Pair Feeding, but Not Insulin, Phloridzin, or Rosiglitazone Treatment, Curtails Markers of β-Cell Dedifferentiation in db/db Mice

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β-Cell failure is a hallmark of type 2 diabetes. Among several cellular biological mechanisms of cellular dysfunction, we and others have recently proposed that dedifferentiation of β-cells can explain the slowly progressive onset and partial reversibility of β-cell failure. Accordingly, we provided evidence of such processes in humans and experimental animal models of insulin-resistant diabetes. In this study, we asked whether β-cell dedifferentiation can be prevented with diet or pharmacological treatment of diabetes. db/db mice, a widely used model of insulin-resistant diabetes and obesity, were either pair fed or treated with the Sglt inhibitor phloridzin, the insulin sensitizer rosiglitazone, or insulin. All treatments were equally efficacious in reducing plasma glucose levels. Pair feeding and phloridzin also resulted in significant weight loss. However, pair feeding among the four treatments resulted in a reduction of β-cell dedifferentiation, as assessed by Foxo1 and Aldh1a3 immunohistochemistry. The effect of diet to partly restore β-cell function is consistent with data in human diabetes and provides another potential mechanism by which lifestyle changes act as an effective intervention against diabetes progression.

In type 2 diabetes, insulin resistance usually precedes the inception of hyperglycemia (1,2). In the early stages, pancreatic β-cells compensate for the elevated insulin demand to maintain euglycemia. But, as the disease progresses, hyperglycemia becomes increasingly hard to manage (3), in part because of β-cell failure, which attenuates islet insulin secretion (4).

Various mechanisms of β-cell failure have been proposed, including glucolipotoxicity (5), oxidative stress (6,7), endoplasmic reticulum stress (8,9), apoptosis (10,11), or inflammation (6,12). Recently, we and others described β-cell dedifferentiation as a mechanism of β-cell failure in humans (13) and animal models (14–16). We showed that diabetic β-cells lose their identity as insulin-secreting cells and reactivate endocrine progenitor markers, including Neurogenin3 (Neurog3). Dedifferentiated β-cells also give rise to α-cells or other islet cell types.

If β-cells in the diabetic islet are dedifferentiated, and not dead, the question arises of whether β-cell dedifferentiation is reversible. In rodents, there are precedents showing that β-cell redifferentiation can be achieved using insulin or calorie restriction (15–17). In type 2 diabetes, it is well-known that patients’ β-cell function can be preserved by diet (18) or by insulin treatment (19,20). Although in the past insulin secretagogues have been suspected to accelerate β-cell failure, newer medications of this class appear to be more durable in this regard (21–23). But the effects of these treatment modalities on β-cell dedifferentiation are still unclear.

Specifically, the relative roles of normalizing glycemia versus reducing insulin resistance have been debated. Modest hyperglycemia has been known to profoundly affect β-cell performance (24); yet, it is undisputable that reducing the “afterload” of insulin resistance also has beneficial effects on the preservation of β-cell function (2). To answer this question, in the current study we aimed to assess the effect of different diabetes therapies on β-cell dedifferentiation in a mouse model of insulin-resistant, obese diabetes: db/db. We treated animals with either food restriction by pair feeding or the sodium/glucose cotransporter (Sglt) inhibitor phloridzin, the insulin sensitizer rosiglitazone, and...
insulin. Thereafter, we analyzed basic metabolic parameters and β-cell dedifferentiation markers.

RESEARCH DESIGN AND METHODS

Animals
B6.BKS(D)-Lepr<sup>db</sup>/+ heterozygous mice (db/+; and db/db) were purchased from The Jackson Laboratory (Sacramento, CA) and bred to homozygosity to obtain wild-type, db/+; and db/db mice. All mice were fed normal chow except in the experiments with rosiglitazone (see below) and maintained on a 12-h light/dark cycle (lights on at 7:00 A.M.). Eight- to 12-week-old db/db mice and littermates were subjected to drug treatment or pair feeding and killed after 1 month unless otherwise indicated. Average daily food intake in ad libitum–fed db/db mice was 8.5 g, and body weight averaged 55–65 g during treatment. The Columbia University Institutional Animal Care and Utilization Committee approved all experiments.

Study Design
Before treatment, fasting blood glucose and body weight were measured in all db/db mice, which were then randomly assigned to control and treatment groups. Likewise, lean littermates were assigned in a blinded manner. Phloridzin (Sigma-Aldrich, St. Louis, MO) was dissolved in 40% propylene glycol in saline and injected subcutaneously once daily at a dose of 0.2 g/kg (25). Rosiglitazone (Sigma-Aldrich) was added to normal chow at a dose of 0.15 g/kg. Mice were fed with either regular or rosiglitazone-supplemented chow. The estimated dose of rosiglitazone was 20 mg/kg/day (26).

Sustained-release insulin implants, LinBit (15,27), and control implants (Limshin Canada, Inc., Ontario) were placed subcutaneously under the interscapular skin of db/db mice. The manufacturer’s recommended dosage was adopted. Estimated insulin release from implants was ~0.7 units/24 h. For pair feeding, all the animals were housed individually and fed by food hopper. Cumulative food intake of individual db/+ mice was calculated from the total food hopper’s weight every 24 h. The next day, the same amount of chow was provided to the paired db/db animal. Ad libitum–fed db/db mice and controls had free access to food during the experiment. A subgroup of animals did not respond to pair feeding with lower glycemia and were designated as non-responder (NR) animals.

Metabolic Analyses
Animals were fasted for 5 h before measurement of blood glucose and plasma insulin unless otherwise indicated. We performed glucose tolerance tests in overnight-fasted mice by intraperitoneal injection of glucose (1.2 g/kg). We measured insulin by ELISA (Mercodia, Winston Salem, NC).

Immunofluorescence
Tissues were fixed with 4% paraformaldehyde and embedded in Tissue-Tek O.C.T. Compound to obtain frozen sections. We applied heart-perfused fixation and antigen retrieval to detect transcription factors (Nacalai USA Inc., San Diego, CA) (14). Anti-insulin (category no. A056401-2; Dako, Carpinteria, CA), anti-glucagon (category no. G2654; Sigma-Aldrich), anti-Neurog3 (category no. 2011; Beta Cell Biology Consortium), anti-Pdx1 (category no. 56798; Cell Signaling Technology, Danvers, MA), and anti-Aldh1a3 (category no. NBP2-15339; Novus Biologicals, Littleton, CO) were used as primary and Alexa Fluor–conjugated goat antibodies as secondary (Jackson ImmunoResearch Laboratories and Molecular Probes) antibodies. For Foxo1 immunostaining, we used a mixture of rabbit polyclonal antibodies, containing antibody nos. 9462 (Cell Signaling Technology), sc-11350 (Santa Cruz Biotechnology, Dallas, TX), and 3587 (28). For Neurog3 immunostaining, we permeabilized the cell membrane by 0.3% TritonX. We used confocal microscopy and Laser Scanning Microscope Software (Zeiss LSM 510 and 710) to survey colocalization and capture images. We performed the quantification in a blinded fashion using the Cytoskeleton PL function of the HALO software as previously described (13). The density of immunostaining in the captured images was measured by ImageJ. We defined as “weakly positive staining” a 70% decrease from the maximum density in each islet and as “strongly positive” any cell above this arbitrary threshold.

RNA Analysis
We used an RNeasy Mini Kit (Qiagen, Valencia, CA) to isolate total RNA from islets and High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Waltham, MA) to carry out reverse transcription. We performed quantitative real-time PCR with goTaq qPCR Master Mix (Promega, Fitchburg, WI) on a Bio-Rad CFX96 real-time PCR system. The relative gene expression levels were determined by the ΔΔCt method using tubulin as a reference gene. The primer sequences are as follows: α-tubulin, forward 5′-GCC TTT GTG CAC TGG TAT GTG-3′ and reverse 5′-CCA AAT CCA CAC CCC CCT-3′; Mafa, forward 5′-ATC ACT CTG CCC ACC AT-3′ and reverse 5′-CCG CCA ACT TCT CGT ATT TC-3′; and Neurod1, forward 5′-GCC CAG CTT AAT GCC ATC TTT-3′ and reverse 5′-CAA AAG GCC TGC CTG TCG TAA-3′.

Statistical Analyses
Sample sizes were estimated from the expected effect size based on previous experiments (13,14). We present data as the means ± SEM. We used two-tailed Student t test for data analysis, unless otherwise indicated in the figure legends, and the customary threshold of P < 0.05 to declare statistically significant differences.

RESULTS
Characterization of db/db Mouse Islets
Db/db mice on a C57BL/6J background develop hyperglycemia and hyperinsulinemia at 2–3 months of age (29). We assessed expression of insulin, Foxo1, and the newly identified marker of human and murine β-cell dedifferentiation, Aldh1a3, by immunohistochemistry of islets from ad libitum–fed, 16- to 20-week-old db/db mice. Consistent with
prior data from us (13,14,30) and others (17), the number of β-cells expressing cytoplasmic Foxo1 decreased by 26%, whereas the percentage of insulin-positive/Aldh1a3-positive cells increased from 3 to 76% (Fig. 1A–D). We detected Neurog3-positive cells in db/db mice (Supplementary Fig. 1B and C). Pdx1 was expressed at similar levels in β-cells in db/db and lean mice (Supplementary Fig. 2A), whereas MafA and NeuroD1 RNA expression was significantly decreased (16% and 68%, respectively) (Supplementary Fig. 2B). Based on these results, β-cells in 16- to 20-week-old db/db mice appear to be in the early stages of dedifferentiation. Next, we measured glucagon-immunoreactive cells and found that the percentage of glucagon-positive cells decreased from 13 to 7% (Fig. 1E and F) (P = 0.0001) when normalized by the increased islet size in db/db mice. On the other hand, weakly insulin-positive cells increased from 3 to 17% in db/db mice (Fig. 1F) (P, 0.0001). In addition, the percentage of insulin- and glucagon-negative cells increased from 19 to 25% (P = 0.001). As the percentage of somatostatin- and pancreatic polypeptide–positive cells was unchanged (data not shown), the likely cause of the increase in hormone-negative cells is a rise in dedifferentiated cells, as shown previously (14). Despite the fact that the percentage of glucagon-positive cells decreased and hormone-negative cells increased, the α-cell–to–β-cell ratio decreased because of the greater number of insulin-positive cells in the larger db/db islets (Fig. 1F). As previously described (31), the total number of islet cells increased in db/db mice (Fig. 1G).

**Pair Feeding Reverses β-Cell Dedifferentiation**

We analyzed the effect of diet on β-cell dedifferentiation in pair-feeding experiments. Food restriction is known to lower blood glucose and increase insulin and Foxo1 expression in db/db islets (32). We randomly assigned animals to the pair-fed and ad libitum groups. Pair feeding resulted in an ~50% reduction of food intake compared with ad libitum db/db mice (Fig. 2A). After one month of pair feeding, we observed improved glucose tolerance (Fig. 2B and C) and increased plasma insulin levels (Fig. 2D) in a subgroup of animals (responder [R]). Body weight decreased steadily during pair feeding but did not normalize to db/+ mouse.

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**Figure 1**—Characterization of db/db islets. A: Representative images of Foxo1/insulin coimmunostaining. B: Number of Foxo1-positive cells among insulin-positive cells. C: Representative images of Aldh1a3/insulin coimmunostaining. D: Number of Aldh1a3-positive cells among insulin-positive cells. E: Representative images of glucagon/insulin coimmunostaining. F: Percentage of glucagon-positive and insulin-positive (strong and weak) cells. G: Islet cell number in db/db animals and controls. Data show means ± SEM. n = 11 for lean group and 14 for db/db. P values were calculated by t test for A–F and Mann-Whitney U test for G (P < 0.0001). ***P < 0.001 between the denoted pairs. Gcg, glucagon; Ins, insulin.
Figure 2—Effect of pair feeding on markers of β-cell dedifferentiation. A: Average daily food intake per mouse. B: Intraperitoneal glucose tolerance test after 4 weeks of pair feeding. C: Area under the curve (AUC) of intraperitoneal glucose tolerance test shown in B. D: Fasting plasma insulin in the different treatment groups after 4 weeks of pair feeding. E: Body weight in the various treatment groups during pair feeding. F and G: Representative images of Foxo1 (F) or Aldh1a3 (G) and insulin coimmunostaining in islets of the three groups. H: Number of Foxo1-positive/insulin-positive cells. I: Number of Aldh1a3-positive/insulin-positive cells. J: Total cell number per islet in the various groups after pair feeding. K: Representative image of glucagon/insulin coimmunostaining. L: Percentage of glucagon-positive and insulin-positive cells. Data show means ± SEM. (n = 6 for db/+; 4 for pair-fed R; 3 for pair-fed NR; 6 for ad lib itum db/db). P values were calculated by t test for A–I and L and Mann-Whitney U test for I. *P < 0.05, **P < 0.01, and ***P < 0.001 between the denoted pairs. ad lib, ad libitum; Gcg, glucagon; Ins, insulin.
levels (Fig. 2E). A subgroup of animals with hyperglycemia experienced a weight loss that was out of proportion to the pair feeding and appeared to have developed a state of relative insulin deficiency (designated NR).

When we analyzed islets by immunohistochemistry, we found a 17% decrease in Foxo1-positive β-cells that was prevented after pair-feeding regardless of the response on glucose (Fig. 2F and H). The percentage of Aldh1a3-expressing β-cells decreased from 80 to 25% and 38% in the R and NR groups, respectively (Fig. 2G and I) (P < 0.001). Neurog3-positive cells were absent (Supplementary Fig. 1D), and MafA and NeuroD1 expression levels increased in pair-fed db/db mice (Supplementary Fig. 2B). The number of islet cells, a measure of islet size, was smaller in the pair-fed NR group compared with the ad libitum db/db group (Fig. 2J). In the pair-fed R group, the percentage of strongly insulin-positive cells per islet increased (56% to 68%, P = 0.01) while that of α-cells didn’t change (2% to 4%) (Fig. 2L). In contrast, the percentage of weakly insulin-positive cells and potentially dedifferentiated insulin- and glucagon-negative cells decreased 18% to 13% and 32% to 17%, respectively (P = 0.03 and P = 0.003) (Fig. 2K and M). In the pair-fed NR group, the percentage of non-α/non-β did not change (32% in ad libitum group to 29%), while that of α-cells increased (3% to 16%) (P < 0.001).

**Phloridzin Does Not Affect β-Cell Dedifferentiation Markers in db/db Mice**

Phloridzin, a natural flavonoid found in apple bark that inhibits Sglt, thus increasing urinary glucose loss, can normalize glycemia in diabetic rodents, including leptin-deficient mice (33). The relative contribution of hyperglycemia and insulin resistance to the pathogenesis of β-cell dedifferentiation remains unknown. We reasoned that, if hyperglycemia played a dominant role in this process, normalization of plasma glucose by phloridzin should improve β-cell dedifferentiation. Thus, we treated hyperglycemic 8- to 12-week-old db/db mice with phloridzin or vehicle for 1 month (Fig. 3A). After 30 days of treatment, phloridzin halted the progression of hyperglycemia in db/db mice (Fig. 3B), without altering plasma insulin levels (Fig. 3C), and reduced body weight (Fig. 3D). In contrast, vehicle-injected animals developed significant hyperglycemia (Fig. 3B). Despite the lower blood glucose, phloridzin did not curtail islet expansion (Fig. 3E) or affect Foxo1 (Fig. 3F and G) or Aldh1a3 (Fig. 3H and I) expression. Phloridzin slightly increased the percentage of α-cells in db/db mice (Fig. 2J and K), but the change was not statistically significant (6% to 13%, P = 0.06); in addition, it had no effect on weakly insulin-positive cells or insulin- or glucagon-negative cells. In sum, phloridzin surprisingly failed to prevent dedifferentiation markers, despite its profound antihyperglycemic effect.

**Effects of Rosiglitazone on β-Cell Dedifferentiation**

Given the failure of the glucose-lowering agent phloridzin to prevent β-cell dedifferentiation, we asked whether increasing insulin sensitivity would affect this process. To answer this question, we carried out a 2-month course of rosiglitazone treatment in db/db mice and examined metabolic and islet parameters (34). After a 2-month treatment, rosiglitazone improved glucose tolerance in wild-type and db/db mice (Fig. 4A and B), without altering body weight (Fig. 4C), and substantially reduced hyperinsulinemia (Fig. 4D). The percentage of Foxo1-positive β-cells increased from 56 to 85% (Fig. 4F and G), but that of Aldh1a3-positive cells did not change, even though it showed a trend toward a modest decrease (Fig. 4H and I). Rosiglitazone did not change the percentage of glucagon-positive, or weakly insulin-positive, cells or the non-α-cell-to–non-β-cell ratio in db/db islets (Fig. 4J and K).

**Effects of Insulin Treatment on β-Cell Dedifferentiation Markers**

Finally, we investigated the effect of insulin therapy on β-cell dedifferentiation in db/db mice. Although db/db mice have severe insulin resistance, insulin administration can be effective in lowering blood glucose levels (35,36). After implantation, blood glucose levels in ad libitum-fed animals fell by ~50% for ~2 weeks (Fig. 5A). The number of cells per islet was slightly increased in the insulin-treated group compared with controls but not significantly so (Fig. 5B). Foxo1 expression in β-cells was decreased slightly in the insulin group (Fig. 5C and D). Aldh1a3 expression remained high (Fig. 5E and F). α-Cell and weakly insulin-positive cell percentage remained the same after insulin implant, as did the non-α-cells/non-β-cells (Fig. 5G and H).

**DISCUSSION**

In the current study, we report the effect of different diabetes treatments on β-cell dedifferentiation. In pair-fed db/db animals, β-cell markers such as insulin, Foxo1, MafA, and NeuroD1 were restored and the dedifferentiation marker, Aldh1a3, was suppressed. These results are compatible with previous data (17). The percentage of weakly insulin-positive cells decreased, as did the percentage of non-β-cells/non-α-cells, a surrogate of dedifferentiated cells. And there was no increase in α-cells. We also found increased plasma insulin levels, which may reflect improved β-cell function or number, as suggested in a recent study (37). These results suggest that calorie restriction can prevent, and possibly reverse, β-cell dedifferentiation. In a previous study, food restriction in db/db mice restored insulin secretion from isolated islets, improved insulin sensitivity, and decreased islet inflammation (32,37). Whether inflammation and dedifferentiation are mechanistically related is unknown. Since Foxo, a driver gene of β-cell fate, is regulated by cytokine signaling (38), the two processes likely overlap.

We have proposed that there are several stages to β-cell dedifferentiation (14). In the first phase, Foxo1 translocates to the nucleus in response to various noxious cues (hyperglycemia, hyperlipidemia, cytokines). Over time, Foxo1 expression decreases as a result of deacetylation, and this is associated with increased expression of the progenitor cell
marker Aldh1a3 and decreased markers of mature β-cells such as Pdx1, NeuroD, and MafA. In the final stages, Neurog3-positive endocrine progenitor-like cells can be found in the islet, as dedifferentiated cells convert to other cell types. By this measure, the islets of mice chosen for the current study are in the early phases of dedifferentiation, as they have low MafA and NeuroD, but are still Pdx1 positive; in addition, Neurog3-positive cells are rare. It should be noted that Aldh1a3 is a marker of a heterogeneous cell population that includes both Pdx1-positive and Neurog3-positive subsets (39).

In clinical practice, evidence that diet improves β-cell function in type 2 diabetes is overwhelming (18). The

Figure 3—Phloridzin fails to normalize markers of β-cell dedifferentiation. A: Blood glucose after a 5-h fast in wild-type (w) and db/db mice before treatment ($P = 0.0065$). B: Fold change of blood glucose after a 5-h fast during phloridzin (Ph) treatment ($P = 0.0072$ in phloridzin-treated animals vs. controls). C: Fasting plasma insulin after phloridzin treatment. D: Changes in body weight after phloridzin treatment in db/db animals ($P = 0.0032$ in phloridzin vs. vehicle). E: Number of cells per islet after phloridzin treatment in both wild-type and db/db mice. F: Representative image of Foxo1/insulin coimmunostaining. G: Number of Foxo1-positive/insulin-positive cells. H: Representative image of Aldh1a3/insulin coimmunostaining. I: Number of Aldh1a3-positive/insulin-positive cells. J: Representative image of glucagon/insulin coimmunostaining. K: Proportion of glucagon-positive and insulin-positive cells. Data show means ± SEM. ($n = 3$ for each group.) $P$ values were calculated by $t$ test for B, C, and F–K; paired $t$ test for D; and Mann-Whitney $U$ test for A and E. **$P < 0.01$ and ***$P < 0.001$ between the denoted pairs. AU, arbitrary units; Gcg, glucagon; Ins, insulin; ve, vehicle.
Diabetes Prevention Trial has consistently demonstrated the superiority of diet and exercise to pharmacological treatment. But we also know that diet alone as a treatment ceases to be efficacious in the more advanced stages of diabetes (40). In humans, reversal of β-cell dysfunction by diet appears to be limited to the first decade of disease (41). Interestingly, severely hyperglycemic db/db mice appear to be resistant to pair feeding. The reasons for the failure to respond to pair feeding in a subset of animals may have to do with more advanced disease and functional exhaustion of the endocrine pancreas. In future studies, it will be important to perform a time course to determine whether there is a "point of no return" to restore β-cell dedifferentiation.

Sglt2 inhibitors have made an impact on diabetes treatment, in part due to their ability to forestall cardiovascular mortality (42). In humans, there is evidence that Sglt2 inhibitors protect against β-cell failure (22). In db/db mice, phloridzin normalized blood glucose, decreased body weight, and improved insulin levels. However, rosiglitazone failed to normalize markers of β-cell dedifferentiation.

**Figure 4**—Rosiglitazone fails to normalize markers of β-cell dedifferentiation. A: Intraperitoneal glucose tolerance test after rosiglitazone treatment. B: Area under the curve (AUC) of intraperitoneal glucose tolerance test shown in panel A. C: Body weight after rosiglitazone treatment. D: Plasma insulin levels after rosiglitazone treatment. E: Total islet cell number after rosiglitazone treatment (rosiglitazone vs. vehicle, P = 0.0159). Representative image of Foxo1/insulin communostaining (F) and cell number (G). Representative image (H) and number (I) of Aldh1a3-positive/insulin-positive cells. Representative image (J) and proportion (K) of glucagon/insulin-positive cells. Data show means ± SEM. (n = 3–4 for each group.) P values were calculated by t test for A–D and F–K and Mann-Whitney U test for E. *P < 0.05 and **P < 0.01 between the denoted pairs. cont, control; Gcg, glucagon; Ins, insulin; Rosi, rosiglitazone; w, wild type.
weight, and improved insulin secretion during glucose tolerance tests without changes to plasma insulin levels or insulin sensitivity (43). But it did not affect β-cell dedifferentiation markers in the current study. In fact, it even tended to increase islet α-cell percentage, possibly as a compensatory response to the lowering of hyperglycemia. These results suggest that alleviating hyperglycemia does not suffice to restore β-cell dedifferentiation. This could be due to the greater effect of pair feeding on lipid levels (32) compared with phloridzin treatment (44), acting possibly to relieve metabolic inflexibility or glucolipotoxicity (30). Alternatively, phloridzin may differ from selective Sglt2 inhibitors used in the clinic. Interestingly, the latter have been found, unlike phloridzin, to increase islet β-cell proportion (43).

Thiazolidinediones (TZDs) primarily act by ameliorating insulin resistance in insulin target tissues. In addition, there is evidence for a direct role of Ppard in β-cells (45), and we have suggested that this is partly due to an effect on lipid disposition (30). Rosiglitazone has been shown to reduce insulin resistance and hyperlipidemia (46) and to improve insulin secretion during glucose tolerance tests in db/db mice (47). In the latter study, rosiglitazone also increased islet size and β-cell number. Consistently, we found that it improved glucose tolerance and reduced plasma insulin level, probably as a result of decreased afterload on β-cells. It also restored Foxo1 expression in β-cells but failed to affect other parameters examined. These data are consistent with the findings in insulin-treated animals, in which a reduction of the secretory workload of β-cells was not sufficient to restore β-cell dedifferentiation. It is noteworthy that treating insulin resistance without changes to the diet had a lesser impact on β-cell dedifferentiation than diet alone. The dissociation between increased Foxo1 levels and failure to decrease Aldh1a3 in rosiglitazone-treated db/db islet could be interpreted to suggest that rosiglitazone has a partial effect.

TZDs have been reported to improve β-cell function in humans (21,23), but there are no data on dedifferentiation. Thus, it cannot be concluded from these data whether this reflects intrinsic differences among different species or experimental models or a basic mechanism of action. For instance, β-to-α ratio is lower in human islets (48), and humans might respond less to the relative hyperglucagonemia caused by TZDs, compared with mice.

Insulin treatment of db/db had the least effect on β-cell dedifferentiation in the current study. Db/db mice have

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Figure 5—Insulin fails to normalize markers of β-cell dedifferentiation. A: Time course of changes in blood glucose during insulin (Ins) treatment in db/db mice (P = 0.0096 control [cont] vs. insulin). B: Islet cell number after 30-day treatment (P = 0.0875 control vs. insulin). C: Representative image of Foxo1/insulin coimmunostaining. D: Number of Foxo1-positive/insulin-positive cells. E: Representative image of Aldh1a3/insulin coimmunostaining. F: Number of Aldh1a3-positive/insulin-positive cells. Representative image (G) and proportion (H) of glucagon/insulin-positive cells. Data show means ± SEM. (n = 2–3 for each group.) P values were calculated by ANOVA for A, Mann-Whitney U test for B, and t test for E–H. *P < 0.05. AU, arbitrary units; Gcg, glucagon.
severe insulin resistance and slow-releasing insulin pellets may not have delivered a sufficient amount of insulin to overcome it. In fact, glucose levels did not achieve normal values in the current study. Previous work has shown that insulin supplementation was effective to reverse β-cell dedifferentiation in a model of primary β-cell failure (15). Also, longer (for 12 weeks) and more aggressive insulin treatment successfully restored insulin secretion from isolated islets in db/db mice (35). In clinical practice, early intervention with insulin tends to preserve β-cell function and even achieve transient remission (20, 21, 49).

The other limitation of our study is that we did not use lineage tracing to mark dedifferentiated cells, so we cannot unequivocally assess whether we reversed, as opposed to simply prevented, dedifferentiation. Further work will be required to address this point. Another limitation is that we did not test glucagon-like peptide 1 agonist or dipeptidy l peptidase 4 inhibitors, both of which are known to restore β-cell function and reduce hyperglucag onemia (21, 50). A further study is planned to address this question.

In conclusion, we show a remarkable effect of diet on β-cell dedifferentiation in obese mice. Our results strengthen the concept of a central role of nutrition in diabetes management and raise the question of alternative pharmacological approaches to preserving β-cell function and forestalling progression of diabetes.

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**Author Contributions.** E.I. designed and performed experiments, analyzed data, and wrote the manuscript. J.Y.K.-M. designed and generated mice. D.A. designed experiments, oversaw research, and wrote the manuscript. D.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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