Disruption of Growth Factor Receptor–Binding Protein 10 in the Pancreas Enhances β-Cell Proliferation and Protects Mice From Streptozotocin-Induced β-Cell Apoptosis

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Defects in insulin secretion and reduction in β-cell mass are associated with type 2 diabetes in humans and understanding the basis for these dysfunctions may reveal strategies for diabetes therapy. In this study, we show that pancreas-specific knockout of growth factor receptor–binding protein 10 (Grb10), which is highly expressed in pancreas and islets, leads to elevated insulin/IGF-1 signaling in islets, enhanced β-cell mass and insulin content, and increased insulin secretion in mice. Pancreas-specific disruption of Grb10 expression also improved glucose tolerance in mice fed with a high-fat diet and protected mice from streptozotocin-induced β-cell apoptosis and body weight loss. Our study has identified Grb10 as an important regulator of β-cell proliferation and demonstrated that reducing the expression level of Grb10 could provide a novel means to increase β-cell mass and reduce β-cell apoptosis. This is critical for effective therapeutic treatment of both type 1 and 2 diabetes.

To maintain glucose homeostasis, pancreatic β-cells are able to adapt their insulin-secretory capacity in response to altered physiological and pathological demands. A key mechanism contributing to this adaptability is enhanced insulin secretion from existing β-cells and increased β-cell mass (1,2). β-cell mass is the sum of β-cell size, the rate of cell proliferation/differentiation, and the difference between β-cell genesis and apoptosis (3). Reduction in β-cell mass is associated with type 2 diabetes in humans (4). Therefore, expansion of the β-cell mass from endogenous sources, either in vivo or in vitro, represents a highly significant research area for developing specific treatment of human diabetes.

Growth factor receptor–binding protein 10 (Grb10) is a pleckstrin homology and Src homology 2 (SH2) domain-containing protein that interacts with several receptor tyrosine kinases, including the insulin receptor (IR) and the IGF-1 receptor (IGF1R) (5,6). The interaction between Grb10 and these receptors negatively regulates insulin and IGF-1 signaling in cultured cells (7–9) and in vivo (10,11). Grb10 negatively regulates insulin or IGF-1 signaling by binding to the kinase domain of the tyrosine-phosphorylated IR or IGF1R, mainly via its SH2 domain and partly a region between the pleckstrin homology domain and the SH2 domain (BPS) (9,12–14). Grb10 is expressed in insulin target tissues such as fat and muscle, but the highest expression of this protein is in pancreas of adult mice (11). Although disruption of the Grb10 gene in all tissues (except the brain) significantly increases the size of pancreas in mice (11,15), the underlying mechanisms are unknown. It is also unclear whether Grb10 has an autonomous role in regulating pancreatic cell mass and β-cell function. In this study, we report the establishment and characterization of the first tissue-specific Grb10 knockout mice. In addition, we demonstrate that pancreas-specific deletion of the Grb10 gene enhanced β-cell proliferation and improved glucose tolerance under high-fat diet (HPD) feeding condition. Knockout of Grb10 in the pancreas protects mice from streptozotocin (STZ)-induced apoptosis, suggesting that suppressing the expression levels of Grb10 in the pancreas could be a promising approach for increasing β-cell mass and improving β-cell function.

RESEARCH DESIGN AND METHODS

Generation of Grb10 conditional knockout mice in the pancreas. A 100-kb region of the Grb10 gene was isolated from a mouse bacterial artificial chromosome library (Invitrogen/Life Technologies, Grand Island, NY) and the targeting vector, in which exon 3 of the Grb10 gene is flanked by loxP sites (Grb10loxP), was constructed by using standard molecular biology techniques. The targeting vector was linearized with KpnI and electroporated into the 129/SVEV embryonic stem cell line. Positive clones were selected by G418 (300 μg/mL) and ganciclovir (2 μmol/L) double resistance and verified by Southern blot. Clones of Grb10loxP/loxP embryonic stem cells that underwent homologous recombination and possessed a single integration event were used to microinject C57BL/6 blastocysts. Chimeric mice were mated with C57BL/6 mice, and germline transmission of the floxed Grb10 allele was confirmed by Southern blot and PCR genotyping. The Neo cassette was removed by crossing the floxed mice carrying the targeting vector with FLPe mouse (The Jackson Laboratory) expressing FRT in their germline. Grb10 is an imprinted gene and is paternally expressed in the brain and maternally expressed in all other tissues (16). Thus, female homozygous Grb10 floxed mice were used to breed with male heterozygous PDX-Cre mice, which led to the generation of pancreas-specific Grb10 knockout mice, hereafter referred to as pGrb10KO mice, and the Grb10 flox−− wild-type (WT) littermates. Because Grb10...
expression in the brain is paternally imprinted, our breeding strategy does not affect Grb10 expression in the brain. All animals were housed in specific pathogen-free facilities, maintained on a 12-h light/dark cycle, and fed standard rodent chow at the Department of Laboratory Animal Resources in the University of Texas Health Science Center at San Antonio and the Animal Care and Use Committee of the Second Xiangya Hospital of Central South University. All protocols for animal use were approved by the University of Texas Health Science Center at San Antonio Animal Care and Use Committee and the Second Xiangya Hospital Animal Care and Use Committee of Central South University.

**Body weight, food intake, body composition, glucose, and insulin tolerance tests.** Male mice were fed an ad libitum diet (19% protein; 5% fat; 5% carbohydrate) for 56 days. Male mice (7–12 weeks old; 25–30 g body weight) were anesthetized by injection of 0.3 mL of ketamine (I.M. 79±2.3 mg/kg body weight) and xylazine (10 mg/100 g body weight). An indwelling catheter was inserted in the right internal jugular vein 4 to 5 days before clamp experiments. Mice were housed in individual cages and monitored for postoperative recovery and weight gain. Following overnight fast, a 2-h hyperglycemic clamp was conducted in conscious mice with a variable infusion of 20% glucose to raise and maintain plasma glucose concentration at ~300 mg/dL. Blood samples (40 μL) were collected at 5–10-min intervals for the measurement of plasma glucose and insulin levels.

**Islet isolation and islet viability testing.** Mouse islets were isolated by collagenase digestion of the pancreas according to the procedures described (17–19). In brief, overnight-fasted male pGrb10KO mice and WT littermates (2–6 months old) were anesthetized by intraperitoneal injection of avertin (1 mL/40 g body weight). Mouse pancreases were inflated by injection of 3 mL of a collagenase P solution (Sigma Chemical, St. Louis, MO). 1 mg/mL in Hank's buffered salt solution (HBSS) was used to digest the pancreas for 20 min at 37°C with vigorous agitation. Mouse islets were removed and incubated at 37°C for about 12 min to allow complete digestion. Digestion was stopped by the addition of 30 mL of Hank's buffer solution, followed by washing the islets with 20 mL RPMI-1640 medium three times. Isolated islets were selected with the aid of a pipette under a stereo microscopic view from the medium and maintained in RPMI-1640 with or without 10% FBS or HEPES-buffered Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.19 mM MgCl2, 1.19 mM NaHCO3, 25 mM NaHPO4, and 10 mM HEPES [pH 7.4]) containing 0.5% BSA at 37°C.

For insulin and IGFI-1 signaling studies, the freshly isolated islets (30 islets/experiments) were serum-starved for 2 h and then treated with or without 100 nM insulin for 30 min or 100 nM IGF-I for 30 min. For mammalian target of rapamycin (mTOR) signaling studies, freshly isolated islets (50 islets/experiment) were serum-starved for 2 h and then treated with 100 nM insulin with or without 100 nM IGF-I for 30 min. Islets were lysed, and insulin-stimulated phosphorylation of Akt at Thr2451 and Ser473, extracellular signal–regulated, mitogen-activated protein kinase 1 and 2 (ERK1/2) at Thr202/Tyr204, p70S6K at Thr389, and 4E-BP1 at Thr37/46, as well as the protein levels of these insulin/IGF-1 or mTOR signaling molecules, were determined by Western blot using specific antibodies. Antibodies and phosphoantibodies to Akt, ERK1/2, S6K, and 4E-BP1 were purchased from Cell Signaling Technology or Millipore (for ERK1/2). The antibody to Grb10 was described previously (8). Wortmannin was purchased from Sigma Chemical.

For insulin granule counting, islets were isolated, fixed with 1% glutaraldehyde, and then embedded in epon. Blocks of tissue (0.1–1.5 mm3) were osmicated in 0.1 M sodium cacodylate buffer (pH 7.4) containing 1% OsO4 for 1 h at room temperature, rinsed in cacodylate buffer, and then rinsed in distilled water before they were stained in 2% uranyl acetate double-distilled water for 1 h. Sections were dehydrated in graded alcohol and placed in 100% propylene oxide. The blocks were incubated in epon-propylene oxide (1:1) overnight at room temperature and then changed to 100% epon and polymerized overnight. Sections (90 nm) were cut on a Reichert-Jung Ultracut E microtome, collected on slot grids, and stained with 2% uranyl acetate and lead citrate. The pictures were captured by Jeol1230 Transmitted Electronic Microscope (JEOL, Peabody, MA).

**Islet proliferation assays.** Male mice (2 months old; n = 3 to 4/group) were injected intraperitoneally with 100 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Roche Molecular Biochemicals, Indianapolis, IN) and killed 16 h after injection. Pancreases were isolated, fixed, and sectioned. Pancreas slices were analyzed by double staining with anti-BrdU (Millipore) and anti-insulin (Santa Cruz Biotechnology) antibodies. Insulin-positive β-cells (>2,500 β-cells/pancreas) were counted, and the percentage of BrdU- and insulin-double positive nuclei to total insulin-positive nuclei was determined for both pGrb10KO mice and their WT control mice. Quantification of total BrdU-positive cell numbers was done using an unbiased stereological method to ensure that the same BrdU-labeled cell is not counted twice on adjacent sections and that the area of the pancreatic sections counted for each animal are consistent.

**Immunohistochemistry of pancreas.** The immunohistochemistry experiments were carried out as previously described (20), with some modifications. In brief, pancreata obtained from 10-week-old male pGrb10 KO mice and their Grb10 WT littermates, cleared of fat and spleen, were fixed and processed in 4% paraformaldehyde (Electro Microscopic Sciences, Fort Washington, PA). The fixed tissues were embedded in Optimal Cutting Temperature Compound (Ted Pella, Inc.) and cut into 10-μm sections using a cryostat or a microtome. The sections were rehydrated, permeabilized, and stained with specific antibodies (Grb10 [homemade], 1:400; Insulin [Santa Cruz Biotechnology], 1:100; Grb10 WT, 1:100; β-cells [Cell Signaling Technologies], 1:50). The cell nuclei were determined by 4',6-diamidino-2-phenylindole staining.

The analysis of islet mass and β-cell size. Male mouse pancreases were cut into 10-μm sections systematically through pancreatic head-to-tail axis, and sections were selected between every 200 μm. Eight sections per mouse were picked. Mouse pancreatic slices were stained with an anti-insulin antibody. Images of pancreas slices were viewed by an Olympus inverted microscope (IX70 Olympus) and captured with a Spot II digital camera (Olympus, Japan). All insulin-positive β-cell clusters (islets) were loosely traced, and the insulin-immunoreactive areas were determined by use of the threshold option (21). Total tissue areas were quantified with the threshold option to select the stained areas but not unstained areas (white space). The islet area (in square micrometers) and the area of each section were determined with Image J software (National Institutes of Health). Four to eight sections of each pancreas were analyzed by acquiring images from eight nonoverlapping fields of 1.5 × 103 μm2. Analyses of β-cell area and size were performed using Image J software (National Institutes of Health) or the Image-Pro Plus software ((Version 5.0, Media Cybernetics, Inc.). β-cell mass was calculated by insulin-immunopositive area/total pancreatic area times weight. (17,22,23).

**STZ-induced β-cell apoptosis and transferase-mediated dUTP nick-end labeling assays.** Male WT and pGrb10KO mice (2 months old) were injected once per day with freshly prepared STZ solution (0.5% in 0.1 M sodium citrate [pH 4.5]) for 5 days (75 mg/kg i.p.). Blood glucose levels and body weight were examined every 3 days at the same time (12 P.M. every day). Twenty-two days later, mice were killed, and serum insulin levels, islet mass, and pancreatic weight of the mice were determined. For acute STZ treatment experiments, male mice were injected with STZ twice daily (at 0 and 24 h) and killed 16 h later. Pancreas sections of the pancreas were labeled with an in situ cell death detection kit (Roche) and insulin antibody, and STZ-induced apoptosis was determined by immunofluorescence using a Nikon TE2000 microscope (Nikon). Four mice per group were used for transferase-mediated dUTP nick-end labeling (TUNEL) assays, and at least five islets (500–1,000 β-cells) were counted for each mouse.

**Statistics.** Quantification of the relative increase in insulin-stimulated protein phosphorylation was performed by analyzing Western blots using Scion Image software and was normalized with the amount of protein expression in each experiment. Statistical analysis of the data was performed by using unpaired Student t test or ANOVA (one-way repeated-measure ANOVA or two-way ANOVA with post hoc Bonferroni test). Statistical significance was set at P values of <0.05, <0.01, and <0.001.

**RESULTS**

**Generation and characterization of pGrb10KO mice.** Grb10 floxed construct was generated by insertion of loxp sites at both ends of exon 3 of the Grb10 gene (Fig. 1A). exon/intron boundary analysis revealed that deletion of the exon causes a frameshift and thus a null mutant of Grb10. The floxed Grb10 allele was confirmed by Southern blot (Fig. 1B) and PCR genotyping (data not shown). The Neo cassette was removed by crossing the floxed mice with the FLP mice expressing FRT in their germline (The

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Grb10 is an imprinted gene and is maternally expressed in peripheral tissues (6). Thus, we bred female homozygous Grb10 floxed mice with male heterozygous PDX-Cre+/− mice, which led to Cre-mediated deletion of the Grb10 gene specifically in the pancreas but not other tissues including the brain (pGrb10KO; Fig. 1C and Supplementary Fig. 1B and C). Western blot analysis also revealed that Grb10 is highly expressed in mouse pancreas and islets (Fig. 1C and Supplementary Fig. 1A). By immunohistochemistry studies, we found that Grb10 protein is expressed in islets and neighboring cells in the pancreas, and its signal is highly colocalized with insulin-staining cells (Fig. 1D, top panel). This further confirms that Grb10 is expressed in pancreatic β-cells. No Grb10 signal was detected in the pancreatic section of the pGrb10KO mice (Fig. 1D, bottom panel), demonstrating the successful deletion of the Grb10 gene in the pancreas of the knockout mice. Consistent with the finding of Smith et al. (10), our breeding strategy had no effect on Grb10 expression in the hypothalamus (Supplementary Fig. 1B and C).

Pancreas-specific knockout of Grb10 had little effect on mouse body composition, food intake, and body weight compared with WT littermates (Supplementary Figs. 2 and 3), but significantly increased pancreas tissue weight (Fig. 2A). Whereas the β-cell size did not differ between these two strains fed with normal chow diet, the male pGrb10KO mice displayed more β-cell mass than WT mice (Fig. 2B and C). By 4′,6-diamidino-2-phenylindole/insulin/BrdU triple staining of pancreatic sections, we found that the rate of BrdU incorporation into β-cell cells was quite slow in WT...
Pancreas-specific disruption of the Grb10 gene improves glucose tolerance and insulin secretion. Under normal chow-feeding conditions, the pGrb10KO mice showed a slightly lower fasting blood glucose levels and displayed better glucose tolerance compared with the WT littermates (Fig. 3A). However, there was no overall significant difference in insulin tolerance between pGrb10KO and WT littermates. On an HFD, the pGrb10KO mice and their WT littermates showed similar body weight, food intake, and body composition (Supplementary Fig. 3). Although insulin tolerance tests showed similar insulin tolerance between the two strains (data not shown), the pGrb10KO mice displayed significantly better glucose tolerance compared with WT littermates (Fig. 3B), suggesting improved insulin secretion in the pGrb10KO mice. Under normal diet, both WT and pGrb10KO mice exhibited relatively normal glucose levels. Although pGrb10KO mice showed slightly lower glucose levels, they were not hypoglycemic (Fig. 3A). After challenged with 60% HFD for 16 weeks, WT mice showed high glucose levels, but pGrb10KO mice still maintain comparably lower serum glucose (Fig. 3B). Consistent with these findings, hyperglycemic clamp studies revealed that pGrb10KO mice exhibited higher glucose infusion rate (GIR) compared to wild-type littermates (Fig. 3C). The insulin secretion rate of the pGrb10KO mice was also higher than that of the wild-type control mice during the hyperglycemic clamp studies (Fig. 3D).

Targeted deletion of the Grb10 gene in pancreas enhances β-cell mass and insulin/IGF-1 and mTOR signaling in islets. To elucidate the mechanism by which disruption of Grb10 expression leads to increased insulin secretion in mice, we examined total β-cell mass and individual β-cell size. The male pGrb10KO mice displayed higher β-cell mass than that of the WT littermates (−0.003 vs. 0.001–0.0015 g/mouse) (Fig. 4A). Consistent with these results, transmission electron microscopy studies showed increased insulin granules in β-cells of the pGrb10KO mice compared with WT littermates (Fig. 4B; 12.47 ± 0.34/µm² vs. 9.25 ± 0.52/µm²; P < 0.001). However, the sizes of individual β-cells are similar between these two strains (Fig. 4C), suggesting that the increased β-cell mass is mainly due to enhanced cell proliferation in the pGrb10KO mice. This finding is also consistent with the results from the BrdU staining experiments (Fig. 2D). By Western blot, we found that insulin and IGF-1 had a greater effect on the phosphorylation of Akt Thr⁴⁰⁸ and ERK1/2 Thr²⁰²/Tyr²⁰⁴ in islets isolated from HFD-fed pGrb10KO mice compared with their WT littermates (Fig. 4D). Insulin-stimulated phosphorylation of Akt Thr⁴⁰⁸ and Ser⁷²⁷, ERK1/2 Thr²⁰²/Tyr²⁰⁴, p70S6K Thr³⁸⁹, and 4E-BP1 Thr³⁷/4⁶ was enhanced in pGrb10KO mice compared with WT mice and could have been blocked by wortmannin (Fig. 4E). Similar results were also observed in mice fed with normal diet (data not shown).

Pancreas-specific knockout of Grb10 protects mice from STZ-induced diabetes. To determine whether pancreas-specific deletion of the Grb10 gene has a beneficial effect on diabetes, we investigated the effect of low-dose STZ treatment on β-cell damage in 2-month-old pGrb10KO mice and WT littermates. STZ treatment greatly increased serum glucose levels in WT mice, and the STZ-induced hyperglycemic effect was significantly reduced in the pGrb10KO mice (Fig. 5A). Pancreas-specific knockout of Grb10 also significantly protected mice from STZ-induced body weight loss (Fig. 5B), which is a typical characteristic of diabetes. Furthermore, the male pGrb10KO mice showed
and measured using a glucometer. Blood glucose was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min after intraperitoneal injection of glucose (2 g/kg body weight) in 3-month-old normal chow diet male pGrb10KO and WT control mice (n = 9) fed with 60% HFD for 16 weeks. Blood glucose after intraperitoneal injection of glucose (2 g/kg body weight) was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min.

**FIG. 3.** Pancreatic knockout of Grb10 increases glucose tolerance and insulin secretion. **A:** GTT was performed in 5-month-old male pGrb10KO mice (n = 9) and age-matched WT littermates (n = 8) fed with normal diet. Blood glucose was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min after intraperitoneal injection of glucose (2 g/kg body weight) and measured using a glucometer. **B:** GTT was performed on overnight-fasted male pGrb10KO mice and WT littermates (WT: n = 11; pGrb10KO: n = 9) fed with 60% HFD for 16 weeks. Blood glucose after intraperitoneal injection of glucose (2 g/kg body weight) was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min. **C:** The glucose infusion rates in 3-month-old normal chow diet male pGrb10KO and WT control mice were determined by hyperglycemic clamp experiments (n = 4/group). **D:** Insulin levels of male pGrb10KO and WT littermates during hyperglycemic clamp experiments (n = 4/group). The data represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (one-way repeated-measure ANOVA).

**DISCUSSION**

Increasing β-cell mass is one of the mechanisms by which glucose homeostasis is maintained in vivo in response to physiological (i.e., growth and gestation) and pathophysiological (i.e., obesity and insulin resistance) stimuli. Unlike other terminally differentiated cell types, pancreatic β-cells retain their ability to proliferate under both physiological and pathophysiological conditions (3). In the progression of diabetes, insulin secretion is increased, and a large amount of the increase in the first stage of this compensation process is due to increased β-cell mass, which is mainly achieved through an increase in β-cell number (24).

The gene encoding for Grb10 is imprinted in mammalian cells, and its expression is defined by its parental origins (25). The maternal allele determines Grb10 expression in all tissues except the brain, and the paternal allele determines Grb10 expression in the brain. Grb10 is expressed in insulin target tissues such as fat and muscle in vivo. The unique structure of the SH2 domain of Grb10 confers its binding specificity to either the insulin or the IGF1R (13) and thus its negative role in insulin or IGF-1 signaling. Ablation of Grb10 in the brain engenders increased social activities of mice (15). Maternal Grb10-deficient animals show enlarged body size and organ size, which enhances insulin signaling and sensitivity (11). In contrast, overexpression of Grb10 in mice led to postnatal growth retardation and insulin resistance (26) or glucose intolerance (27). Dual ablation of Grb10 and Grb14 has no additive effects on insulin signaling and body composition (28).

In the current study, we have used a tissue-specific knockout strategy to demonstrate for the first time that Grb10 plays an important autonomous role in the negative regulation of pancreas mass and β-cell proliferation (Fig. 2A and C). The pancreas contains multiple cell types including the exocrine (acinar) cells and endocrine cells that included insulin-secreting β-cells. We found that Grb10 disruption, specifically in the pancreas, results in an enlarged pancreas (Fig. 2A), which is consistent with previous findings that Grb10 negatively regulates tissue growth (26, 29). Our results also showed pancreas-specific knockout of Grb10 increased β-cell mass (Fig. 2C). However, because β-cells make up <2% of the pancreas, it is likely that the increased β-cell mass cannot account for the doubling of pancreas mass, suggesting that Grb10 also plays a role in regulating the mass of other cells in the pancreas. It remains to be established whether the enhanced islet mass and β-cell function is due to altered communication between β-cells and neighboring cells. However, some early studies showed that enhanced acinar mass is negatively correlated with insulin secretion and islet mass (30, 31). In contrast, we have demonstrated that pancreas-specific deletion of the Grb10 gene protects mice from STZ-induced β-cell apoptosis (Fig. 5). Because Grb10 is a negative regulator of insulin and IGF-1 signaling, it is most likely that the increased β-cell proliferation and enhanced resistance to STZ-induced apoptosis are due to increased insulin/IGF-1 signaling or its mediated downstream signaling events. Consistent with more pancreas weight than that of the WT littermates (Fig. 5C). In addition, the male pGrb10KO mice displayed relatively higher plasma insulin levels in response to STZ treatment (Fig. 5D) and were protected from STZ-induced loss of β-cells (Fig. 5E). Consistent with these findings, TUNEL assays showed that the pGrb10KO mice had lower STZ-induced β-cell apoptosis compared with WT littermates (Fig. 5F).

**FIG. 3.** Pancreatic knockout of Grb10 increases glucose tolerance and insulin secretion. **A:** GTT was performed in 5-month-old male pGrb10KO mice (n = 9) and age-matched WT littermates (n = 8) fed with normal diet. Blood glucose was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min after intraperitoneal injection of glucose (2 g/kg body weight) and measured using a glucometer. **B:** GTT was performed on overnight-fasted male pGrb10KO mice and WT littermates (WT: n = 11; pGrb10KO: n = 9) fed with 60% HFD for 16 weeks. Blood glucose after intraperitoneal injection of glucose (2 g/kg body weight) was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min. **C:** The glucose infusion rates in 3-month-old normal chow diet male pGrb10KO and WT control mice were determined by hyperglycemic clamp experiments (n = 4/group). **D:** Insulin levels of male pGrb10KO and WT littermates during hyperglycemic clamp experiments (n = 4/group). The data represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (one-way repeated-measure ANOVA).
this, we found that insulin- and IGF-1–stimulated phosphorylation of Akt and ERK1/2 was increased in β-cells isolated from pGrb10KO mice compared with that in the WT littermates (Fig. 4D). However, it has been shown that β-cell–specific disruption of the genes encoding components in the insulin signaling pathway, including the IR (32), the IGF1R (33,34), phosphoinositide-dependent kinase-1 (34), and Akt (36,37), had little effect on β-cell proliferation. Thus, it is possible that Grb10 negatively regulates other signaling pathways in addition to the insulin/IGF-1 signaling pathways that regulate β-cell proliferation. Consistent with this view, we found that the mTOR signaling pathway is increased in islets of the Grb10 knockout mice compared with WT littermates (Fig. 4E). There is some evidence showing that the mTOR signaling pathway plays a key role in the regulation of β-cell mass, survival, and insulin production (40–42). Thus, the increased mTOR signaling may contribute to the enhanced β-cell proliferation.

**FIG. 4.** Pancreatic-specific knockout of Grb10 increased β-cell mass, insulin granule numbers, and insulin/IGF-1 signaling in islets. pGrb10KO mice and WT littermates were fed with 60% HFD for 16 weeks. A: Average β-cell mass between male pGrb10KO (n = 4) and WT littermates (n = 3). B: Insulin granule numbers in islets of male pGrb10KO (n = 4) and WT littermates (n = 3) were detected. The numbers of docked granules were measured in a cell-surface area of 100 μm². Scale bars, 2 μm. The data represent mean ± SEM. C: The average β-cell size was analyzed from male pGrb10KO and WT littermates (n = 4/group; >500 cells were counted per slide) using Image-Pro Plus software (Version 5.0; Media Cybernetics, Inc). D: Insulin signaling and IGF-1 signaling in β-cell mass. Islets were isolated from HFD-fed male pGrb10KO and WT control mice (n = 5/group), serum starved for 2 h, and treated with insulin (100 nmol/L) or IGF-1 (100 nmol/L) for 30 min. Phosphorylation and the protein levels of Akt Thr⁹⁶⁸ and ERK1/2 Thr²⁰²/Tyr²⁰⁴ were determined by Western blot using specific antibodies as indicated. E: mTOR signaling in β-cell mass. Islets were isolated from male pGrb10KO and WT control mice (n = 5/group), serum starved for 2 h, and treated with insulin (100 nmol/L) with or without wortmannin (100 nmol/L) for 30 min. Phosphorylation and the protein levels of Akt Thr⁹⁶⁸ and Ser⁴⁷³, ERK1/2 Thr²⁰²/Tyr²⁰⁴, p70S6K Thr³⁸⁹, and 4E-BP1 Thr³⁷/4⁶ were determined by Western blot using specific antibodies as indicated. Data were quantified by using the Scion Image program (Scion Corporation, Frederick, MD). Black bars, Grb10 flox⁺⁻; white bars, pGrb10 KO. The data represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (t test).
and mass. However, how knocking out of Grb10 increases mTOR signaling remains unknown. Grb10 has recently been identified as a direct substrate of mTOR, and the phosphorylation plays a feedback role in the negative regulation of the phosphatidylinositol 3-kinase (PI3K) and ERK1/2 pathways (38,39). Because mTOR functions downstream of the PI3K/Akt signaling pathway, it is possible that reducing the negative regulation of Grb10 on PI3K/Akt signaling leads to enhanced mTOR signaling. However, it is also possible that Grb10 may have a direct effect on mTOR. We are currently investigating these possibilities.

It is well-established that β-cells have low antioxidant protection capability and are prone to apoptosis induced by oxidative stress and inflammation (43,44). In fact, pancreatic β-cell apoptosis has been recognized as a key event in type 1 diabetes (45). Our results show that pancreas-specific disruption of Grb10 protects mice from STZ-induced apoptosis (Fig. 5F). Because Grb10 is highly expressed in pancreas (11) and islets (Fig. 1C and D), and this protein negatively regulates insulin/IGF-1 signaling in cells (7–9) and in vivo (10,11), which are known to exert an antiapoptotic function, it is possible that the high expression level of Grb10 in β-cells plays a causative role in the low antioxidant protection capability. Thus, reducing Grb10 expression in β-cells would provide an approach to reduce the rate of oxidative stress-induced apoptosis.

Our results show that knockout of Grb10 enhances insulin signaling in isolated islets and protects mice against STZ-induced apoptosis (Figs. 4D and 5F), suggesting that Grb10 has an autonomous effect on β-cell function and survival. However, the finding that Grb10 is expressed not only in insulin positive β-cells but also in other cells of the islets (Fig. 1D) suggests that Grb10 may have an additional function. It is well-established that the pancreas contains multiple cell types including the exocrine (acinar) cells and endocrine cells such as the glucagon-producing α-cells, the insulin-producing β-cells, the somatostatin-producing δ...
cells, and the pancreatic polypeptide- and ghrelin-producing ε cells (46). A number of studies have demonstrated that hormones and other paracrine factors from neighboring cells play critical roles in regulating insulin release from β-cells (47–49). Although it remains to be established whether Grb10 is expressed in all of these cells and is involved in the communication between β-cells and neighboring pancreatic cells, it is possible that in addition to an autonomous mechanism, Grb10 may regulate β-cell function and survival by a nonautonomous mechanism in vivo. Further studies will be needed to test this possibility.

In summary, our study has identified Grb10 as a key negative regulator of β-cell proliferation, function, and survival. β-cell failure, which is largely due to impairment in cell proliferation and increased cell death, is a key component in the pathogenesis of both type 1 and type 2 diabetes. Therefore, increasing β-cell mass from endogenous sources represents a highly significant research area for developing specific therapeutic treatment for human diabetes. Approaches to suppress Grb10 function in β-cells may thus be an effective strategy for treating type 1 and type 2 diabetes.

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J.Z. designed and performed research, analyzed data, and wrote the manuscript. N.Z., N.M., and R.A.D. helped with the clamp study. M.L., L.Z., W.H., Z.X., and J.L. performed research. X.Li and J.M.C. helped in the generation of the Grb10 floxed mice. Z.Z. and X.-Y.Lu analyzed data. F.L. supervised the project, designed the research, analyzed data, and wrote the manuscript. F.L. 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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