrix (ECM)-coated cell dishes (Novarmed, Jerusalem, Israel) and kept until monolayer formation (5–7 days). Proliferation assessment was performed following Exendin-4 treatment (100 mM; 72 h). ECM plates were fixed with 4% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS) (3×), and permeabilized with 0.3% Triton X-100 and 1% BSA (pH 7.4) for 30 min at room temperature. Monolayers were then incubated in the presence of rabbit TdT.
anti-Ki67 antibody (diluted 1:500) for 1 h at room temperature. Following washes with PBS (3×), monolayers were incubated in the dark with a goat anti-rabbit IgG antibody coupled to Alexa-Fluor 568 (diluted 1:400) for 1 h at room temperature. Finally, after three washes with PBS, cells were mounted on a coverslip with Vectashield-DAPI (Reactolab SA, Servion, Switzerland) and observed with a Zeiss Axi-ovision Fluorescence microscope. Quantification was made by counting K67-positive cells using the ImageJ software (http://rsweb.nih.gov/j). Previous studies, using co-staining for Glut2, showed that in these culture conditions 95% of proliferating cells were beta-cells (β-cells) [22].

2.9. β-Cell purification
Pancreatic β-cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM of glutamine, and 1% pen/strep with Exendin-4-Cy3 (100 nM) for 30 min at 37°C. Single-cell preparations were then obtained by incubating islets for 1.5 min at 37°C, with gentle pipetting, in Ca2+ and Mg2+ free PBS containing 0.24 mM of ethylenediaminetetraacetic acid (EDTA) and 0.025% trypsin. Digestion was stopped by adding RPMI 1640 supplemented with 10% FBS, 1 mM of glutamine and 1% pen/strep. Dispersed islet cells were resuspended in 300–500 μL of Krebs 1% BSA pH 7.4 supplemented with 2.5 mM of glucose, 5 mM of EDTA and 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI). β-cells were purified by fluorescence-activated cell sorting using a 4-laser MoFlo ARIA II STRISIQ EQ™ cell sorter (Beckman Coulter, Indianapolis, USA) using a 100-μm nozzle. After gating out the doublets and dead cells (DAPI positive), Cy3-positive cells detected using the 561 nm laser were selected.

2.10. Histomorphometric and immunofluorescence detection
Histomorphometric and β-cell proliferation analysis were performed using 5-μm-thick sections from 4% paraformaldehyde (PFA)-fixed and paraffin-embedded pancreata. β-cell mass was measured by histomorphometry [21,23]. Briefly, for each condition, 6 sections per pancreas and 4–6 pancreata were analyzed, representing a total of >500 islets. β-Cell surface area was measured using the ImageJ software. β-cell mass was calculated by multiplying the β-cell area by the pancreas weight. For β-cell proliferation and α-cell mass measurements, pancreas sections were incubated overnight at 4°C with a rabbit anti-Ki67 antibody (diluted 1:100) and guinea pig anti-glucagon antibody (diluted 1:500) and washed 3 times with PBS. The sections were then incubated for 1 h with Alexa-Fluor 568-labeled goat anti-rabbit IgG antibody and Alexa-Fluor 488-labeled goat anti-guinea pig IgG antibody, washed with PBS and then mounted with Vectashield containing DAPI. Ki67-positive cells were counted over islet areas defined by glucagon staining in 50–100 islets. The analysis was performed using the ImageJ software. Only non-glucagon, Ki67-positive cells were counted. Because β-cells form the majority of the islets non-α cells, we considered the Ki67-positive cells as representing proliferating β-cells. Preceding experiments also showed that the percent of Ki67/Glut2 double-positive cells was only marginally different from the number of total Ki67-positive cells [22,24]. Glucagon staining was used for α-cell mass measurements.

For immunofluorescence detection of islet Glut2, insulin, and glucagon, mice were fixed by intracardiac perfusion with cold 4% PFA. Pancreata were then excised, incubated in 5% PFA for 4 h and overnight in a 30% sucrose solution before being frozen at −80°C in optimal cutting temperature (OCT) compound. Twenty-micrometer cryosections were prepared, blocked in PBS supplemented with 3% BSA and 0.3% Triton X-10,0 and then incubated at 4°C overnight with rabbit anti-Glut2 (diluted 1:500), guinea pig anti-insulin (diluted 1:500), and guinea pig anti-glucagon (diluted 1:500). Primary antibodies were then detected with an Alexa-Fluor 488-labeled goat anti-rabbit IgG antibody or a goat anti-guinea pig IgG antibody (diluted 1:200).

2.11. RNA sequencing analysis
Integrity of islet RNA was verified on a fragment analyzer (Agilent Technologies, Inc., Santa Clara, CA 95051, USA), and all preparations had an RNA quality number (RQN) between 8.2 and 9.3. RNA-seq libraries were prepared using 250 ng of total RNA and the Illumina TruSeq Stranded mRNA reagents (Illumina; San Diego, California, USA). Cluster generation was performed with the resulting libraries using the Illumina TruSeq SR Cluster Kit v4 reagents and sequenced on the Illumina HiSeq 2500 using TruSeq SBS Kit v4 reagents. A sequencing depth of 31–46 million reads using 125 bp single-end reads was used. Reads were mapped with STAR-2.5.3a software [25] to the M. musculus-mm 15 reference genome. Read counts for each gene were generated with HTSeq-0.9.1 software [26] using as reference the annotation index GRCm38.83 from ENSEMBL. Counts were filtered, excluding those genes with less than one count per million in two libraries, and normalized using trimmed mean normalization method (TMM) with ‘edgeR’ [27]. Pathway enrichment analysis of the genes differentially expressed between Ctrl and Klf6KO mice was performed using the Kyoto Encyclopedia of Genes and Genomes KEGG and gene ontology (GO) databases and the R library “clusterProfiler”. P-values were adjusted for multiple comparisons using the Benjamini Hochberg procedure (Benjamini and Hochberg 1995). Terms with an adjusted p-value ≤0.05 were considered overrepresented.

2.12. Data representation and statistics
Results are expressed as mean ± SEM with significant p-values ranging from 0.05 to 0.001 (*P < 0.05; **P < 0.01; ***P < 0.001). Statistical analyses performed on the presented data were done with Prism 6.0 using Student’s t-test, and two-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison test.

3. RESULTS
3.1. Klf6 as a potential regulator of β-cell adaptation to metabolic stress
To identify novel genes potentially involved in the control of β-cell mass and function in response to metabolic stress, we previously performed a study where mice from six different strains were fed with a regular chow (RC) or a HFHS diet for 2, 10, 30, and 90 days [14]. At each time point, mice were tested for multiple metabolic phenotypes, and their islets were isolated for RNASeq analysis. WGCNA led to the identification of gene modules, i.e., groups of genes whose expression is similarly regulated across all mouse groups. These modules were then correlated with the measured phenotypes, and the results are represented as a heat map (Supplementary Fig. 1a and Ref. [14]). Here, we further investigated the Thistle 3 module, which showed strong correlation with plasma insulin levels at 0, 15, and 90 min of an oral glucose tolerance test. This module contains 114 genes, and the transcription factor Klf6 (Krüppel-like Factor 6), is among the genes that were most negatively correlated with insulinemia and strongly associated with module membership (Supplementary Fig. 1b). To have an indication of a potential association of KLF6 with diabetes in human islets, we checked its level of expression in a large transcriptomic database of islets from brain-dead, non-diabetic and type 2 diabetic donors [26]. This data showed statistically significantly higher
expression of KLF6 mRNA in type 2 diabetic islets (log2-fold-change = 0.27, adj. p-value 0.015) (Supplementary Fig. 1c).

3.2. Generation of β-cell-specific Klf6 conditional knockout mice
To investigate the role of Klf6 in β-cells, we generated mice with a β-cell-specific inactivation of this gene by crossing Klf6fl/fl mice [20] with Ins1Cre/+ mice [19] to generate Klf6fl/fl;Ins1Cre/+ (βKlf6KO) mice and Klf6fl/fl;Ins1Cre/+ (Ctrl) mice. Figure 1 shows that Cre-mediated recombination resulted in the inversion of exons 2 and 3, leading to inactivation of Klf6. Genotyping was performed with the primers indicated in Figure 1A, and recombination was observed only in islets (Figure 1B). Quantitative RT-PCR analysis showed a 40% reduction of Klf6 expression in islets isolated from βKlf6KO mice (Figure 1C). This indicated that Klf6 was also expressed in non-β-cells. Quantitative RT-PCR analysis performed with RNA extracted from cell sorter purified β- and non-β-cells shows a 3-fold higher expression of Klf6 in non-β-cells (Supplementary Fig. 2), in agreement with published data [29]. Thus, the remaining Klf6 expression in islets from βKlf6KO mice is consistent with it being expressed at a 3-fold higher level in non-β-cells, which represent 30% of islet cells. No gross morphological difference could be observed between Ctrl and βKlf6KO mice (Supplementary Fig. 3).

3.3. Normal glucose homeostasis, β-cell mass, and insulin secretion in βKlf6KO mice
We first assessed glucose homeostasis as well as β-cell mass and function in regular chow (RC)-fed βKlf6KO mice. These mice had similar body weight, fasted, fed, and fasted/refed glycemia, as well as plasma insulin and glucagon levels as their control littermates (Supplementary Figs. 4a–c). Their total pancreas and isolated islet insulin content (Supplementary Figs. 4e and f) were similar to that of Ctrl mice. GSIS assays using isolated islets from both types of mice yielded identical results (Supplementary Figs. 4g) and i. p. glucose tolerance tests performed on 12- and 36-week-old male and female mice were also indistinguishable (Supplementary Figs. 4h–k). Pancreatic weights (not shown) and islet morphologies (Supplementary Fig. 5) were indistinguishable between genotypes.

3.4. Reduced β-cell mass expansion in βKlf6KO mice in response to pregnancy
Because inactivation of Klf6 did not impact β-cell mass and function in non-challenged conditions, we tested whether metabolic stress would reveal a role for Klf6. In a first set of experiments, we analyzed β-cell proliferation and mass during gestation, an insulin-resistant condition associated with compensatory β-cell growth [30]. Pancreatic weights (not shown) and islet morphologies (Supplementary Fig. 5) were indistinguishable between genotypes at 14 days of pregnancy. We analyzed Ki67 staining in β-cells at day 14 and β-cell mass at day 18 of pregnancy. Ctrl and βKlf6KO mice displayed similar blood glucose and plasma insulin levels throughout gestation (Figure 2A,B) but β-cell proliferation increased by 6.5-fold in Ctrl mice as compared to 4-fold in βKlf6KO (Figure 2C,D). As a result, β-cell mass increased by 2.3-fold in Ctrl mice as compared to 1.7-fold in βKlf6KO (Figure 2E). The difference in β-cell mass was related to a higher number of islets per pancreas in Ctrl mice rather than to a change in islet size distribution (Figure 2F,G).

3.5. Reduced β-cell mass expansion in βKlf6KO mice in response to high-fat high-sucrose diet
In the second approach, we fed 6-week-old Ctrl and βKlf6KO male and female mice for an additional 22 weeks with a RC or an HFHS diet. Feeding a high-energy diet induced a similar increase in body weight, glucose intolerance, and in vivo GSIS in both types of mice (Supplementary Fig. 6). Pancreatic weights (not shown) and islet morphologies (Supplementary Fig. 5) were indistinguishable between genotypes at the end of the HFHS feeding period. β-cell proliferation was, however, markedly lower in male and female βKlf6KO mice as compared to Ctrl mice (Figure 3A,E), and this was associated with
Figure 2: Reduced β-cell Mass Expansion During Pregnancy in βKlf6KO Mice. (a) Blood glucose and (b) plasma insulin levels of 10–12-week-old female mice taken in non-pregnant mice and mice at 14 and 18 days of pregnancy (n = 4–6). (c) Quantification of Ki67-positive β-cells over islet cell surface in non-pregnant and 14-day pregnant mice (n = 5–8). (d) Representative immunofluorescence detection of Ki67 in Ctrl and βKlf6KO mice. Scale bar: 50 μm. (e) β-cell mass in response to pregnancy (day 18). (f) Islet density and (g) size distribution following 18 days of gestation (n = 5). Error bars represent SEM. Statistical analyses were performed using a two-way ANOVA (Bonferroni’s post-hoc test) *P < 0.05; **P < 0.01; ***P < 0.001.
Together, the above data showed impaired diet feeding, or induced by pharmacological inhibition of the insulin receptor. This indicates that Klf6 is necessary for the full compensatory β-cell proliferation to insulin resistance.

3.6 Reduced β-cell mass expansion in βKlf6KO mice during S961 treatment

Insulin resistance induced by chronic delivery of the insulin receptor antagonist S961 [31] leads to rapid development of hyperglycemia and hyperinsulinemia. Here, we treated Ctrl and βKlf6KO male mice for 2 and 7 days with saline or S961. This induced a strong hyperglycemia from the first day of treatment until the end of the experiment in both groups of mice (Figure 4A). Insulinemia was similarly elevated in Ctrl and βKlf6KO mice at day 1 of treatment but was reduced by half in βKlf6KO mice at day 7 (Figure 4B), at which time the total pancreatic insulin content was also reduced by 50% in the pancreata of βKlf6KO mice (Figure 4C). The pancreatic glucagon contents (Figure 4D), body weights (Figure 4E), and pancreatic weights (not shown) remained identical in S961-treated Ctrl and βKlf6KO mice. However, unexpectedly, the β-cell section area was reduced by 40% by S961 treatment in both groups of mice (Supplementary Fig. 7), indicating that S961 treatment also induced a reduction in β-cell volume.

We next assessed β-cell proliferation and mass in male mice treated for 7 days with saline or S961. Figure 5A shows that the proliferation rate was increased by 5-fold in Ctrl mice and by 3-fold in βKlf6KO mice. This led to a β-cell mass increase in S961-treated mice that reached 2.5 mg/pancreas in Ctrl mice but only 2.0 mg/pancreas in βKlf6KO mice (Figure 5B). This lower β-cell mass was due to a decrease in the total number of islets, which displays the same size distribution as in S961-treated Ctrl mice (Figure 5C,D). Together, the data showed impaired β-cell mass expansion in βKlf6KO mice in response to insulin resistance of pregnancy, HFHS diet feeding, or induced by pharmacological inhibition of the insulin receptor.

3.7 Klf6 protects β-cells against dedifferentiation

To identify the changes in transcript expression resulting from Klf6 inactivation in β-cells, we performed RNASeq analysis of islets isolated from Ctrl and βKlf6KO male mice treated for 2 days with saline or S961. We selected this treatment time as we searched for early transcriptional events leading to defective β-cell adaptation and tried to minimize the changes in gene expression due to long-term metabolic alterations. In this series of treated mice, the glycemia was already markedly increased after 1 day of treatment and reached the usual maximal value of 30 mM at day 2 (Supplementary Fig. 8a). Plasma insulin levels were very high at day 1 of treatment in both Ctrl and βKlf6KO mice but, at day 2, they were already reduced by 20% in βKlf6KO mice as compared to Ctrl mice (Supplementary Fig. 8b).

Analysis of genes differentially expressed in islets from saline or S961-treated Ctrl and βKlf6KO mice is presented as volcano plots (Figure 6A). The complete lists of differentially expressed genes are presented in Suppl. Tables 1 and 2. GO analysis of genes differentially regulated in islets from saline-treated Ctrl and βKlf6KO mice (Figure 6B, Supplementary Tables 3a and b), revealed that most pathways over-represented in knockout islets were associated with cell cycle regulation ("mitotic cell division", "regulation of cell cycle process", etc.) and a few with hexose metabolic processes. The absence of Klf6 did not lead to impaired proliferation under unchallenged conditions in vivo, yet islets from βKlf6KO mice had lower Ki67 staining than Ctrl islets when cultured as monolayers on an extracellular matrix in the presence or absence of Exendin-4 (Supplementary Fig. 9). In the hexose metabolic G0 term, 3 glycolytic genes controlling the same step were present, Pfkfb2 (6-phosphofructo-2-kinase/Fructose-2,6-Bisphosphatase 2), reduced by 34% in βKlf6KO compared to Ctrl islets, the bifunctional enzyme that produces and hydrolyzes the...
allosteric glycolysis activator fructose 2,6-bisphosphate (Fru-2,6-P2), Fbp2 (fructose bisphosphatase 2), increased by 2.4 fold, which catalyzes the hydrolysis of Frc-1,6-P2 into Frc-6-P, and Tigar (P53 Induced Glycolysis Regulatory Phosphatase), reduced by 30%, an enzyme that hydrolyzes both Frc-1,6-P2 and Frc-2,6-P2 into Frc-6-P thus increasing the reverse glycolytic pathway potentially leading to increased production of NADPH through the pentose phosphate shunt [32–34].

GO analysis of genes differentially expressed in islets from Ctrl and bKlf6KO mice under S961 treatment (Figure 6C, Supplementary Tables 4a and b) revealed enrichment in cell division genes (“mitotic nuclear division”, “regulation of cell cycle process”) and a striking increase in terms related to glucose metabolism and insulin secretion (“hormone transport”, “hormone secretion”, “exocytosis”, “glucose metabolic process”, and others) (Figure 6C).

A more detailed analysis of the genes that were dysregulated in islets from bKlf6KO as compared to Ctrl mice following S961 treatment revealed: (Figure 7A) i) a 30% reduced expression of genes controlling different steps in GSIS, including Slc2a2, Slc30a8, Igf1r, Ffar1, Vdac1d, Atp2a2, Vamp2, and Sytl4; ii) a reduced expression of Pdx1, Nkx6.1, Foxa2, and Foxo1, essential mature β-cell transcription factors [7,35–37]; iii) an increased expression of several mRNAs encoding dedifferentiation markers, including Gast, Aldh1a3, Serpina7, Vim, and

Figure 4: Impaired Insulinemic Response to Insulin Resistance in bKlf6KO Mice. Twelve-week-old male Ctrl and bKlf6KO mice were treated with saline or the insulin receptor antagonist S961 for 7 days. (a) Glycemia (n = 7–10) (b) insulinaemia at days 1 (n = 9–15) and 7 (n = 9–14). (c) Pancreatic insulin and (d) glucagon contents at day 7 of treatment (n = 9–11) (e) body weight (n = 4–10). Statistical analyses have been performed by two-way ANOVA (Bonferroni’s post-hoc test). ***P < 0.001. Statistical analyses in c and d were done using a two-tailed unpaired Student’s t-test. Error bars represent SEM.

Figure 5: Reduced β-cell Mass Expansion in S961-Treated bKlf6KO Mice. (a) Quantification of Ki67-positive β-cells over islet cell surface in mice treated with saline or S961 for 7 days (n = 5–7). (b) β-cell mass in response to saline and S961 treatment. (c) islet density and (d) size distribution of islets following S961 treatment for 7 days (n = 4–5). Statistical analyses were performed using a two-way ANOVA (Bonferroni’s post-hoc test) *P < 0.05; **P < 0.01; ***P < 0.001. Error bars represent SEM.
Figure 6: Transcriptomic Analyses of Islets from Mice Treated with Saline and S961. Ctrl and Klf6KO mice were treated with saline or S961 for 2 days before islet harvesting. (a) Volcano plots showing the number of islet genes differentially expressed between genotypes (Ctrl and Klf6KO) following treatment. Selected gene ontology analysis of differentially expressed genes following (b) saline and (c) S961. *genes associated with metabolic processes.
Slc18a2 as well as iv) expression of the “disallowed genes”, Ldha, Hk1, Slc16a2, oat, Igfbp4, Mylk, Ly6a, Pcolce, Parp3, and Ndg2 [1,7,36–43]. After 7 days of treatment with S961, the expression of Ins1, Ins2, Slc2a2, and Slc30a8, as determined by RT-QPCR analysis, was still more reduced in islets from βKlf6KO than in Ctrl mice (Figure 7B). Immunofluorescence microscopy detection of insulin and Glut2 confirmed lower expression of both proteins in islets of S961-treated βKlf6KO as compared to Ctrl mice (Figure 7C). The endocrine markers Foxo1 and Adlh1a3 were, however, no longer differentially expressed between Ctrl and βKlf6KO islets (Figure 7B). Immunostaining for Adlh1a3, a protein induced in a subset of dedifferentiated β-cells, was similarly increased in islets from both types of mice after S961 treatment (Supplementary Fig. 10). Finally, chromogranin A showed homogenous expression over the whole islets, although the staining intensity was lower after S961 than after saline treatment (Figure 7E). There was no difference in chromogranin A mRNA expression between islets from Ctrl and βKlf6KO mice treated with saline or S961 as indicated by the RNASeq data (not shown).

As previous reports indicated that hyperglycemia induces trans-differentiation of β-cells into α-cells [7,44], we measured α-cell mass in the pancreas of Ctrl and βKlf6KO mice after 7 days of saline or S961 treatment. Figure 8A, B shows that the α-cell mass was not increased by S961 treatment in Ctrl mice but was increased by 60% in islets from S961-treated βKlf6KO mice, and this corresponded to a 60% percent increase in islet glucagon mRNA (Figure 8C). Ki67 staining also revealed a higher proliferation rate of α-cells in S961-treated βKlf6KO mice than in Ctrl mice (Figure 8D).
To determine whether the increased number of α-cells can be accounted for by transdifferentiation of β-cells, we generated ins1Cre;Rosa26tdTomato and βKlf6KO, Rosa26tdTomato mice and treated them for 7 days with S961. We then quantified the number of glucagon positive cells also expressing tdTomato. Approximately 4% of α-cells in ins1Cre;Rosa26tdTomato mice were also positive for tdTomato and 8% in βKlf6KO; Rosa26tdTomato mice (Figure 8E). Thus, S961 treatment induced a larger number of β-cells to transdifferentiate into glucagon-producing cells in βKlf6KO mice than in Ctrl mice, suggesting that the increase in α-cell mass may result from both β-cell transdifferentiation and increased proliferation of α-cells.

4. DISCUSSION

Here, we show that Klf6 is required for the full proliferation response of β-cells to the insulin resistance of pregnancy, HFHS diet, and induced by insulin receptor antagonism. When insulin resistance is associated with marked hyperglycemia, as in S961-treated mice, Klf6 also protects β-cells against dedifferentiation and reduces their transdifferentiation into α-cells. Klf6 is, thus, a transcription factor that is required for preserving mature β-cell functional adaptation to insulin resistance.

Klf6 is a member of the Krüppel-like family of transcription factors that contains 18 isoforms [45]. These have various functions in cell cycle progression, apoptosis, and cancer development [46–49], and Klf6 is also associated with non-alcoholic fatty liver disease [50,51] and diabetic nephropathy [52,53]. Here, we identified Klf6 through a systems biology approach aimed at identifying novel regulators of β-cell adaptation to metabolic stress [14]. We found that Klf6 expression is not required for the normal development of adult β-cell mass and function nor for their functional stability over time when mice are fed RC. Only when an additional stress is imposed, such as during gestation when insulin resistance develops but mice remain normoglycemic, does the absence of Klf6 lead to impaired β-cell proliferation and mass expansion. In HFHS-fed mice, glucose intolerance and in vivo glucose-stimulated insulin secretion evolved similarly in βKlf6KO and Ctrl mice, but β-cell proliferation and mass were significantly less induced in βKlf6KO mouse islets. Here, we fed the mice with an HFHS diet for 22 weeks a period of time that may not be sufficient for the β-cell mass expansion defect observed to cause more severe glucose intolerance in βKlf6KO mice than in Ctrl mice. When βKlf6KO mice were treated with S961, a condition that induces rapid and massive hyperglycemia [31,54], reduced β-cell proliferation, and lower mass expansion was associated with a rapid decline in plasma insulin as compared to S961-treated Ctrl mice. Thus, combining insulin resistance with severe hyperglycemia induces stronger β-cell dysfunctions in βKlf6KO mice highlighting the important protective role of Klf6 in these conditions.

To obtain insights into the gene network controlling defective β-cell adaptation in βKlf6KO mice, we performed islet transcriptomic analysis and searched for genes differentially expressed between Ctrl and βKlf6KO mouse islets in saline or S961 treated mice. In saline treated mice, comparative transcriptomic analysis showed that the differentially expressed genes were enriched in genes controlling the cell cycle. Most of these genes were upregulated in βKlf6KO islet cells.

Figure 8: Increased α-cells Mass and β-cell Transdifferentiation in S961-Treated βKlf6KO Mice. Ctrl and βKlf6KO mice were administered S961 or saline for 7 days. (a) Immuno-fluorescence of glucagon-producing cells. Scale bar: 50 μm. (b) Measurement of α-cell mass (n = 3–5). (c) Islet gcg mRNA expression (n = 9–13). (d) Quantification of K67-positive α-cell over islet cell surface (n = 3–5). (e) Lineage tracing experiments were performed using Ctrl and βKlf6KO expressing tdTomato from the Rosa26 locus. Quantification of glucagon-positive cells expressing tdTomato in Ctrl and βKlf6KO following S961 treatment for 7 days with an example of immunostaining (n = 4–7). Statistical analyses were performed using two-way ANOVA (Bonferroni post-hoc) *P < 0.05, **P < 0.01, ***P < 0.001. Statistical analysis in c was done using two-tailed unpaired Student’s t-test. Error bars represent SEM.
even though these cells have lower proliferation capacity than those in Ctrl islets as tested in islets cultured as monolayers. Nevertheless, Cdkn3, a cell cycle inhibitor [55], was overexpressed 6-fold in Klf6KO islets and this may explain the reduced β-cell proliferation capacity. On the other hand, 3 glycolytic genes were also dysregulated, Pkb2, Fbp2, and Tig. These genes control the production and hydrolysis of fructose-1,6-bisphosphate, a key regulatory step in glycolysis and neogluconeogenesis. Activating the neogluconeogenic direction of this pathway also leads to increased activity of the pentose phosphate pathway, a supplier of Nicotinamide adenine dinucleotide phosphate (NADPH) required for protection against oxidative stress and for the biosynthesis of new molecules necessary for cell proliferation [32—34]. Thus, in regular feeding conditions and when normoglycemia is preserved, Klf6 mainly controls the expression of proliferation genes and of a small group of glycolytic genes possibly required for supporting proliferation through redirecting glucose carbons to biosynthetic activities. In the present study, we have not analyzed whether any of these genes are direct or indirect transcriptional targets of Klf6. However, a study performed in primary mouse oligodendrocyte progenitors and immature oligodendrocytes identified genes that were direct targets of Klf6-binding [56]. Among those, the following were also found to be dysregulated in islets from Klf6KO mice treated with S961: Ly6a, Oat, and Slc16a2 (disallowed genes), Serpina7 and Vim (dedifferentiation markers), and Slc30a8, gene linked to insulin secretion. Klf6KO mice and Ctrl mice under S961 treatment display severe insulin resistance and hyperglycemia. These lead to more important differences in their islets transcriptional program with several groups of genes associated with β-cell dedifferentiation in addition to the “cell cycle” and “glucose metabolism” genes as described in diabetic mice [7,57] and hyperglycemic rats [6]. These changes include down-expression of genes controlling GSIS, reduced expression of genes associated with differences in their islets transcriptional program with several groups of genes that negatively interfere with GSIS. These observations therefore suggest that Klf6 is a critical guardian of β-cell mass and identity when insulin resistance and hyperglycemia develop. In this context, our observation that KLF6 expression is increased in islets from type 2 diabetic organ donors suggests that, in humans, its induction aims at protecting β-cells against dedifferentiation. Another aspect of hyperglycemia-induced dedifferentiation is the capacity of β-cells to transdifferentiate into α-cells [7,44]. We show that S961 treatment induces an approximately two-fold higher rate of β-cell transdifferentiation into α-cells when Klf6 is absent. Thus, our data demonstrates that Klf6 is required for the normal proliferative response of β-cells induced by insulin resistance to protect β-cells against dedifferentiation induced by severe insulin resistance and hyperglycemia and to limit β-cell transdifferentiation into α-cells. It is interesting to compare the role of Klf6 with that of Foxo1. In their landmark paper, Talchai et al. [7] report that β-cell specific Foxo1−/− mice display reduced β-cell mass, increased β-cell apoptosis, and impaired fasting glucose with hypoinsulinaemia and hyperglycaemia only when metabolically challenged by multiple pregnancies or aging. Inactivation of Foxo1 is associated with compensatory increased expression of Foxa3 and Foxa4 and mice with triple Foxo knockout developed glucose intolerance due to reduced insulin secretion when fed with RC [7,57]. Thus, in analogy with the Foxo transcription factor studies, inactivation of Klf6 does not induce any islet phenotype under RC, but genetic inactivation of additional Klf family members may generate a stronger diabetic phenotype. Indeed, Klf10 [58] and Klf11 [59] have been reported as potential additional regulators of β-cell function. It is also interesting to note that Klf6 expression in purified mouse β-cells has been found to decrease with age [60], potentially explaining the reduced proliferation capacity of old β-cells [61].

5. CONCLUSION

In conclusion, our present study identifies Klf6 as a transcription factor that controls β-cell proliferation induced by the insulin resistance of pregnancy, the consumption of high-energy-containing food, and induced by pharmacological induction of insulin resistance. In the latter condition, which is also associated with strong hyperglycemia, Klf6 also restricts β-cell dedifferentiation and transdifferentiation into glucagon-producing α-cells. Thus, Klf6 expression has a protective effect against type 2 diabetes development, and its overexpression may protect against appearance of this disease.

AUTHOR CONTRIBUTIONS

B.T. conceived the experiments and secured funding. B.T. and C.D. designed the experiments and wrote the manuscript. C.D., D.T., A.P., B.T. conceived the experiments and secured funding. B.T. and C.D. performed the experiments. A.R.S.A and M.I. performed RNA-Seq data analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.02.001.

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