Intermittent protein restriction improves glucose homeostasis and restores damaged islet β cells in diabetic mice

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Abstract

Diabetes is caused by an interplay between genetics and environmental factors, tightly linked to lifestyle and dietary patterns. In this study, we explored the effectiveness of intermittent protein restriction (IPR) in diabetes control. IPR drastically reduced hyperglycemia in diabetic mice and enhanced β cell function and β cell proliferation in pancreatic islets. IPR also elevated insulin signaling in peripheral tissues with increased circulating FGF21 level. The changes of methionine/leucine are associated with the intervention activity of IPR. Single-cell RNA sequencing revealed that IPR reversed diabetes-mediated β cell reduction and immune cell accumulation in the islets of diabetic mice. The sequencing analysis also identified a small group of highly proliferative β cells that was elevated by IPR. As IPR is not based on calorie restriction and is highly effective in diabetes control and β cell protection, it has great potential to be widely applied to diabetic human subjects in the future.
Introduction

Diabetes mellitus, especially type 2 diabetes (T2D), is one of the biggest challenges in the 21st century due to its devastating effect on lives and health management. As T2D is mainly caused by a sedentary lifestyle, combined with consumption of foods with high energy density, various intervention strategies have been proposed with a focus on changing lifestyle and diet\(^1\), \(^2\). For example, Mediterranean and DASH diets that emphasize consumption of plant-based foods with reduction of animal-based foods have been widely used in the management of T2D\(^3\), \(^4\). In a study which explored the interactive effects of three macronutrients (\(i.e.,\) protein, fat and carbohydrate) on food intake, cardiometabolic phenotype, and longevity in mice, it was found that reduction of protein intake is crucial to improve metabolic health and longevity\(^5\). Many other studies also pinpoint to reduced protein consumption as a contributing factor to longevity and improvement of metabolic health\(^6\)-\(^8\). For example, an early study indicated that consumption of a low-protein diet at 0.8 g/kg lean body mass for consecutive 7-days can significantly reduce postabsorptive and average daily blood glucose concentrations in persons with T2D\(^9\). Furthermore, it was recently found that restriction of a few key essential amino acids improves glucose homeostasis. Restriction of methionine in mice can enhance insulin-mediated suppression of hepatic glucose production and elevate insulin signaling in the liver\(^10\). The insulin-sensitizing effect of methionine restriction was later found to be mediated by an increase in FGF21 expression in the liver\(^11\), \(^12\). In addition to methionine, branched-chain amino acids (BCAAs) were also recognized to regulate glucose metabolism. Increased BCAA consumption contributes to the development of obesity-associated insulin resistance in rats\(^13\). Restriction of BCAAs in rats was also found to improve insulin sensitivity in the skeletal muscle through an increase in efficiency of fatty acid oxidation and acyl-glycine export\(^14\). Deprivation of leucine, one of BCAAs, could enhance insulin sensitivity in the liver via GCN2/mTOR/S6K1 and AMPK pathways\(^15\).
In addition to a strategy of restriction in protein or amino acids, fasting or calorie restriction has long been used as an effective means to extend longevity and improve metabolic health including diabetes(16). In recent years, periodic fasting (PF) or intermittent fasting (IF) have been widely used to improve metabolic health and aging(17, 18). Lately, intermittent dietary restriction using a fasting-mimicking diet (FMD) has been used to improve type 1 and type 2 diabetes(19, 20). In particular, intermittent application of FMD was proposed to promote regeneration of β cells in the pancreatic islets(19).

Although continuous protein/amino acid restriction and intermittent fasting have been shown to improve glycemic control in diabetes, it has not been explored before whether periodic protein restriction alone is sufficient in improving diabetes. In this study, we analyzed the intervention activity of intermittent protein restriction (IPR) on diabetes in mice. We found that IPR is highly effective in improving glucose homeostasis by promoting β cell regeneration in the islets, different from continuous protein restriction. Moreover, single-cell RNA sequencing of the islets demonstrated that reduction of β cells and accumulation of immune cells in the islets of the db/db mice were reversed by IPR. As this dietary strategy is not based on calorie restriction, we believe that it has an invaluable potential to be applied to diabetic patients in the future.
Results

Intermittent protein restriction improves hyperglycemia in diabetic mice

As continuous application of a low-protein diet might compromise immune and liver functions, as well as cause protein loss especially under diabetic condition (21), we explored the effect of IPR as a diabetes intervention. At first, we tried to identify an optimal low-protein diet that could lower blood glucose level in a streptozotocin (STZ)-induced type 1 diabetes mouse model. Each week, the STZ-treated mice were fed with diets with various protein concentrations for 3 days, followed by normal chow for 4 days (Figure 1A & 1B). Surprisingly, IPR with 5% of dietary protein gave rise to the best result in improving hyperglycemia (Figure 1C). We next investigated the effect of the duration of IPR. Application of 5% protein diet for 3 days in each week resulted in optimal lowering of blood glucose in the STZ-treated mice (Figure 1D & 1E). We also tested the efficacy of adding a high-protein diet following the low-protein diet by analyzing the “therapeutic” effect of two diets, a 3-4 diet (low-protein diet for 3 days followed by normal chow for 4 days) and a 3-2-2 diet (low-protein diet for 3 days followed by high-protein diet for 2 days and normal chow for 2 days) (Figure 1F & 1G). In this experiment, the dietary intervention was only given for the first 4 weeks. Intermittent application of both the 3-4 and 3-2-2 diets significantly improved hyperglycemia even after the intervention was stopped, with the 3-2-2 diet performing better than the 3-4 diet (Figure 1G). The blood HbA1c level, as well as results of GTT and ITT tests performed at the end of the experiment, further corroborated the finding that IPR had a long-lasting effect on improving glycemic control and insulin sensitivity (Figure 1H-1J).

We next tested the “therapeutic” effect of the 3-4 and 3-2-2 diets in db/db mice, a classical T2D mouse model that develops diabetes due to morbid obesity caused by deficiency of leptin receptor (Figure 1K). IPR significantly reduced the fasting blood glucose level of the mice (Figure 1L). In particular, the 3-2-2 diet had a better effect than the 3-4
diet in decreasing hyperglycemia (Figure 1L). The intervention effect of IPR was also very fast, as the diet began to reduce the fasting blood level in one week during both early and late intervention periods (Figure 1L). Consistently, the blood HbA1c level was reduced by both 3-2-2 and 4-3 diets (Figure 1M). Glucose tolerance and insulin sensitivity of the db/db mice were also markedly improved by IPR, while the 3-2-2 diet had a better effect than the 4-3 diet (Figure 1N & 1O). Taken together, these results indicate that intermittent use of a low-protein-diet, especially when combined with a high-protein diet, had a rapid and powerful effect in improving hyperglycemia in both type 1 and type 2 diabetes mouse models.

**Intermittent protein restriction improves β cell function and increases proliferation of β cells in diabetic mice**

Diabetes is caused by two major mechanisms: damage of β cells in the islets and insulin tolerance in peripheral tissues mainly including the liver and skeletal muscle. We first analyzed the β cell function by measuring glucose-stimulated insulin secretion (GSIS) using freshly isolated islets from db/db mice. The 3-2-2 diet robustly improved GSIS (Figure 2A), indicating that this diet scheme was able to recover the function of β cells in the islets. Immunofluorescent staining also revealed that both 3-2-2 and 3-4 diets prevented the loss of β cells in db/db mice and also increased the number of β cells in STZ-treated mice (Figure 2B & SFigure 1).

We reasoned that the increased β cell numbers upon IPR was caused by an increase of cell proliferation. To test this hypothesis, we analyzed the proliferation rate of the islet cells by different strategies. Analysis with EdU incorporation in the STZ-treated mice revealed that intermittent application of 5% protein diet led to the highest cell proliferation rate in the islets among the diets with various protein concentrations (Figure 2C). Consistently, the serum C-peptide level, an indicator of the secretory capacity of β
cells, was highest in the mice taking IPR with 5% protein diet (Figure 2D). The number of Ki67-positive cells was also increased by the 3-2-2 diet in the islets of the db/db mice (Figure 2E), together with an increase in the serum C-peptide level (Figure 2F). Most importantly, we found that continuous application of a low-protein diet could not elevate the Ki67-positive cells in the diabetic mice, drastically different from the effect of the 3-2-2 diet (Figure 2G). However, continuous feeding with a low-protein diet reduced blood glucose level of the mice (SFigure 2).

We next used a genetic lineage tracing strategy to investigate whether the recovery of the β cells by the 3-2-2 diet was caused by neogenesis from non-β cells(22). Transdifferentiation of β cells from non-β cells led to temporary co-expression of mTomato and GFP, resulting in yellow-colored cells (Figure 2H). As expected, the 3-2-2 diet reduced hyperglycemia of the mice that were treated with STZ and fed with high-sucrose high-fat diet (HSHFD) (SFigure 3). However, we did not find any yellow-colored cells after the use of the 3-2-2 diet, although the number of β cells was profoundly increased by the diet (Figure 2I), indicating that the restoration of damaged β cells by IPR is likely caused by enhanced proliferation of existing β cells but not neogenesis from non-β cells.

**Intermittent protein restriction reduces gluconeogenesis and improves insulin sensitivity**

We next analyzed the effect of IPR on the peripheral tissues. In the liver, both 3-2-2 and 3-4 diets reduced the expression of PEPCK, a rate-limiting enzyme for gluconeogenesis, at both the mRNA and protein levels (Figure 3A & 3B), indicating an inhibition in gluconeogenesis. The phosphorylation of AKT in the skeletal muscle was enhanced by IPR (Figure 3C), indicating an increase in insulin signaling. We further tested the effect of the 3-2-2 diet in the wild type mice. As expected, the 3-2-2 diet improved glucose tolerance and insulin sensitivity in the mice (SFigure 4). The mRNA levels of PEPCK and G6Pase in the liver were reduced by the 3-2-2 diet (Figure 3D). Pyruvate tolerance test (PTT), an indicator
of gluconeogenesis, was reduced by the 3-2-2 diet (Figure 3E), together with a reduction of PEPCK protein level in the liver (Figure 3F). The insulin-stimulated AKT phosphorylation was enhanced by the 3-2-2 diet in the skeletal muscle (Figure 3G), along with enhancement of GLUT4 translocation to the plasma membrane (Figure 3H).

To identify the potential mechanism underlying the insulin-sensitizing effect of IPR, we analyzed the blood level of FGF21, a key circulating protein that was shown to be upregulated by protein restriction and regulate insulin sensitivity (23, 24). In STZ-treated mice, use of 5% protein diet for 3 days a week significantly elevated the blood FGF21 level (Figure 3I). In db/db mice, both 3-2-2 and 3-4 diets robustly increased the blood FGF21 level (Figure 3J). Taken together, these results suggest that IPR is capable of enhancing insulin sensitivity of peripheral tissues likely via elevation of circulating FGF21 level.

Methionine and leucine mediate the intervention activity of intermittent protein restriction

Application of low-protein diet was expected to reduce the levels of essential amino acids as they cannot be synthesized de novo in animals. We thus measured the blood amino acid levels in the mice. In db/db mice, both the 3-2-2 and 4-3 diets reduced the blood levels of a number of essential amino acids, including methionine, valine, isoleucine, and leucine (Figure 4A). We next explored which essential amino acid was likely contributing to the beneficial effect of IPR. In the low-protein diet, we added individual essential amino acids for the first 3 days each week (Figure 4B). Addition of leucine or methionine, but not other amino acids, negated the glucose-lowering effect of the low-protein diet in STZ-treated mice (Figure 4C). Conversely, deprivation of methionine and/or leucine for 3 days each week recapitulated the glucose-lowering effect of the low-protein diet in STZ-treated mice (Figure 4D & 4E). These results thus supported the idea that reduction of methionine and/or leucine are associated with the improvement of glucose homeostasis by IPR.
Single cell RNA sequencing of the islets reveals that diabetes-mediated endocrine cell reduction and immune cell accumulation in the islets are reversed by intermittent protein restriction

We next used single cell RNA sequencing (scRNA-seq) to analyze the islet cells of the mice. Fresh islets were isolated from the four groups of mice: wild type mice as a normal control; early stage db/db mice at a time right before the dietary intervention; and late stage db/db mice which either received or did not receive the 3-2-2 diet (Figure 5A). As expected, the 3-2-2 diet reversed hyperglycemia of the db/db mice at the late stage (Figure 5B). We sequenced a total of 22,749 cells that passed quality control from the islets of the 4 groups of mice. The dataset was visualized using t-distributed stochastic neighbor embedding (t-SNE) (25); 23 distinct clusters were identified (Figure 5C, SFigure 5, & SFigure 6). Two prominent features of the cell type changes were found during the progression of diabetes in db/db mice: gradual loss of endocrine cells and accumulation of immune cells including B cells, T cells, macrophages, and innate lymphoid cells (ILCs) in the islets (Figure 5D). Strikingly, the 3-2-2 diet almost completely reversed the loss of endocrine cells and accumulation of immune cells (Figure 5D). Overall, endocrine cells dropped from 89.3% in normal control to 58.2% in early stage db/db and 21.9% in late stage db/db (STable 1). However, the percentage of endocrine cells was restored to 84.3% by the 3-2-2 diet (STable 1). The percentage of B cells and T cells was 31.9% and 30.9% respectively in late stage db/db mice but decreased to 0.2% and 0.3% respectively by the 3-2-2 diet (STable 1). We next verified that changes of immune cells of the islets by staining with antibodies against CD3 (a T cell marker), CD19 (a B cell marker), and CD11c (a macrophage marker). We found that the accumulation of these immune cells in db/db mice was prevented by the 3-2-2 diet (Figure 5E). Furthermore, flow cytometry analysis revealed that the increase of CD45-positive cells in db/db mice was reversed by the 3-2-2 diet (Figure 5F), thus providing
further evidence that immune cell accumulation in the islets of the T2D mice was prevented by IPR.

Diabetes and intermittent protein restriction alter the molecular signatures of endocrine cells

We further analyzed the endocrine cells from the dataset of scRNA-seq and identified a total of 17 subgroups (clusters 0-16) (Figure 6A, SFigure 7-9). Along with the progression of diabetes in db/db mice, the numbers of all four endocrine cell types (β, α, δ and PP) gradually declined (Figure 6B). The 3-2-2 diet robustly elevated the number of β cells and slightly increased the numbers of the other three types of endocrine cells (Figure 6B-5F, and STable 1). The percentage of β cells decreased from 52.1% in normal control to 36.1% in early stage db/db mice and 15.0% in late stage db/db mice (STable 1). However, β cells were restored to 69.5% after the 3-2-2 diet (STable 1). We next analyzed the endocrine cell subgroups in detail. Clusters 2 and 10 were exclusively from the normal control and characterized by the highest expressions in Ins1, Ins2, Mafa, and Nkx6-1 among all the 17 subgroups (Figure 6C, 6G & 6H), similar to previous reports based scRNA-seq using islets (26-28). Generally, it was difficult to distinguish between the β cells from the early stage and late stage db/db mice. Most of the β cells from db/db mice had declined expressions of Ins1 and lost expression of Mafa, such as in clusters 3, 7, 8, 11 (Figure 6C, 6G & 6H), while they had an increase in lapp expression (Figure 6G). On the other hand, cluster 0 was the major subgroup of β cells in db/db mice after intervention with the 3-2-2 diet (Figure 6C). The expression levels of Ins1, Ins2, Mafa, and Nkx6-1 in this cluster were higher than those of β cells from the db/db mice without the dietary intervention (Figure 6C, 6G & 6H), indicating a functional recovery of the β cells after the 3-2-2 diet.

We also analyzed the expression profile of Ucn3 that was proposed to mediate a somatostatin-dependent feedback loop in insulin secretion (29). Ucn3 had the highest
expression in the β cell subgroups from normal mice, while the β cells from db/db mice all had a reduced expression in Ucn3 (Figure 6G). In addition, Ucn3 was undetectable in α, δ, and PP cells (Figure 6G). However, we did not find any “virgin” β cell subgroup that had low expression of Ucn3 but with concomitant high expressions of Ins1, Ins2 and Mafb (Figure 6G & 6H). A previous study proposed that these “virgin” β cells constitute an intermediate stage during transdifferentiation from α cells to functional β cells(30).

Our data revealed two distinct subgroups for both α and δ cells (Figure 6D & 6E). The α cells in cluster 1 were exclusively from normal mice and the db/db mice after IPR, while the cells in cluster 6 were mainly from db/db mice without the dietary intervention (Figure 6D). Both subgroups of α cells had similarly high level of Gcg and transcription factors Arx and Irx2 (Figure 6G & 6H). However, Mafb expression was slightly reduced in cluster 6 (Figure 6H). The two subgroups of δ cells were similar in the expression of a few critical genes such as Sst, Pdx1, Hhex, and Neurog3 (Figure 6G & 6H). In addition, we only identified one group of PP cells in our dataset (Figure 6F). In general, PP cells had high expression of most of the major hormones including Ins1, Ins2, Iapp, Sst, Ppy, and Pyy (Figure 6G). In our analysis, it was difficult to identify the specific transcription factors that determine the identity of PP cells (Figure 6H). Overall, PP cells had moderate to high expression of Nkx6-1, Pdx1, Etv, Neurod1, and Pax 6 (Figure 6H).

Consistent with our finding that IPR could increase the proliferation of β cells in the islets (Figure 2), we identified a group of highly proliferative β cells in our dataset. Cluster 14 had high expression of numerous markers of cell proliferation such as Mki67 (Ki67), Stmn1 and Cdk1 (SFigure 10). In addition, cluster 14 had relatively high expression of Hmgb1 (high mobility group box 1) and Hmgb2 (high mobility group box 2), indicating that the DNA binding proteins encoded by these genes are likely associated with the high proliferative activity of this subgroup of β cells (SFigure 10). It is noteworthy that this subgroup of β cells was barely detected in diabetic mice (Figure 6C). The percentage of
these cells was 0.8% in normal mice and 1.5% in db/db mice after the dietary intervention (Table 1), indicating that IPR increased this subgroup of β cells.

Different from a previous study (31), we did not find that β cells can be categorized to four subgroups based on the expression of St8sia1 and Cd9 (SFigure 11). In addition, a previous study indicated sub-clustering of β cell into five subgroups due to differential expression of Rbp4, Ffar4 (GRP12), Id1, Id2 and Id3(27). However, we could not find differential expression of these genes in the β cells in our dataset (SFigure 11).

**Single cell secretome analysis of the endocrine cells**

To elucidate the intercellular crosstalk among the endocrine cells, we analyzed the expression profiles of 650 receptors and 974 ligands using a secretome database as previously described(32). We first examined the ligand profile of the endocrine cells. As expected, all the β cell subgroups had high expression in Ins1, Ins2, and Iapp. Both the α cell subgroups had high expression in Gcg; and the δ cell subgroups had high expression in Sst; and the PP cells had high expression in both Ppy and Pyy (Figure 6G & SFigure 12).

Interestingly, the β cell subgroups from db/db mice had relatively higher Iapp level than the β cell subgroups from normal mice (Figure 6H & SFigure 12), indicating the development of diabetes is likely associated with an increase in Iapp expression, consistent with the notion that amyloid formation by IAPP can lead to β cell death and dysfunction(33). We also noted that a few β cell subgroups, mainly clusters 5, 9, 11, and 16, had moderate expression of hormones of other endocrine cells, such as Gcg, Ppy, Pyy, and Sst, indicating the highly heterogeneous feature of β cells in terms of secretion of major endocrine hormones.

In addition to Gcg, a few ligand genes were relatively rich in α cells, including Ttr (transthyretin), Gpx3 (glutathione peroxidase 3), Sfrp5 (secreted frizzled related protein 5), and Wnt4 (Figure 6A, SFigure 12). In addition to Sst, δ cells also had high expression of
other ligands such as App (amyloid precursor protein), Cd24a (CD24a antigen), Ly6h (lymphocyte antigen 6 complex, locus H), and Rbp4 (retinol binding protein 4).

We identified that clusters 11 and 16 of β cells, cluster 6 of α cells, and cluster 13 of δ cells had relatively high expression of numerous ligands that were characteristic of acinar cells (Figure 7A, SFigure 12), such as Cela3b (chymotrypsin like elastase 3B), Clps (colipase), Cpa1 (carboxypeptidase A1), Cpb1 (carboxypeptidase B1), Pnlip (pancreatic lipase), and Rnase1 (ribonuclease A family member 1). Such findings indicate that these subgroups of endocrine cells acquired the features of acinar cells in secretion of digestion enzymes. Most of these cells, especially those in clusters 11, 6 and 13, were from db/db mice at early stage of diabetes. These cells likely represent the cell types that are undergoing dedifferentiation from endocrine cells to acinar cells during the progression of diabetes. Consistently, the acinar markers were also detected in β cells of islets from T2D patients (34).

We also uncovered other features in ligand expression in the endocrine cells. In particular, Cck (cholecystokinin) and Gast (gastrin) were mainly expressed in β cell subgroups from db/db mice (Figure 7A, SFigure 12), including clusters, 3, 7 and 11, suggesting that the expression of these two genes is likely associated with the development of diabetes. Both CCK and gastrin peptides are classical gastrointestinal peptide hormones. It has been reported that bioactive CCK and gastrin peptides can stimulate islet cell growth as well as secretion of insulin and glucagon (35). These peptides secreted by the β cells in db/db mice may possess a compensatory effect due to functional decline during diabetes progression.

Regarding the receptor expression profiles of the endocrine cells, β cells had relatively high expression of Ntrk2 (neurotrophic receptor tyrosine kinase 2), Prlr (prolactin receptor), and Ptprn (protein tyrosine phosphatase receptor type N) (Figure 7B). Interestingly, the expression levels Ntrk2 and Ptprn were reduced in clusters 2 and 10, the subgroups of β
cells exclusively from normal mice, indicating that an increase in the expression of these two genes could be associated with the development of diabetes in db/db mice. PTPRN is also called IA-2 and is one of the major autoantigens found in type 1 diabetes. Autoantibodies against IA-2 can be detected years before clinical onset and is now used as a predictive marker for high-risk subjects(36). As the β cells from db/db mice had an elevated expression of PTPRN, it will be of interest to explore whether the autoantibody-mediated immune response can also contribute to the pathogenesis of T2D. As we detected robust immune cell infiltration in the T2D diabetic mice (Figure 5), we postulate that autoimmunity likely plays an important role in diabetes progression at least in the db/db mouse model.

However, Prlr was highly expressed in clusters 2, 10, 14 (Figure 7B), suggesting that a high Prlr expression level is a feature of functional β cells. In agreement with our observation, deletion of prolactin receptor in mice leads to gestational diabetes(37), accompanied by reduction of β cell proliferation and failure to expand β cell mass during pregnancy.

Compared to other endocrine cell types, δ cells had relatively high expression in numerous receptors (Figure 7B), such as Ffar4 (free fatty acid receptor 4), Folr1 (folate receptor alpha), Gabrb3 (gamma-aminobutyric acid type A receptor subunit beta3), Itgb1 (integrin subunit beta 1), Itpr1 (inositol 1,4,5-trisphosphate receptor 1), and Ramp1 (receptor activity modifying protein 1, also a receptor for IAPP) (38).

In addition to δ cells, cluster 15 also had relatively high expression levels of numerous receptors (Figure 7B & SFigure 12). In particular, cluster 15 had the highest expression in Adgrl1 (adhesion G protein-coupled receptor L1), Adgrl3 (adhesion G protein-coupled receptor L3), Csf2ra (colony stimulating factor 2 receptor subunit alpha), Gabbr1 (gamma-aminobutyric acid type B receptor subunit 1), Gcgr (glucagon receptor), Ghr (growth hormone receptor), Gipr (gastric inhibitory polypeptide receptor), Gria2 (glutamate
ionotropic receptor AMPA type subunit 2), Grik5 (glutamate ionotropic receptor kainate type subunit 5), Il1r1 (interleukin 1 receptor, type I), Il6ra (interleukin 6 receptor, alpha), Insrr (insulin receptor related receptor), Itgav (integrin subunit alpha V), Prlr (prolactin receptor), Ptprn2 (protein tyrosine phosphatase receptor type N2), and Robo2 (roundabout guidance receptor 2). Therefore, cluster 15 likely represents the β cell subgroup that plays a central role in communicating with hormones, growth factors, inflammatory factors, and other microenvironment cues.

**Pseudotime analysis reveals β cells transition by intermittent protein restriction**

We further subdivided the β cells in the scRNA-seq dataset and identified a total of 16 subgroups (Figure 8A). Clusters 3, 7, 9, 15 of which mainly existed in normal control (Figure 8B, SFigure 13). Clusters 0, 4, and 10 were mainly from the db/db mice after the 3-2-2 diet intervention (Figure 8B, SFigure 13), indicating these β cells had molecular signatures different from the β cells from the normal control. Clusters 5, 8 and 12 were mainly composed of β cells from early stage db/db mice, while clusters 2 and 13 were mainly from the late stage db/db mice (Figure 8B, SFigure 13).

Although cell clustering is a very useful tool to identify subtypes of cells, it is difficult to reconstruct the cell states in a continuous process. Thus, we employed a trajectory analysis to understand the pseudotime transition of the β cells. First, we investigated the directionality of the β cells via examining spliced and unspliced mRNA rations (RNA velocity). The velocity map revealed the β cells of cluster 0, representing β cells from the diet intervention group, were moving toward cells of cluster 7, which were from normal mice (Figure 8C). Likewise, cells from cluster 10, also exclusively from the IPR group, were moving toward cells in cluster 15 that were mainly from normal control (Figure 8C). These observations indicated that the β cells from db/db mice after the dietary intervention were undergoing a transition toward “normal” β cells. On the other hand, the cells from cluster
5, mainly representing the β cells in early stage db/db mice, were moving toward cluster 2, largely composed of β cells from late stage db/db mice (Figure 8C), indicating a transition of β cells during the progression of diabetes.

The trajectory of pseudotime analysis constituted one decision point and three states (Figure 8D). The β cells from the normal control were “moving” from state 1 to state 2, with most of the cells staying in state 1 (Figure 8E & 8F). In early stage db/db mice, a few β cells were “moving” to state 3, while many β cells from the late stage db/db mice had “moved” to state 3 (Figure 8E & 8F), indicating a dynamic transition of β cells during the progression of diabetes. Intriguingly, the β cells from the diet intervention group were mostly staying in state 1 and state 2, similar to the cells of the normal control (Figure 8E & 8F). These results, therefore, provided additional evidence that IPR is able to prevent the deterioration of β cells during the progression of diabetes.
Discussion

Our data demonstrate that IPR is able to recover damaged β cells in diabetic mice. Combining the results of genetic lineage tracing and scRNA-seq results, we propose that regeneration of β cells after the dietary intervention is not likely caused by trans-differentiation or neogenesis from non-β cells. Consistent with our predictions, we did not observe any increase in Neurog3 (Ngn3) expression after the 3-2-2 diet (Figure 5H), and Neurog3 was considered as the critical transcription factor driving β cell regeneration after intermittent fasting(19). Currently, there exists two major theories about β cells regeneration, i.e., reactivation of ductal progenitor cells (or transdifferentiation from non-β cells) and duplication of pre-existing β cells(39, 40). In this study, we identified a subgroup of highly proliferative β cells that were increased by IPR. Consistently, we found that β cell proliferation is significantly increased by IPR (Figure 2). We thus postulate that this subgroup of highly proliferative β cells might contribute to recovery of functional β cells after IPR intervention. If this were the case, exploiting this group of highly proliferative β cells can be considered as a promising strategy for the treatment of diabetes in the future.

It is noteworthy that it was previously found that Fltp (Cfap126) is a marker that subdivides endocrine cells into two subpopulations and distinguishes proliferation-competent β cells from mature β cells(41). However, we did not find that Fltp was differentially expressed in the different β cell subgroups (SFigure 11). In particular, Fltp was not highly expressed in cluster 14 (SFigure 11). In addition, Lifr (LIF receptor) was only slightly expressed in clusters 2 and 10 (SFigure 11), the β cell subgroups that were exclusively from normal mice. However, Lifr expression was not detected in cluster 14 either, indicating that the highly proliferative subgroup of β cells identified in this study is not likely associated with LIF responsiveness, different from what was previously reported(42). In our study, we found that cluster 14 had high expression of Hmgb1 and
Hmgb2 (SFigure 10), indicating that these two DNA binding proteins might initiate a transcriptional program that gives rise to the identity of this group of cells.

Due to the complexity of cell composition in the islet, scRNA-seq possesses a unique power to elucidate the cellular heterogeneity and identify new subgroups of cells (43, 44). The analyses using scRNA-seq allowed us to discover the uniqueness and potential function of different types of endocrine cells in the islet. By analyzing secretome with the scRNA-seq dataset, we found that α cells had high expression of a few ligand genes such as Ttr, Gpx3, Sfrp5, and Wnt4 (Figure 7A, SFigure 11). Similar to IAPP, transthyretin is involved in amyloid formation in the islet (45). Therefore, α cell-secreted transthyretin could potentially affect β cell functions via transthyretin-mediated amyloid formation. Sfrp5, a soluble modulator of Wnt signaling, was reported to have an inhibitory effect on β cell proliferation (46). On one hand, GPX3 catalyzes the reduction of organic hydroperoxides and hydrogen peroxide by glutathione and may protect islet cells against oxidative damage. On the other hand, WNT4 was reported to regulate β cell proliferation (47). Overall, these α cell-secreted ligands may actively regulate the functions of β cells through secretion of ligands. δ cells also had high expression of ligands such as App, Cd24a, Ly6h, and Rbp4 in addition to Sst. APP is involved in the formation of amyloid beta (Aβ) that is involved in plaque formation Alzheimer's disease. It was recently found that APP likely plays a role in pancreatic function as App knockout mice showed increased insulin secretion in response to glucose (48). RBP4, primarily secreted by adipocytes, links diet to inflammation and metabolic health (49). It will be of interest to investigate whether RBP4 secreted by α cells have a functional impact on other endocrine cells in the islets.

The secretome analysis results are consistent with some of previous reports demonstrating that δ cells are rich in receptor expression. For example, Ffar, also called GPR120, was found to be preferentially expression in δ cells and regulates somatostatin secretion in islets (50). Intriguingly, δ cells had high expression of folate receptors. Although
the function of folate receptors in the islets was unknown, we postulated that folate, as an important vitamin, may regulate the functions of δ cells. The function of GABRB3 in islets is currently unknown. However, it was also reported to exist in δ cells in another scRNA-seq study using human islets(51). Heterozygous deletion of Itpr1 in mice disrupted glucose homeostasis(52), suggesting such receptor in δ cells is implicated in modulating the insulin secretory function of β cells. In all, our study is consistent with previous reports to illustrate that δ cells are rich in different types of receptors and thereby this group of cells may function as an integration hub to coordinate metabolic signals and relay to functional changes of the pancreatic islet(51).

Both continuous protein restriction and intermittent fasting have been shown to promote longevity and metabolic health(6, 7, 19). We proposed IPR strategy in this study. Our IPR strategy combined the benefits of protein restriction and intermittent fasting, while avoiding the harmful effects of continuous protein restriction on immune and liver functions. The molecular mechanisms of IPR to control diabetes are multifaceted. The inhibition on the hepatic glucose production may be contributed by reduction of methionine intake as previously reported(10). The stimulation of insulin sensitivity is likely caused by decreases in methionine and leucine as previously reported(10, 15). The insulin-sensitizing effect of methionine reduction was reported to be mediated by an increase in FGF21 expression in the liver(11, 12). We also found that hepatic FGF21 was increased by IPR (Figure 3I & 3J). The stimulation of β cell regeneration in the islets is likely caused by the intermittent pattern of IRP, similar to the case of intermittent fasting(19).

Consistently, we found that continuous protein restriction did not increase β cell proliferation in the islets (Figure 2G). We also did a preliminary experiment to compare IPR with intermittent fasting. In STZ-treated mice, IPR performed better in glycemic control than in intermittent fasting (SFigure 14). In addition, IPR appeared to have more beneficial effects than intermittent fasting in the elevation of serum FGF21 level and the increase of
β proliferation as measured by EdU assay (SFigure 14). Nevertheless, more stringent experiments will be needed in the future to compare IPR with different types of fasting as well as to optimize IPR for diabetes intervention.

In summary, our study indicated that intermittent application of a low-protein diet, especially when combined with a high-protein diet, may stand out as a potentially well accepted and highly effective strategy for the control of diabetes in the future. First, it is not a fasting-based strategy that is commonly used in practice. A non-fasting diet scheme may have better compliance in diabetic patients compared to any types of fasting. Second, intermittent use of a low-protein diet is better than chronic/continuous protein restriction that might be detrimental to physiology and health (e.g., affecting immune system, liver function, wound healing, and skeletal muscle maintenance). Third, the nutritional strategy proposed in this study is akin to “killing two birds with one stone”, as it can recover β cell function and promote glucose homeostasis in peripheral issues at the same time. Fourth, the alternating use of a low-protein diet followed by a high-protein diet might strongly activate a regenerative program in the pancreas to promote rapid repair of damaged β cells of diabetes, a function not found with continuous protein restriction. Fifth, the proposed intervention strategy in this study is low-cost and can be easily applied to people at any economic level. Considering the multiple benefits of this intervention strategy, clinical studies are urgently needed to validate its effectiveness in diabetic patients.
Material and Methods

Mouse models

Eight-week-old male C57BL/6 mice and C57BL/ksJ-db (db/db) mice were purchased from SLAC (Shanghai, China) and maintained in pathogen-free conditions on a 12h light/dark cycle at the animal facility of Shanghai Institute for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS). For type 2 diabetes mouse model, db/db mice were fed with normal chow ad libitum until they became hyperglycemia (>11.1 mmol/l). All mice were weighed at the beginning of the study and randomly allocated to different experimental groups. For STZ-induced type 1 diabetes mouse model, low-dose streptozotocin (STZ) (40 mg/kg) was injected intraperitoneally for four consecutive days. These mice were weighed and fasted 8 h prior to STZ injection. STZ was purchased from Sigma-Aldrich (Cat. No. S0130, MA, USA) and was dissolved in sodium citrate buffer (pH 4.5). An equal volume of citrate buffer was injected into control mice. 10% sucrose water was provided to prevent hypoglycemia after STZ injection. Nine days after STZ injection, all mice became hyperglycemia and were randomly allocated to different experimental groups. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) with an approval number 2010-AN-8.

Mouse diet

The normal chow was purchased from Shanghai Pu Lu Teng Biological Technology Co., Ltd (Shanghai, China) and its nutrition composition is 26% protein, 60% carbohydrate, and 14% fat. The low-protein diet used in most of the experiments was purchased from Jiangsu Synergetic Pharmaceutical Bioengineering Co., Ltd (Jiangsu, China) and its composition was 5% protein, 71% carbohydrate, and 10% fat. The diets containing different protein
concentrations (0%, 2.5%, 5%, 7.5%, 10%, 20%, and 40%) were purchased from FBSH Biotechnology Co., Ltd (Shanghai, China). The diet deprived of leucine and/or methionine was purchased from Dyets, Inc. (Wuxi, China) with a composition of 17% protein, 71% carbohydrate, and 12% fat. The diet was given to the mice every morning during the experiment.

Genetic lineage tracing to analyze β cell neogenesis

B6.129(Cg)-Gt (ROSA)26Sortm4(Actb-tdTomato, -EGFP)Luo and B6.Cg-Tg(Ins2-cre)25Mgn/J mice were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China) (22). They were crossed to each other to generate the INS\(^{Cre}\); mTmG (INS\(^{Cre}\)mTmG) strain. The mice used were heterozygous for both Cre and mTmG in our experiments. The mice were fed with a high sucrose, high fat diet (HSHFD) that contained 40% of sucrose and 40% of fat at 4-week-old for a total of 6 weeks. The mice were then weighed and randomly allocated to different experimental groups. Low dose streptozotocin (STZ) (40 mg/kg) was injected intraperitoneally for four consecutive days. These mice were weighed and fasted 5 h prior to STZ injection. Nine days after STZ injection, all mice became hyperglycemia and then treated with different dietary schemes.

Blood glucose and C-peptide measurement

All mice were fasted for 6 h in the morning (starting at 9 am) before blood glucose measurements. Blood glucose was measured through the tail vein using the One Touch UltraEasy Blood Glucose Monitoring System (Lifescan, Milpitas, CA, USA). Serum C-peptide levels were determined by a murine enzyme-linked immunosorbent assay kit (Crystal Chem Inc., Downers Grove, USA) according to manufacturer’s instructions.

Blood HbA1c measurement
After the mice were euthanized, whole blood was immediately collected from the orbital sinus into EDTA-K2-treated tubes (Kangjian Medical, Shanghai, China). The tubes were then centrifuged at 3,400 rpm for 20 min, and the plasma supernatant was collected for uses. Excluding those for immediate use, all plasma samples were stored at −80°C. Blood HbA1c level was detected by Mouse GHbA1c ELISA KIT (mlbio, Shanghai, China).

**Glucose tolerance testing (GTT) and insulin tolerance testing (ITT)**

Mice were caged individually and fasted for 4 h (starting at 9 am) for ITT in the morning and fasted overnight for GTT. Glucose (1g/kg) or insulin (1 unit/kg) was injected intraperitoneally. Blood glucose levels were measured from 0 min to 120 minutes after the injection.

**Pyruvate tolerance testing (PTT)**

Mice were caged individually and fasted overnight (16 h). Sodium pyruvate (2 g/kg) was injected intraperitoneally. Blood glucose levels were measured from 0 min to 120 minutes after injection.

**Serum amino acid analysis**

Mice serum was collected as previously described. The serum samples were precipitated and diluted, followed by labeling with aTRAQ™ Reagent△8. After that, the labeled samples were combined with a TRAQ™ Reagent△8 internal standard. The samples were introduced into Agilent 1200 LC system (CA, USA). AB SCIEX 4000 QTRAP LC-MS/MS system with TurbolonSpray ion source (Foster City, California, USA) were used for LC-MS/MS analysis. Internal standard was used to quantify the results.

**Ex vivo glucose-stimulated insulin secretion (GSIS) assay with perfusion and incubation**
Islets of each group of mice were isolated and incubated in RPMI1640 medium overnight. At least 60 hand-picked islets from each mouse were placed into Bio-Gel P-4 bead chambers for perfusion GSIS analysis. An automated perfusion system (Biorep Perfusion System; BioRep, Miami, USA) was employed in the assay. Islets were first equilibrated in Krebs-Ringer bicarbonate HEPES buffer containing 2.8 mM glucose for 30 min and then perfused at a flow rate of 0.1 mL/min using the following buffers, KRBH with 2.8 mM glucose for 10 min, then KRBH with 20 mM glucose for 35 min. The perfusate was collected every minute in an automatic fraction collector for insulin measurement by ELISA.

**Real-Time PCR**

Total RNA was extracted from frozen tissues using Trizol reagent according to the manufacturer’s instructions. The purity and concentration of the total RNA were determined by a NanoDrop spectrophotometer (ND-1000, Thermo Fisher, MA, USA). 2μg of total RNA was reverse transcribed using FastQuant RT kit (with gDNA) (TIANGEN BIOTECH CO., LTD, Beijing, China). Real-time PCR was carried out in an ABI 7900HT Fast Real-Time PCR System (AB Applied Biosystems, Warrington, UK) with SYBR Green PCR master mix (AB Applied Biosystems) and gene-specific primers. The sequence and GenBank accession number for the forward and reverse primers used to quantify mRNA were listed in the table. The following conditions were used for real-time PCR: 95°C for 10 min, then 95°C for 15 s, and 60°C for 1 min in 30 cycles. The relative changes in gene expression were normalized against Actin mRNA expression.

**Flow cytometry**

The isolated mouse islets were incubated in PBS. The tissues were then filtered through a 40 μm filter, then the cells were collected. After washing with PBS, the suspended cells were stained with mixed cell surface antibodies of APC-Cy™7-Rat anti-mouse CD45 (BD
Finally cells were analyzed by FACS\textsuperscript{Aria} machine (BD Bioscience).

**Antibodies and immunoblotting**

The antibodies were purchased as follows: the antibodies against AKT, p-AKT were from Cell Signaling Technology (Danvers, MA, USA). The antibodies against PEPCK was from Proteintech (Catalog number: 16754-1-AP, Wuhan, China). The protocols for immunoblotting have been described previously (Feng et al., 2007).

**Immunofluorescence analysis**

Mice pancreas samples and muscle tissue were collected, then rinsed in PBS and fixed in 4\% paraformaldehyde overnight before being dehydrated and embedded into paraffin. After that, the tissues were sectioned into slices (4\(\mu\)m), deparaffinized into xylene, and rehydrated via a graded ethanol series (100\%, 90\%, 70\%, 50\%, and 30\%) and PBS. The antigen was retrieved by heat treatment with 0.1 M citrate buffer (pH = 6.0), and the sections were blocked with blocking buffer (PBS + 1\% normal goat serum + 0.1\% triton-100). The following primary antibodies were used: anti-insulin (Catalog number: C27C9, Cell Signaling Technology, Danvers, MA, USA), anti-glucagon (Catalog number: ab10988, Abcam, Cambridge, UK), anti-GLUT4 (Catalog number: NPP1-49533, Novusbio, USA), CD11c (Catalog number: 97585, CST, MA, USA), CD3 (Catalog number: ab135372, Abcam, Cambridge, UK), CD19 (Catalog number: 90176, CST, MA, USA), Ki67 (Catalog number: 550337, Bioscience, San Diego, CA, USA). The nucleus was stained with Hoechst 33342 (Eugene Oregon, USA). The images were captured by a 40\(\times\) objective with an LSM 510 confocal microscope (Zeiss, Jena, Germany).

**BeyoClick\textsuperscript{TM} EdU-555 Cell proliferation assay**
Mice were injected intraperitoneally with 50 mg/kg EdU 6 hours before being sacrificed by CO₂ inhalation. Then mice pancreas samples were collected, then rinsed in PBS and fixed in 4% paraformaldehyde overnight before being dehydrated and embedded into paraffin. After that, the pancreas tissues were sectioned into slices (4 μm), deparaffinized into xylene, and rehydrated via a graded ethanol series (100%, 90%, 70%, 50%, and 30%) and PBS. Then the pancreas β cell proliferation rate was detected by BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 555 (Catalog number: C0075S, Beyotime, Shanghai, China) according to manufacturer’s instructions.

Pancreatic islet single cell isolation

Mice were sacrificed by CO₂ inhalation, which was then immediately followed by pancreatic perfusion. The pancreas was perfused with 2 mL of collagenase P (Roche Diagnostics Corp, Basel, Switzerland, 1 mg/ml) dissolved in HBSS. The collagenase P solution was then injected into the common bile duct under a dissecting microscope. The tissue was digested by incubation at 37°C for 20 min in a total of 2 ml of digestion solution. Digested tissue was shaken vigorously and immediately washed three times in HBSS. Islets were purified using cell strainers and hand-picked under a dissecting microscope. Islets were then washed with HBSS and digested by trypsin for 8 min. After digestion, islet cells were sieved through a 40 μm cell strainer. Dissociated single cells were stained with AO/PI for viability assessment using Countstar Fluorescence Cell Analyzer.

Single-cell Sequencing

The scRNA-Seq libraries were generated using Chromium Single Cell 3’ V3 Reagent Kits and 10 X Genomics Chromium Controller Instrument (10 X Genomics, Pleasanton, CA). First, cells were concentrated to 1,000 cells/μL and about 6,000 cells were loaded into each channel to generate single-cell Gel Bead-In-Emulsions (GEMs). After the RT step, GEMs
were broken and barcoded cDNA was purified and amplified. After that, the amplified barcoded cDNA was fragmented, A-tailed, ligated with adaptors, and index PCR amplified. The final libraries were quantified using Qubit High Sensitivity DNA assay (Thermo Fisher Scientific) and the size distribution of the libraries were determined by a High Sensitivity DNA chip on a Bioanalyzer 2200 (Agilent). All libraries were then sequenced by illumina sequencer (Illumina, San Diego, CA) on a 150 bp paired-end run.

**Single-cell RNA statistical analysis**

The scRNA-seq data analysis was performed by NovelBio Co., Ltd. and NovelBrain Cloud Analysis Platform. We used fastp with a default parameter filtering the adaptor sequence and then removed the low-quality reads in order to achieve the clean data. The feature-barcode matrices were then obtained by aligning reads to the mouse genome (GRCm38 Ensemble: version 92) by using the CellRanger v3.1.0. We then applied the down sample analysis among samples sequenced according to the mapped barcoded reads per cell of each sample and then achieved the aggregated matrix. Cells containing more than 200 expressed genes and mitochondria UMI rate below 20% passed the cell quality filtering and mitochondria genes were removed in this expression table. The Seurat package (version: 2.3.4, https://satijalab.org/seurat/) was used for cell normalization and regression based on the expression table according to the UMI counts of each sample as well as percent of mitochondria rate to obtain the scaled data. PCA was constructed based on the scaled data with the top 2,000 high variable genes, and the top 10 principals were used for t-SNE construction or UMAP construction. Utilizing the graph-based cluster method, we acquired the unsupervised cell cluster result based on the PCA top 10 principals. We calculated the marker genes by the FindAllMarkers function with Wilcox rank sum test algorithm with following criteria: $P$ value < 0.05; lnFC > 0.25; and min.pct >
0.1. In order to identify the cell type, the clusters of the same cell type were selected for t-SNE analysis, marker analysis and graph-based clustering.

**Pseudotime Analysis:**

Single-cell trajectories analysis was performed using the Monocle2 (http://cole-trapnell-lab.github.io/monocle-release) by DDR-Tree and default parameters. Before the Monocle analysis, we selected marker genes of the Seurat clustering result and the raw expression counts of the cells that passed filtering. Based on the pseudotime analysis, branch expression analysis modeling (BEAM Analysis) was used for branch fate determined gene analysis.

**Statistical analysis**

All data were expressed as the mean ± SEM. Significant differences were assessed by one or two-way ANOVA followed by the Student-Newman-Keuls test where appropriate.

**Reagent list**

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Acknowledgment

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Author Contributions

YC conceptualized the project. SW and YC designed the study, analyzed the data and wrote the paper. S.W. performed the experiments. CL, XL, LY, QW, ZM, and TW provided technical and/or conceptual assistance. All authors have read and approved the manuscript.

Declaration of Interests

The authors declare that they have no competing interests.
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Figure 1. Intermittent protein restriction reverses diabetes progression in STZ-treated mice and db/db mice.
(A) The color code of various diets used in the study.

(B, C) The effect of intermittent use of diets with various protein concentrations on glycemic control. Low-dose streptozotocin (STZ) (40 mg/kg) was injected intraperitoneally for four consecutive days into eight-week-old male C57BL/6 mice (n = 5 for each group). A diagram to depict the dietary scheme of each group is shown in A. The mice were fed with the food ad libitum with free access to water. Blood glucose levels were measured on the last day of each cycle and shown in C. Mice were fasted for 6 h (morning fasting) before glucose measurement. The area under the curve (AUC) is shown on the right to indicate the cumulative effect.

(D, E) The effect of the duration of low protein diet on glycemic control. The dietary scheme is shown in D and the fasting blood glucose level measured on the last day of each cycle is shown in E (n = 5 for each group).

(F-J) The intervention activity of IPR in STZ-treated mice. The dietary scheme is shown in F (n = 5 for each group). The fasting blood glucose level measured on the last day of each cycle is shown in G. The blood HbA1c level, glucose tolerance test (GTT) and insulin tolerance test (ITT) were analyzed at the end of the experiment and shown in H, I and J respectively.

(K-O) The intervention activity of IPR in db/db mice. The dietary scheme is shown in K. The db/db mice were fed until they became hyperglycemia and then allocated to 3 groups (n = 5 for each group). The whole experiment lasted 13 weeks and dietary intervention was only applied in the first and last 4 weeks. The fasting blood glucose levels of the mice at the end of each experimental week is shown in L. The blood HbA1c level, GTT and ITT at the end of the experiment are shown in M, N and O respectively.

All the data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ns for non-significant.
Figure 2. Intermittent protein restriction restores β cell function and increases β cell proliferation in the islets of diabetic mice

(A) IPR improves β cell function in db/db mice. Glucose-stimulated insulin secretion (GSIS) of isolated islets. The db/db mice were fed until they became hyperglycemia and then divided into 2 groups with or without intervention. Fresh islets isolated from the mice (n = 3 for each group) were used to measure GSIS.

(B) IPR increases β cell numbers in the islet of db/db mice. Representative immunofluorescence staining of pancreatic sections of db/db mice described in Figure 1K (scale bar, 20 μm). Quantification of β cells and α cells of mice is on the right.

(C, D) The effect of intermittent use of diets with various protein concentrations on β cell proliferation and C-peptide secretion. The STZ-treated mice as described in Figure 1A were used to analyze β cell proliferation by EdU proliferation assay and blood C-peptide level.

(E, F) The effect of the 3-2-2 diet on β cell proliferation and C-peptide secretion in db/db mice. The islets of the db/db mice described in Figure 1K were used to stain for Ki67-positive cells and the blood samples of the mice were used to measure C-peptide level.
Comparison of β cell proliferation between the 3-2-2 diet and continuous application of a low-protein diet. The islets of STZ-treated mice as described in SFigure2 were used to stain for Ki67-positive cells.

Analysis with a genetic lineage tracing experiment. The mechanistic details of the experiment is described in H. Representative immunofluorescence staining images of the pancreatic sections with differentiated β cells (green color) and non-β cells (red color) are shown in I. Note that yellow cells were not detected.

All the data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ns for non-significant.
Figure 3. Intermittent protein restriction reduces gluconeogenesis in the liver and improves the insulin sensitivity in the skeletal muscle of the mice (A-C) Studies in db/db mice. The db/db mice described in Figure 1K was used to measure the expression of gluconeogenic genes in the liver by quantitative RT-PCR (A), immunoblotting (B), and AKT phosphorylation in the skeletal muscle (C).
(D-H) Studies in wild type mice. Eight-week-old male C57BL/6 mice were divided into 2 groups and the intervention was lasted for 2 weeks (n = 5 for each group). Quantitative RT-PCR to detect the expression of genes involved in gluconeogenesis in the liver. Pyruvate tolerance test (PTT) was performed at the end of the experiment. PEPCK protein level in the liver was analyzed by immunoblotting. Phosphorylation of AKT was analyzed by immunoblotting in the skeletal muscle. Immunostaining of GLUT4 (green color) in the skeletal muscle is shown in H (Scale bar, 20 μm).

(I, J) IPR elevated circulating level of FGF21. The serum FGF21 levels in the STZ-treated mice as described in Figure 1D and the db/db mice as described in Figure 1K were measured and shown in I and J respectively.

The data are expressed as the mean ± SEM. *P < 0.05, ** P < 0.01, *** P < 0.001, and ns for non-significant.
Figure 4. Methionine and leucine are associated with the glucose-lowering effect of intermittent protein restriction.
(A) IPR alters the amino acid levels in the blood. The free amino acid levels of the db/db mice as described in Figure 1K were measured at the end of the experiment (n = 5 for each group).

(B, C) Supplementation experiment with essential amino acids. The STZ-treated mice (n = 5 for each group) were fed with normal chow or a low-protein diet as indicated (B). For group 3, individual essential amino acid was added in the first 3 days into the low-protein diet. The fasting blood glucose level was measured on the last day of each experimental week (C). Only the statistic difference between group 2 and group 3 is shown.

(D, E) Amino acid deprivation experiment. The STZ-untreated or treated mice were fed with normal chow or a diet deprived of methionine and/or leucine as indicated (D, n = 5 for each group). The fasting blood glucose level was measured at the end of each experimental week (E). The area under curve is shown on the right to demonstrate the cumulative effect.

The data are expressed as the mean ± SEM. *P < 0.05, ** P < 0.01, *** P < 0.001. Only the P values compared to group 1 for each amino acid is shown in A.
Figure 5. Single cell RNA sequencing reveals that intermittent protein restriction restores \( \beta \) cells and reduces immune cell accumulation in the islets of \( \text{db/db} \) mice

(A) A diagram to depict the 4 types of mice used scRNA-seq. Fresh islets were isolated from 10-week-old male C57BL/6 mice as normal control, \( \text{db/db} \) mice fed with normal chow at 10-week-old (early \( \text{db/db} \) group), \( \text{db/db} \) mice fed with normal chow (sacrificed at 14-week-
old, late db/db group) or fed with 3-2-2 diet for 4 weeks (sacrificed at 14-week-old) as
indicated. The fresh islets were isolated from 3 mice in each group and combined for
scRNA-seq.
(B) Fasting blood glucose levels of the 4 groups of mice.
(C) Projection of all cells (n = 22,749) onto two dimensions using t-SNE based on differential
expression of genes across cells.
(D) Proportional plotting graph of 10 different cell types as percentage of total cells. The
total number of cells in each group is listed.
(E) Analysis of immune cells in the islets of db/db mice. Pancreas sections were used in
immunofluorescent staining with antibodies against CD3 (for T cells), CD19 (for B cells) and
CD11c (for macrophages). The nucleus was stained with Hoechst 33342 (blue color). Only
the merged images are shown here. Scale bar, 20 μm.
(F) Flow cytometry analysis and quantification of CD45+ β cells in the db/db mice fed with
different diet for 4 weeks (n = 3 for each group). Quantification of the results is on the right.
The data are expressed as the mean ± SEM. *P < 0.05, ** P < 0.01, *** P < 0.001, and ns
for non-significant.
Figure 6. Single cell RNA sequencing demonstrates specific changes of endocrine cells during progression of diabetes and after dietary intervention.
(A) Projection of endocrine cells onto two dimensions using t-SNE.
(B) Proportional plotting graph of 4 endocrine cell types as percentage of total cells.
(C-F) Proportional plotting graph of clusters categorized as β cells, α cells, δ cells, and PP cells as percentage of total cells.
(G, H) The violin plot to show the relative expression levels of major hormones (H) and major transcription factors (I) of each cluster of endocrine cells.
Figure 7. Secretome analysis of the endocrine cells

Violin plots to show the relative expression levels of representative ligands (A) and receptors (B) among the 17 clusters of endocrine cells.
Figure 8. Pseudotime analysis with β cell subgroups

(A) Projection of β cells only onto two dimensions using t-SNE.

(B) Proportional plotting graph of 16 clusters of β cells of the four groups mice.

(C) t-SNE plot visualization of β cell clusters annotating monocle pseudo temporal dynamics. RNA velocity field was visualized using Gaussian smoothing on regular grid.

(D) Pseudotime trajectory was reconstructed with the β cells and comprised of 1 branch points (marked with black circle) and 3 states (marked with red lines).

(E) Pseudotime trajectory of the cells from each group of mice.

(F) Pie chart to show the percentage of cluster in each of the state.
Supplemental Figures (there are a total of 14 supplemental figures)

SFigure 1. IPR increases β cells in STZ-treated mice
Representative immunofluorescence staining of pancreatic sections of the STZ-treated mice as described in Figure 1F (left panel). The pancreas sections were stained with antibodies against insulin (green color) and glucagon (red color). The nucleus was stained with Hoechst 33342 (blue color). The quantification of the results is show in the right panel. ** P < 0.01 and ns for non-significant.

SFigure 2. Continuous application of a low-protein reduces blood glucose level
(A) A diagram to depict the type of diet and how different diets were used. Low-dose streptozotocin (STZ) (40 mg/kg) was injected intraperitoneally for four consecutive days into eight-week-old male C57BL/6 mice. The interventions were started after the mice had hyperglycemia. n = 6 for each group. (B) Fasting blood glucose levels of the mice measured at the end of each experimental week. The area under the curve (AUC) is shown on the right. Data are expressed as the mean ± SEM. ** P < 0.01 and *** P < 0.001.
Figure 3. Blood glucose level of the mice used in genetic lineage tracing
The fasting blood glucose level of mice used in Figure 2I. The area under curve (AUC) is shown on the right. Significant differences were assessed by one-way ANOVA followed by the Student-Newman-Keuls test where appropriate. *** P < 0.001.

Figure 4. IPR improves glucose tolerance and insulin sensitivity in wild type mice
Glucose tolerance test (GTT) and insulin tolerance test (ITT) performed with the mice used in Figure 3D. AUC is shown on the right. Significant differences were assessed by one-way ANOVA followed by the Student-Newman-Keuls test where appropriate. * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 5. Representative markers of scRNA-seq for all cells

The t-SNE representation of all cells, colored according to relative expression levels of the representative markers to differentiate among the 23 clusters.
Figure 6. Heatmap to illustrate clustering of scRNA-seq for all cells

Heatmap of cluster-enriched genes of all cells. Each column represents a single cell and each row represents one signature gene. The colors ranging from purple to yellow indicate low to high relative gene expression levels.
**Figure 7.** Representative markers to differentiate among the four types of endocrine cells in scRNA-Seq dataset

The t-SNE representation of four endocrine cell types, colored according to expression levels of 4 representative markers.
Figure 8. Heatmap of cell cluster-enriched genes of endocrine cells

Each column represents a single cell and each row represents one signature gene. The colors ranging from purple to yellow indicate low to high relative gene expression levels.
Figure 9. Representative markers that are differentially expressed among the 17 clusters of endocrine cells

Heatmap of software-selected markers that had differential expression among the 17 clusters of endocrine cells.
**Figure 10. Signatures of cluster 14**

(A) Heatmap of major genes that distinguish cluster 14 from other clusters. (B) Violin plots to show two DNA binding factors that are highly expressed in cluster 14.
**Figure 11. Violin plots to show expression pattern of various genes in the endocrine cells**

Violin plots to show the relative expression of a few genes in 17 clusters of the endocrine cells.
Figure 12. Heatmap of secretome gene expression in endocrine cells

Heatmap of major ligands (a) and receptors (b) in 17 clusters of endocrine cells from the scRNA-seq dataset.
Figure 13. Heatmap of genes that differentiate among the 16 clusters of β cells

Heatmap of representative genes that differentiate among 16 clusters of β cells from the scRNA-seq dataset.
**Figure 14. Comparison of intermittent protein restriction with intermittent calorie restriction (to be determined)**

(A) A diagram to depict the type of diet and how different diets were used. Low-dose streptozotocin (STZ) (40 mg/kg) was injected intraperitoneally for four consecutive days into eight-week-old male C57BL/6 mice. The interventions were started after the mice had hyperglycemia. For the group of calorie restriction, the mice were fed with normal chow at an amount in which the total protein intake was the same as the 3-2-2 group for the first three days every week. n = 6 for each group. (B) Fasting blood glucose levels of mice measured at the end of each experimental week. The area under the curve (AUC) is shown on the right. (C) Serum FGF21 level of the mice. (D) Quantification of EdU+ β cells per islet of mice. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 and ns for non-significant.