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Betatrophin: A Hormone that Controls Pancreatic β Cell Proliferation

Peng Yi,[1] Ji-Sun Park,[1] and Douglas A. Melton[1,*]
[1]Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA
*Correspondence: dmelton@harvard.edu
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SUMMARY

Replenishing insulin-producing pancreatic β cell mass will benefit both type I and type II diabetics. In adults, pancreatic β cells are generated primarily by self-duplication. We report on a mouse model of insulin resistance that induces dramatic pancreatic β cell proliferation and β cell mass expansion. Using this model, we identify a hormone, betatrophin, that is primarily expressed in liver and fat. Expression of betatrophin correlates with β cell proliferation in other mouse models of insulin resistance and during gestation. Transient expression of betatrophin in mouse liver significantly and specifically promotes pancreatic β cell proliferation, expands β cell mass, and improves glucose tolerance. Thus, betatrophin treatment could augment or replace insulin injections by increasing the number of endogenous insulin-producing cells in diabetics.

INTRODUCTION

Diabetes results from dysfunctional carbohydrate metabolism that is caused by a relative deficiency of insulin. It has become a major threat to human health, the prevalence of which is estimated to be 2.8% worldwide (171 million affected) and is predicted to rise to 4.4% (366 million) by 2030 (Wild et al., 2004). Around 10% of diabetics in the United States have type I diabetes, a disease caused by an autoimmune attack on pancreatic β cells and a consequent β cell deficiency. The majority of diabetics are type II, characterized by interrelated metabolic disorders that include decreased β cell function, peripheral insulin resistance, and, eventually, β cell failure and loss or dedifferentiation (Scheen and Lefèbvre, 1996; Talchai et al., 2012). Though the disease can be treated with antidiabetic drugs or subcutaneous insulin injection, these treatments do not provide the same degree of glycemic control as functional pancreatic β cells and do not prevent the debilitating consequences of the disease. Treatments that replenish β cell mass in diabetic patients could allow for the long-term restoration of normal glycemic control and thus represent a potentially curative therapy. Despite the fact that the primary causes for type I and type II diabetes differ, all diabetics will benefit from treatments that replenish their β cell mass.

Though there is evidence that mouse β cells can be derived from rare adult progenitors under extreme circumstances (Xu et al., 2008), the vast majority of new β cells are generated by simple self-duplication (Dor et al., 2004; Meier et al., 2008; Teta et al., 2007). After a rapid expansion in embryonic and neonatal stages, β cells replicate at an extremely low rate (<0.5% divide per day) in adult rodents (Teta et al., 2005) and in humans (Meier et al., 2008). However, pancreatic β cells retain the capacity to elevate their replication rate in response to physiological challenges, including gestation (Parsons et al., 1992; Rieck et al., 2009), high blood sugar (Alonso et al., 2007), pancreatic injury (Cano et al., 2008; Nir et al., 2007), and peripheral insulin resistance (Brüning et al., 1997; Kulkarni et al., 2004; Michael et al., 2000; Pick et al., 1998).

The genetic mechanisms controlling β cell proliferation are incompletely understood. The cell-cycle regulators cyclin D1/D2 and CDK4 promote β cell proliferation (Georgia and Bhushan, 2004; Kushner et al., 2005; Rane et al., 1999), and cell-cycle-related transcription factors such as E2F1/2 are essential for pancreatic β cell proliferation (Fajas et al., 2004; Iglesias et al., 2004). On the contrary, cell-cycle inhibitors, including $p15^{Ink4b}$, $p18^{Ink4c}$, and $p27^{Kip1}$, repress β cell replication (Latres et al., 2000; Pei et al., 2004; Uchida et al., 2005). Other genes reported to regulate β cell proliferation include NFAT, Menin, p53, Rb, and Irs2 (Crabtree et al., 2003; Harvey et al., 1995; Heit et al., 2006; Kubota et al., 2000; Williams et al., 1994).

In addition to the factors listed above, which are expressed in β cells themselves and act in a cell-autonomous fashion, there are several reports showing that systematic or circulating factors can regulate β cell replication and mass. Glucose itself is a β cell mitogen; infusion of glucose in rodents causes a mild increase in β cell replication (Alonso et al., 2007; Bernard et al., 1998; Bonner-Weir et al., 1989). And glucokinase defects significantly decrease the compensatory proliferation of pancreatic β cells in some contexts (Terauchi et al., 2007). In addition, genetic deletion of glucokinase in β cells can reduce replication rates, whereas pharmacological activation of this enzyme increases replication by 2-fold (Porat et al., 2011). Several hormones, including insulin, placental lactogen, and prolactin, also play a role in regulating β cell mass (Bernard et al., 1998; Paris et al., 2003; Parsons et al., 1992; Sachdeva and Stoffers, 2009). The incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent

insulinotropic peptide (GIP) increase insulin secretion and promote β cell replication (reviewed in Drucker, 2006). However, from a therapeutic perspective, the problem with manipulating most of the genes and hormones currently known to impact β cell replication is their lack of β cell specificity and/or the fact that the magnitude of their effect on β cell proliferation is quite modest.

Transplantation studies in mice have shown that insulin resistance results in a circulating islet cell growth factor that is independent of glucose and obesity (Flier et al., 2001). In a telling demonstration, the liver-specific deletion of the insulin receptor results in a dramatic compensatory increase in pancreatic β cell replication (Michael et al., 2000). Similarly, overexpression of a constitutively active MEK1 kinase in mouse liver increases the replication rate in pancreatic β cells and improves glucose tolerance in disease models through an innervation-dependent mechanism (Imai et al., 2008). Precisely how the liver signals pancreatic β cells to proliferate is unknown, but recent work by Kulkarni's group points to the possibility that liver cells secrete a protein that acts directly on islet cells (El Ouaamari et al., 2013; Flier et al., 2001).

In this study, we aimed to identify secreted signals that control pancreatic β cell proliferation. As a first step, we developed a novel insulin resistance mouse model in which β cell replication can be rapidly induced at will. We show that administration of an insulin receptor antagonist induces acute peripheral insulin resistance and leads to a dramatic proliferation in pancreatic β cells and subsequent β cell mass expansion. Using this model, we identified a gene encoding a secreted protein that is expressed in liver and fat and whose expression level is elevated upon insulin resistance. We called this gene betatrophin because its overexpression in mouse liver produces a secreted protein that significantly and specifically promotes pancreatic β cell proliferation and β cell mass expansion and, consequently, improves glucose tolerance.

RESULTS

Administration of an Insulin Receptor Antagonist Induces Insulin Resistance and Pancreatic β Cell Proliferation

Previous work showed that, when the insulin pathway is blocked in vivo in the liver, pancreatic β cell mass expands and there is an increase in insulin secretion as a compensatory response (Brüning et al., 1997; Michael et al., 2000). To investigate the signals that control this type of β cell compensatory growth, we explored a new pharmacological model of severe insulin resistance. S961 is a peptide (43aa) that binds the insulin receptor and antagonizes insulin signaling both in vitro and in vivo in rats (Schäffer et al., 2008). We used osmotic pumps to infuse adult mice with various doses of S961. The data in Figure 1A show that S961 causes hyperglycemia in a dose-dependent manner. A high dose of S961 infused for a week makes the mice glucose intolerant (Figures 1B and 1C), consistent with the fact that S961 blocks the insulin receptor. Plasma insulin levels rise at all doses of the insulin antagonist, presumably due to the compensatory effort of pancreatic β cells (Figure 1D).

To examine whether S961 induces a compensatory β cell proliferation, as seen in other insulin resistance models, the β cell proliferation rate was analyzed by Ki67 and insulin immunofluorescence for all dosage groups following S961 treatment. S961 treatment results in a dramatic increase in β cell proliferation (Figure 2A), which is both immediate and dose dependent (Figure 2B and Figures S1A–S1E available online). The effect of S961 on β cell replication rates is strong but transient: 4 days after S961 is withdrawn, β cell replication rates return to normal (Figure S1F). The proliferation in β cells was confirmed by immunostaining for a nuclear β cell marker (Nkx6.1) and a different cell division marker (PCNA; Figures S2A and S2B). Quantitative PCR analysis of cell-cycle regulators shows that, in pancreatic islets following S961 treatment, the expression level of several cyclins (cyclins A1, A2, B1, B2, E1, and F), CDKs (CDK1 and CDK2), and E2Fs (E2F1 and E2F2) increases, whereas the expression of cell-cycle inhibitors (Cdkn1a, Cdkn1b, and Cdkn2b) decreases (Figure S3A). Even a low dose of S961 (5 nmol/week), which does not detectably alter blood glucose levels, produces a modest but reproducible increase in β cell replication (~4.3-fold increase; Figure 2B). At the highest dose tested, S961 treatment resulted in an ~12-fold increase in β cell replication (Figure 2B), a rate that vastly exceeds any previously reported pharmacological treatment.

The increase in β cell replication rate appears to affect all pancreatic islets equally (Figure S3B) and leads to an increase in total β cell area of ~3-fold within 1 week (Figures 2C–2E), primarily resulting from an increase in islet size (Figure S3C). Though β cell mass expands after S961 treatment, pancreatic insulin content decreases (Figure 2F) possibly because β cells secrete more of their insulin into circulation as a consequence of insulin resistance. And though treatment of mice with a low dose of S961 (2.5 nmol/week) does not produce a detectable increase in β cell proliferation at day 7, as measured by Ki67 (Figure 2B), their β cell mass is nonetheless about 1.5-fold higher than the control. Quantification of average β cell size shows no significant difference between vehicle- and S961-treated animals (Figure S3D). Thus, the increased β cell mass observed at the low dose of S961 (2.5 nmol/week) is not likely due to β cell hypertrophy but, rather, to the result of a transient increase of β cell proliferation prior to day 7 of S961 treatment. The proliferation induced by S961 administration is highly specific to pancreatic β cells. No obvious differences in cell proliferation rates were noticed between control- and S961-treated animals for other pancreatic cell types, including other endocrine cells, exocrine cells, and duct cells, nor for liver, white fat, or brown fat (Figure 2G).

Identification of Betatrophin in S961-Treated Mouse Liver and White Fat

To understand how S961 induces β cell proliferation, we first applied it directly to mouse β cells in vitro to see whether this insulin antagonist works in a β-cell-autonomous manner, but there was no detectable effect (data not shown). Based on this result, we hypothesized that S961 acts indirectly on β cells and analyzed gene expression in tissues involved in metabolic regulation (liver, white fat, and skeletal muscle) in addition to pancreatic β cells themselves to identify potential mediators of the

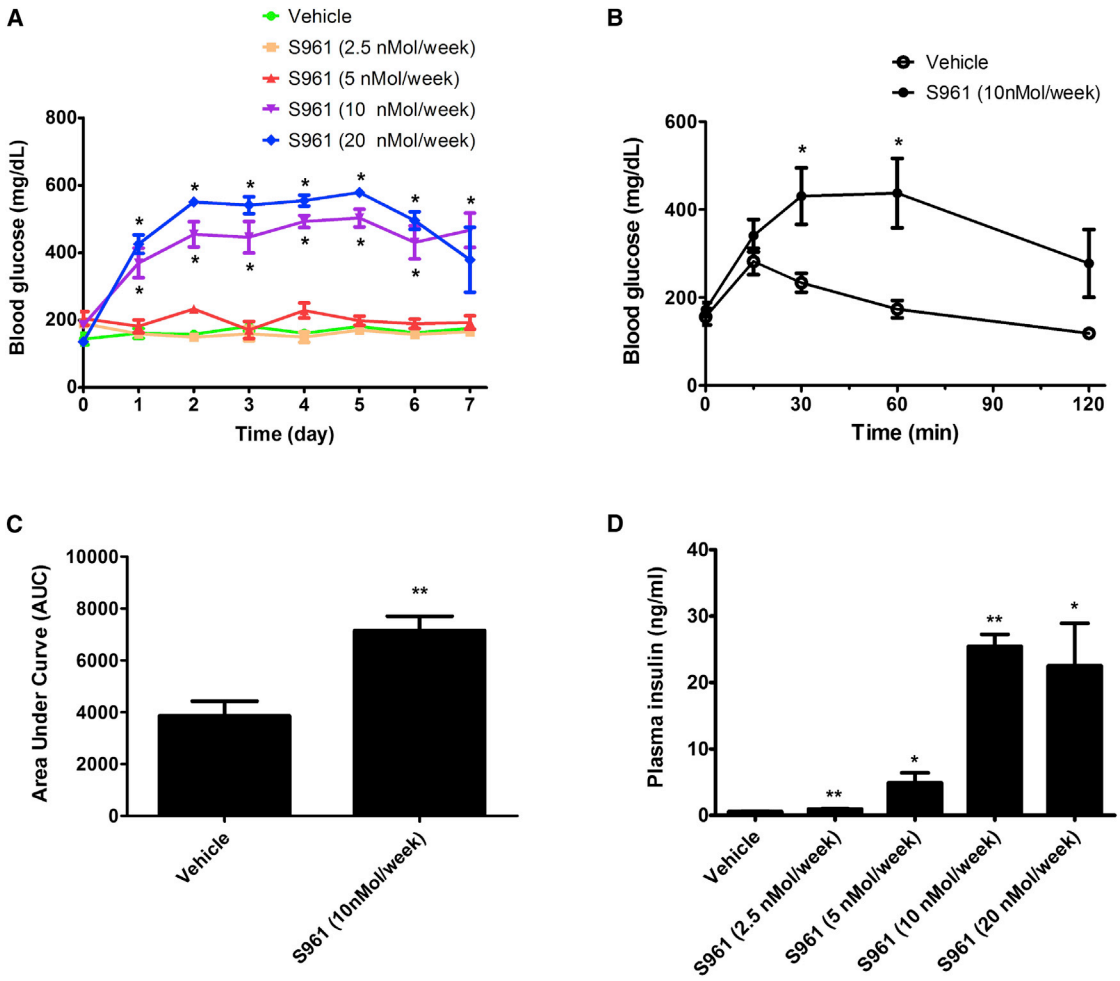

Figure 1. Administration of the Insulin Receptor Antagonist S961 Induces Glucose Intolerance, Hyperglycemia, and Hyperinsulinemia

(A) Continuous treatment of male C57BL/6J mice for 7 days with S961 at different dosages induces hyperglycemia. The fed glucose level is measured daily after pump implantation. n = 4 for each dosage group.

(B) Glucose tolerance test at the end of a 1 week treatment with S961 (10 nMol/week) shows glucose intolerance. n = 4 for each group.

(C) Area under curve (AUC) for the glucose tolerance test shown in (B).

(D) Continuous treatment of S961 by an osmotic pump at different doses induces hyperinsulinemia. n = 4 for each dosage group.

*$p < 0.05$ and **$p < 0.005$ compared to vehicle treatment. Data are represented as mean ± SEM.

effect. Microarray analysis pointed to one gene, which we call betatrophin (Figure 3A). Betatrophin is upregulated in S961-treated liver (~4-fold) and white fat (~3-fold), but its expression is unchanged in skeletal muscle and in pancreatic β cells (Figure 3B) in response to S961.

Betatrophin encodes a predicted protein of 198 amino acids (the mouse gene was previously annotated as Gm6484 and the protein as EG624219; the human gene is annotated as C19orf80 and the protein hepatocellular carcinoma-associated protein TD26 [Dong et al., 2004]). The gene has four exons and lies within the intron of another gene, Dock6, on the opposite strand (Figure S4A). Betatrophin is highly conserved in all mammalian species examined (Figure S4B) but evidently absent in nonmammalian vertebrates and in invertebrates (data not shown).

Betatrophin Is Enriched in Liver and in Fat Tissues, and Its Expression Correlates with High Pancreatic β Cell Proliferation Rates

Betatrophin mRNA is expressed in mouse liver and fat, with minimal expression in other tissues examined (Figure 3C), consistent with previous reports (Quagliarini et al., 2012; Ren et al., 2012; Zhang, 2012). In humans, betatrophin is primarily expressed in the liver (Figure 3D), where betatrophin mRNA levels are >250-fold higher than that found in other tissues tested. Betatrophin protein can also be detected by western blotting in human liver (Figure 4J).

To determine whether betatrophin might be involved in regulating β cell replication in other contexts, we examined betatrophin mRNA expression by quantitative PCR in several physiologically relevant animal models of increased β cell replication.

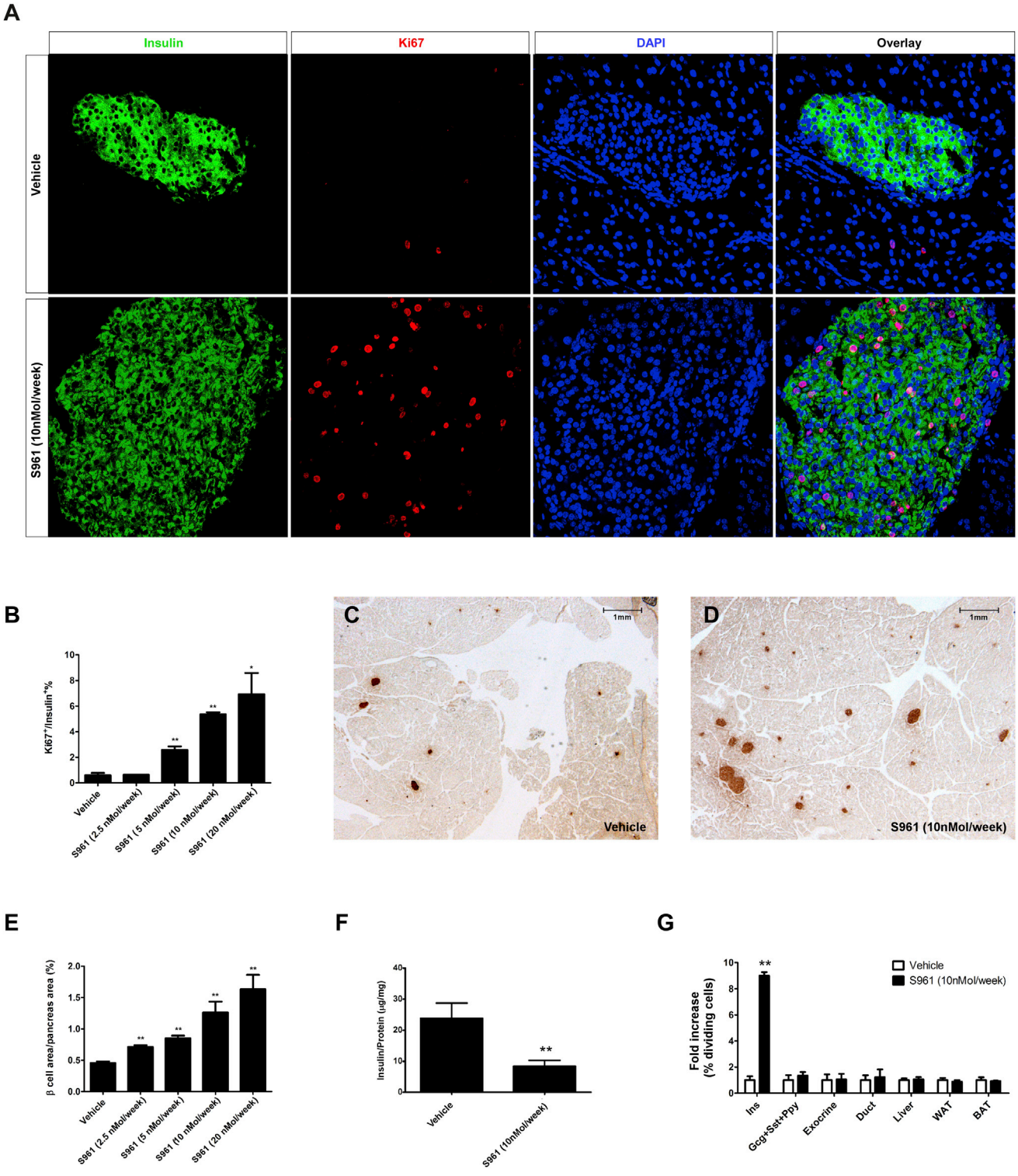

Figure 2. Administration of the Insulin Receptor Antagonist S961 Induces Pancreatic β Cell Proliferation and β Cell Mass Expansion

(A) S961 infused into adult mice at 10 nMol/week for 1 week induces pancreatic β cell proliferation (shown by costaining of Ki67 and insulin).

(B–E) (B) Proliferation rates of pancreatic β cells measured as percentage of dividing β cells 7 days after S961 treatment at different doses. S961 treatment significantly increases β cell area shown by insulin immunohistochemistry (brown) (representative sections shown in C and D; β cell area as a percentage of total pancreas area in E). n = 4 in each dosage group.

(F) Total pancreatic insulin content (normalized by total protein content) in vehicle-treated or S961-treated (10 nMol/week) mice. n = 3 in each group.

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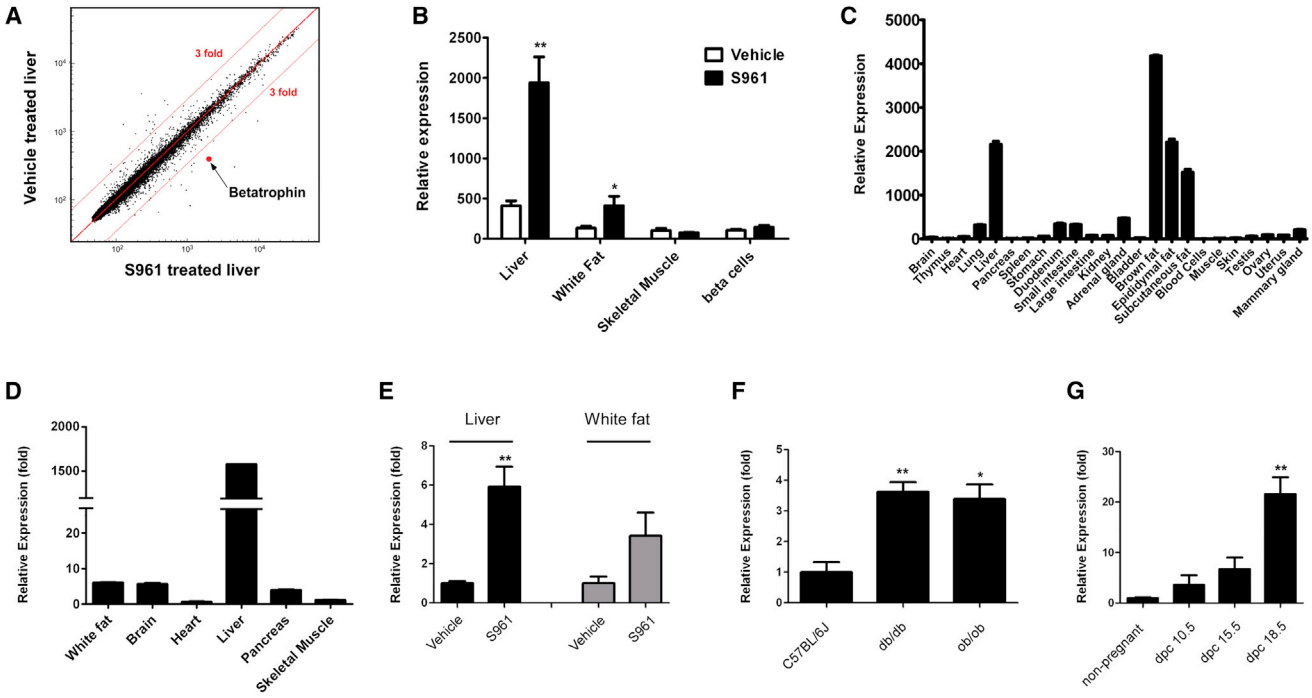

Figure 3. Identification and Expression of Betatrophin

(A) Microarray analysis of livers (n = 4 for each group) following 1 week S961 (10 nMol/week) or vehicle treatment. Candidate genes with at least a 3-fold difference compared to control were chosen. The red dot is betatrophin.

(B) Relative expression of betatrophin mRNA by microarray analysis in liver, white fat, skeletal muscle, and β cells in S961-treated (10 nMol/week) versus vehicle-treated mice (1 week treatment). n = 4 in each group except S961 treated β cells, in which n = 3 (normalized by average RNA expression level in each sample).

(C) Relative expression of betatrophin by real-time PCR analysis in mouse organs/tissues, normalized by total RNA input.

(D) Relative expression of betatrophin by real-time PCR analysis in various human tissues, normalized by total RNA input.

(E) Real-time PCR analysis of betatrophin in liver and white fat samples from S961-treated (10 nMol/week) or vehicle-treated mice (7 days treatment). n = 5 in each group.

(F and G) (F) Livers from C57BL/6J (n = 4), *ob/ob* (n = 4), and *db/db* (n = 4) male mice and (G) livers from C57BL/6J female mice at different gestational stages (n = 3 in each group). Dpc, date postconception.

*p < 0.05 and ** p < 0.005 compared to vehicle treatment or wild-type animals. Data are represented as mean ± SEM. See also Figure S4.

Infusion of the insulin receptor antagonist S961, which causes a dramatic pancreatic β cell proliferation, leads to a 6-fold upregulation of betatrophin in liver and 4-fold in white fat (Figure 3E), consistent with the microarray analysis (Figure 3B). In mouse models of type II diabetes, there is increased pancreatic β cell mass (Bock et al., 2003; Gapp et al., 1983; Tomita et al., 1992; Wang and Brubaker, 2002), and betatrophin mRNA is upregulated 3- to 4-fold in the liver of both *ob/ob* and *db/db* mice (Figure 3F). β cell replication rates also increase during pregnancy (Karnik et al., 2007), and expression of betatrophin mRNA in the liver increases by ~20-fold over the course of gestation (Figure 3G). Finally, specific depletion of β cells with diphtheria toxin leads to increased β cell replication (Nir et al., 2007). This treatment did not stimulate changes in betatrophin mRNA expression in the liver (data not shown). Together, these results indicate that betatrophin expression may contribute to compensatory pancreatic β cell proliferation as a response to physiological challenges, but not as a regeneration response after acute injury.

Betatrophin Encodes a Secreted Protein

How might a protein produced in the liver and fat cause pancreatic β cells to divide? Sequence analysis of mouse and human betatrophin shows a predicted secretion signal at the N terminus and two coiled-coil domains (Figure 4A). To demonstrate that betatrophin is indeed a secreted protein, expression plasmids encoding mouse and human betatrophin, fused with a Myc tag at their C termini (referred to as mbetatrophin-Myc and hbetatrophin-Myc), were prepared and used to transfect tissue culture cells and to express betatrophin in mouse liver by hydrodynamic tail vein injection (Song et al., 2002; Yant et al., 2000; Zhang et al., 1999). Ectopic gene expression in the cell line Hepa1-6 and in liver cells in vivo shows Myc-tagged betatrophin protein in

(G) Replication rates are measured as percentage of cells staining for Ki67 and are shown as fold increase over vehicle treatment. β cells (Ins), non-β-cell endocrine cells (Gcg+Sst+Ppy), exocrine cells, and pancreatic duct cells as well as liver, white fat (white adipose tissue [WAT]), and brown fat (brown adipose tissue [BAT]) after treatment of S961(10 nMol/week) or vehicle treatment. n = 5 for each dosage group.

*p < 0.05 and **p < 0.005 compared to vehicle treatment. Data are represented as mean ± SEM. See also Figures S1, S2, and S3.

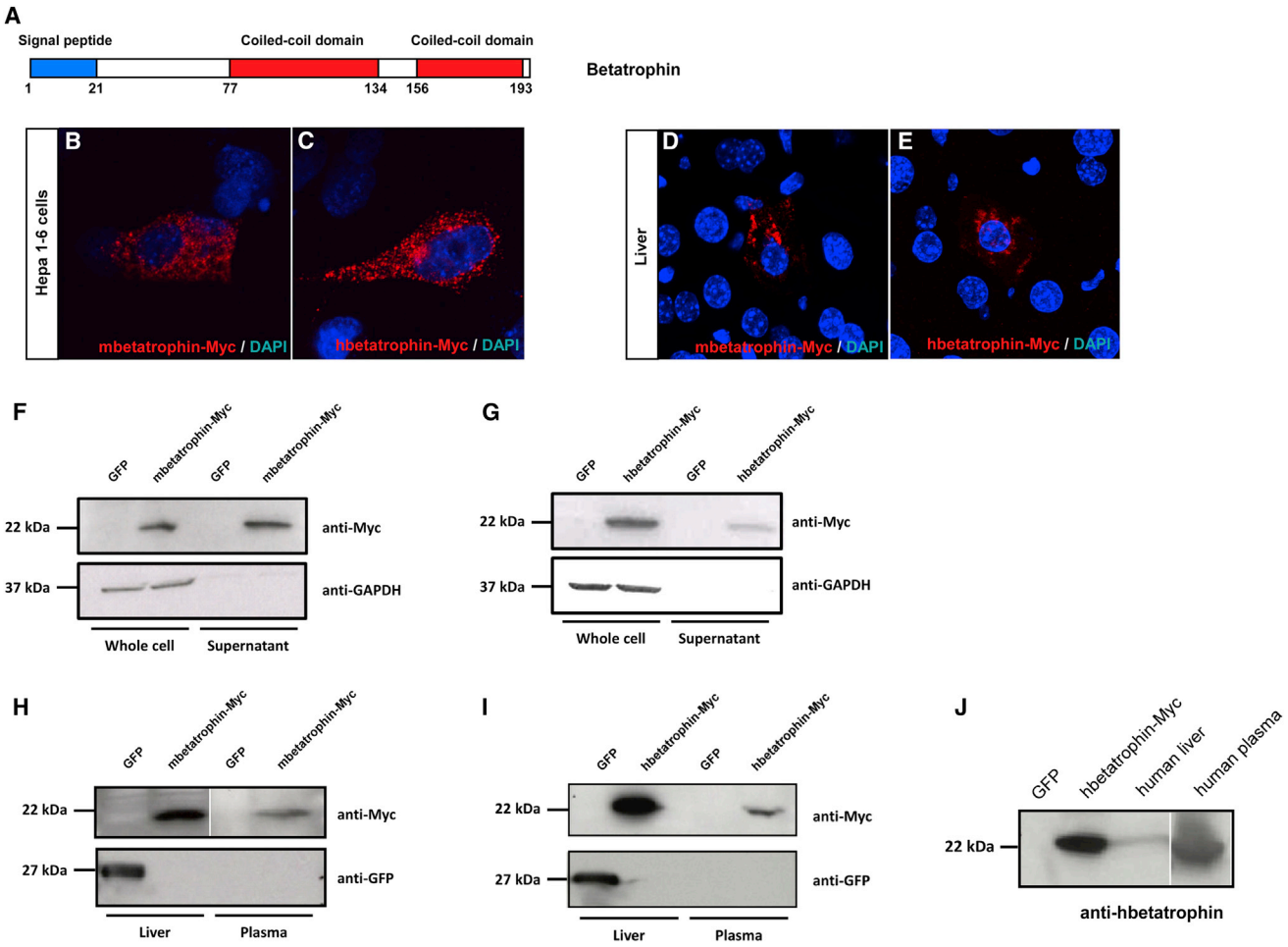

Figure 4. Betatrophin Encodes a Secreted Protein

(A) Predicted domains of the betatrophin protein.

(B and C) (B) Cellular localization of mbetatrophin-Myc or (C) hbetatrophin-Myc protein when transfected into the liver cell line Hepa1-6.

(D and E) (D) Cellular localization of mbetatrophin-Myc or (E) hbetatrophin-Myc when overexpressed in mouse liver through hydrodynamic tail vein injection.

(F and G) Western blots show (F) mbetatrophin-Myc protein or (G) hbetatrophin-Myc in the supernatant following gene transfection into 293T cells. GFP gene transfection and the intracellular GAPDH protein are used as controls.

(H and I) Western blots show (H) mbetatrophin-Myc (I) or hbetatrophin-Myc protein in plasma (3 days after injection) when the gene is overexpressed in mouse liver by hydrodynamic tail vein injection. GFP gene injection is the negative control.

(J) Western blot of human betatrophin in human liver and plasma samples. Cell lysate of hbetatrophin-Myc-transfected 293T cells is the positive control, and cell lysate of GFP-transfected 293T cells is the negative control.

vesicle-like structures, as expected for a secreted protein (mouse, Figures 4B and 4D; human, Figures 4C and 4E). Myc-tagged betatrophin protein is detected in the supernatant of transfected of 293T cells as well as plasma from mice injected with the expression plasmids (mouse, Figures 4F and 4H; human, Figures 4G and 4I). Betatrophin can be detected in human plasma, demonstrating that endogenous betatrophin is a secreted protein in vivo (Figure 4J).

Expression of Betatrophin in Liver Induces Dramatic and Specific Pancreatic β Cell Proliferation and Improves Glucose Tolerance in Mice

To determine whether betatrophin can promote pancreatic β cell proliferation, we used hydrodynamic injection to deliver betatrophin expression constructs to the liver, one of the normal sites of betatrophin expression. Following injection, 5%–10% of liver cells expressed betatrophin (or the control protein, GFP; Figure S5), and this expression persisted for at least 8 days (data not shown). Injection of plasmids encoding betatrophin produces a striking increase in β cell replication (Figure 5A). The β cell proliferation rate in betatrophin-injected animals averaged 4.6%, 17-fold higher than the control (GFP-injected) rate of 0.27% (Figure 5B), with some individual animals achieving replication rates as high as 8.8% (~33-fold increase). The increased proliferation in β cells in betatrophin-injected animals was confirmed by immunostaining for the β cell nuclear marker Nkx6.1 and another cell division marker (PCNA; Figures S2C and S2D). Similar to S961-treated mice,

quantitative PCR analysis also shows that the expression levels of cyclins (cyclins A1, A2, B1, B2, D1, D2, and F), CDKs (CDK1 and CDK2), and E2Fs (E2F1 ad E2F2) increase, whereas cell-cycle inhibitors (Cdkn1a and Cdkn2a) decrease in islets of betatrophin-injected mice compared to control-injected mice (Figure S3E). The increase in β cell proliferation was observed in all islets examined (Figure S3F). The increased rate of proliferation is so dramatic that one can easily identify islets and β cells at low magnification simply by the immunostaining for replication (Ki67; Figure 5C).

The high β cell proliferation rate in betatrophin-injected mice leads to a significant expansion of β cell numbers and total pancreatic β cell mass (Figure 5D). After 8 days, the total pancreatic β cell area in betatrophin-injected mice is 3-fold higher than in control-injected mice (Figure 5E). This increase is the result of having more β cells, which in turn increases islet size (Figure S3G). The total pancreatic insulin content also increases (~2-fold) in betatrophin-injected mice (Figure 5F).

The stimulation in replication caused by betatrophin expression is largely specific for β cells. As shown in Figure 5C and 5G, there is little if any effect on replication in other pancreatic cell types (exocrine, ductal, and non-β-cell endocrine cells) or in other organs (liver, white fat, and brown fat) (Figure 5G).

To evaluate β cell function, we isolated pancreatic islets from control- or betatrophin-injected mice and performed a glucose-stimulated-insulin-secretion (GSIS) analysis. As shown in Figure S6, the GSIS of pancreatic islets from betatrophin-injected mice is indistinguishable from control-GFP-injected mice, suggesting that the normal function of β cells was maintained after the β cell proliferation in betatrophin-injected animals. In addition, a glucose tolerance test was performed in control and betatrophin-injected mice. Mice were fasted for 6 hr before glucose injection, and the data show that betatrophin-injected mice have a lower fasting glucose level (Figure 6A) and improved glucose tolerance compared to control-injected mice (Figure 6A and as shown by area under curve [AUC] in Figure 6B). Betatrophin expression also results in a minor increase in fasting plasma insulin levels (Figure 6C), possibly due to the relative short fasting time or an increased glucose sensitivity.

Because insulin resistance is a potent stimulus known to induce β cell proliferation, it is formally possible that betatrophin may act by first inducing insulin resistance, which in turn leads to compensatory β cell proliferation by some other mechanism. This possibility seems unlikely, as the lower fasting glucose in mice overexpressing betatrophin is inconsistent with an insulin-resistant phenotype. Nonetheless, to rule out this possibility, we performed an insulin tolerance test and found no difference between betatrophin- and control-injected mice, in contrast to S961 administration (10nMol/week), which produces a strong insulin resistance (Figure 6D). These data show that betatrophin promotes β cell replication without insulin resistance.

DISCUSSION

The possibility that the liver produces a signal for β cell proliferation has been suggested before, perhaps most convincingly by Kahn's work on the LIRKO mouse, a liver-specific depletion of the insulin receptor that produces β cell hyperplasia (Michael et al., 2000). Here, using a different method, we show that an insulin receptor antagonist (S961) provides a chemical means of achieving this same phenotype. In a dose-dependent manner, provision of S961 induces a rapid and significant increase in β cell replication and islet growth.

The S961 insulin resistance model enabled us to identify betatrophin. There are three recent reports in which the Gm6484/TD26 gene was identified as a liver- and fat-enriched gene. Those authors pointed to a possible lipoprotein lipase inhibition activity or an effect on serum triglyceride regulation (Quagliarini et al., 2012; Ren et al., 2012; Zhang, 2012) but did not report any effects on pancreatic β cell biology, carbohydrate metabolism, or diabetes. Our findings on betatrophin suggest that this hormone can regulate metabolism by increasing insulin production via an increase in β cell mass.

The upregulation of betatrophin observed during pregnancy and in the *ob*/*ob* and *db*/*db* diabetic mouse models may explain how β cell proliferation and β cell mass are expanded in those circumstances. In other genetic manipulations that increase β cell replication, such as LIRKO and MEK1 mutations (Imai et al., 2008; Michael et al., 2000), it remains to be determined whether betatrophin is similarly upregulated.

The stimulation of β cell replication that we report with S961 and following injection of betatrophin DNA is noteworthy for the rapidity and magnitude of the effect. β cell replication rate is elevated 4-fold during gestation (Karnik et al., 2007), 2- to 4.5-fold with high glucose infusion (Alonso et al., 2007), 2.6-fold with exendin-4 treatment (Xu et al., 1999), 4-fold in a β cell ablation model (Nir et al., 2007), and 6-fold in LIRKO mice (Okada et al., 2007). S961 treatment can increase β cell replication by 12-fold, and providing betatrophin by DNA injection increased replication by an average of 17-fold within a few days, making this an exceptionally potent activity. Together, these results point to the importance of making recombinant betatrophin protein and testing it directly by injection for effects on β cell mass.

We do not yet know the mechanism of action for betatrophin. It may act directly or indirectly on β cells to control their proliferation. Identification of the betatrophin receptor and/or other possible cofactors will help to explain how the liver and fat interact with the pancreas to regulate β cell mass. Nonetheless, identification of betatrophin as a hormone that can exert control on β cell replication and β cell mass opens a new door to possible diabetes therapy.

EXPERIMENTAL PROCEDURES

Reagents and Mice

C57BL/6J, *ob*/*ob*, and *db*/*db* male mice at 8 weeks of age were purchased from Jackson Laboratory. C57BL/6J female mice at 8 weeks of age were obtained from Jackson Laboratory, fertilized, and timed to certain gestational dates in house. For hydrodynamic tail vein injection, ICR male mice at 7 weeks of age were purchased from Taconic. The Hepa1-6 cell line was obtained from ATCC. To make expression plasmids for the GFP control or betatrophin, cDNAs were amplified using PCR from a GFP expression plasmid or mouse/human liver total RNA and subcloned into pT3-EF1α-DEST vector (a sleeping beauty transposon vector) or pCAG-DEST vector. mbetatrophin is mouse, and

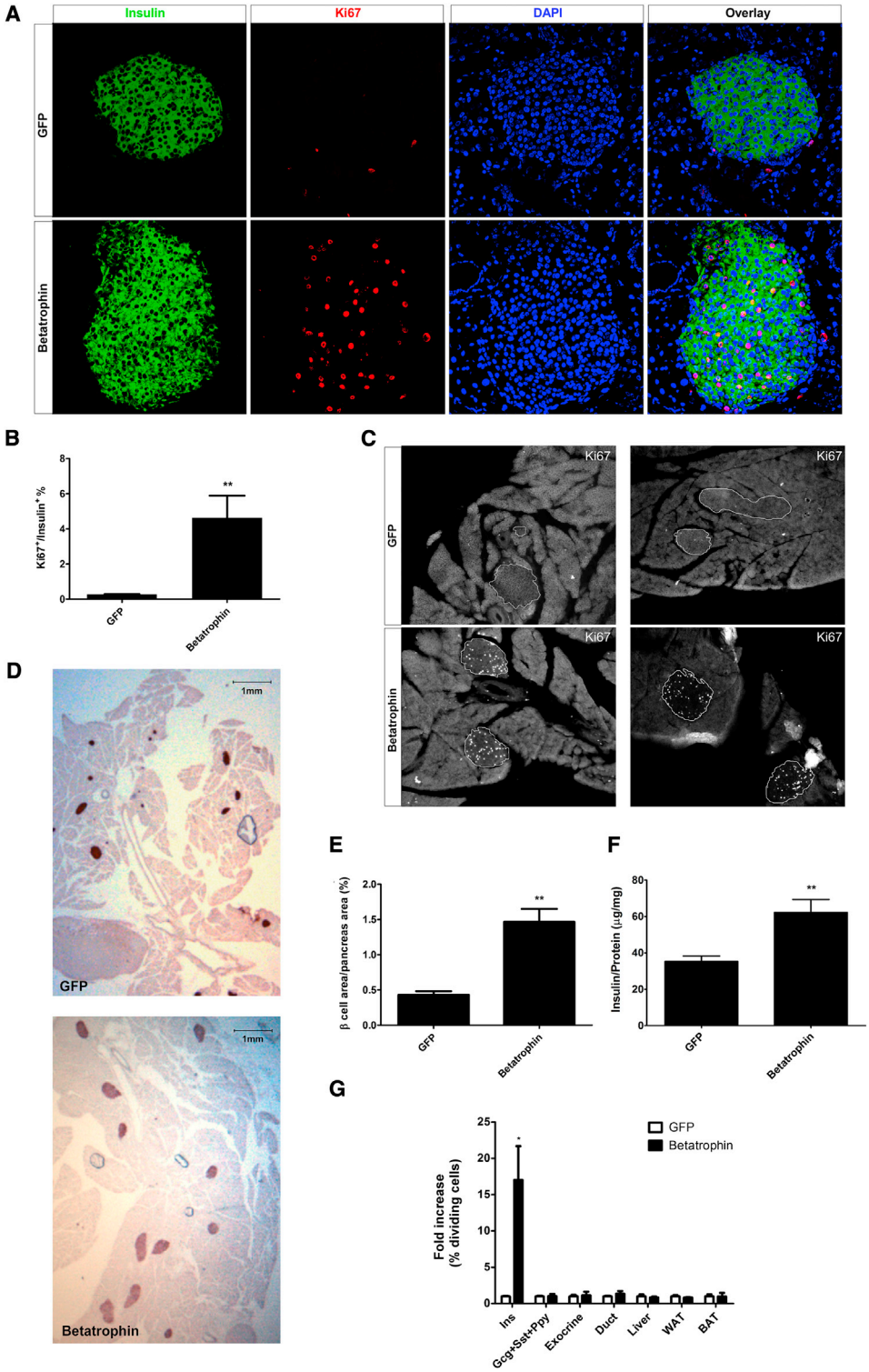

Figure 5. Overexpression of Betatrophin in the Liver Leads to a Specific Pancreatic β Cell Proliferation

(A) Expression of betatrophin in mouse liver by hydrodynamic tail vein injection of mbetatrophin-myc DNA strongly stimulates β cell replication compared to the similarly injected control (DNA-encoding GFP).

(B) Quantification of the Ki67$^+$/insulin$^+$ ratio shows that betatrophin-injected mice (n = 7) have a 17-fold higher rate of β cell replication compared to controls (GFP, n = 5).

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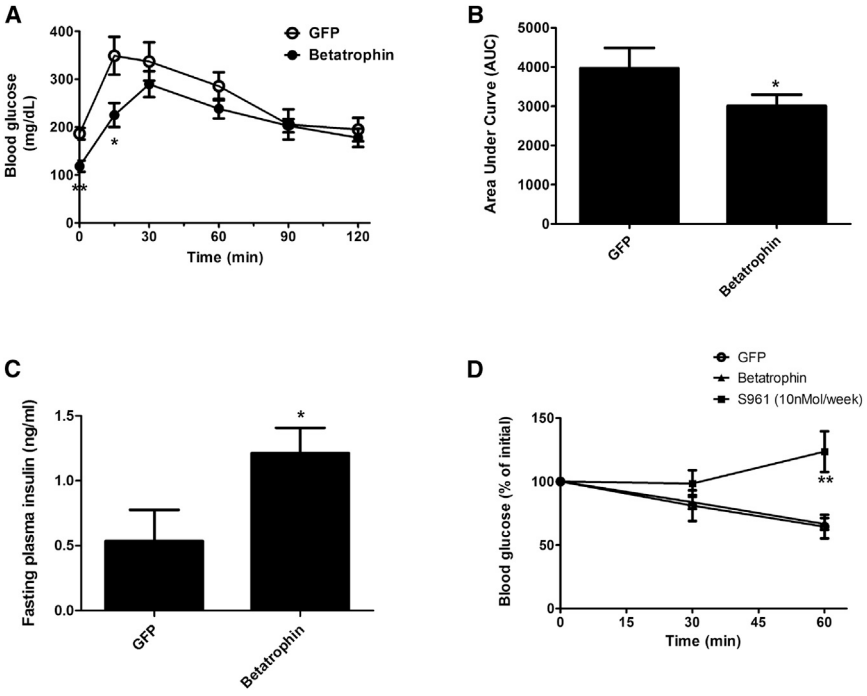

Figure 6. Overexpression of Betatrophin in the Liver Leads to Improved β Cell Function

(A) Glucose tolerance test in GFP-expressing (n = 5) or betatrophin-expressing (n = 7) mice shows a lower fasting blood glucose and an improved glucose tolerance induced by betatrophin expression.

(B) Area under curve (AUC) for the glucose tolerance test shown in (A).

(C) Fasting plasma insulin measurement in GFP (n = 5) or betatrophin (n = 7).

(D) Insulin tolerance tests on GFP-expressing (n = 5) or betatrophin-expressing (n = 4) mice show no sign of insulin resistance, in contrast to S961-treated (10 nMol/week) animals (n = 5), which show severe insulin resistance.

*p < 0.05 and **p < 0.005 compared to control-injected animals. Data are represented as mean ± SEM. See also Figure S6.

hbetatrophin is the human gene. pT3-EF1α-DEST and the transposase-encoding plasmid pCMV-SB100 were gifts from Dr. Aaron Tward. Human RNA, protein, and plasma samples were purchased from BioChain.

S961 Treatment

S961 was received as a generous gift from Dr. Lauge Schäffer (Novo Nordisk) (Schäffer et al., 2008). Vehicle (PBS) or 2.5 nMol—10 nmol S961 was loaded into Alzet osmotic pump 2001 and implanted subcutaneously on the back of mice.

Total Insulin Content Measurement

To measure total insulin content, whole pancreata were dissected and processed according to a standard acid-ethanol extraction protocol. The insulin concentration of the extracts was determined using Mouse Ultrasensitive Insulin ELISA Jumbo (ALPCO). The total protein concentration was measured using a protein assay kit from Bio-Rad.

In Vitro GSIS Analysis

Pancreatic islets were isolated after infusion and digestion of the pancreata by collagenase P (Roche). The islets were cultured overnight to recover in RPMI1640+10% FBS media. About 20 size-matched islets from each mouse were used for the in vitro GSIS assay. After fasting the islets in low-glucose buffer (2.5 mM) for 2 hr, islets were washed and sequentially incubated with 400 μl of low-glucose buffer (2.5 mM), high-glucose buffer (15 mM), and then low-glucose buffer (2.5 mM) with 30 mM KCl. These buffers were collected after incubation and analyzed using the Mouse Ultrasensitive Insulin ELISA Jumbo (ALPCO). Total genomic DNA was extracted from all islets after the GSIS using DNeasy blood and tissue kit (QIAGEN), and the concentration of the genomic DNA was measured using a nanodrop. The insulin content measurement for each sample was normalized to the total genome DNA content.

Immunohistochemistry

Mouse pancreata, liver, and brown fat were fixed in 4% paraformaldehyde for 2 hr at 4°C. The white fat was fixed overnight. Cryosections (10–20 μm) were pretreated with citrate buffer for antigen retrieval according to standard protocols and were immunostained with Guinea pig anti-insulin antibody (Dako), mouse anti-Nkx6.1 antibody (DHSB), rabbit anti-Amylase antibody (Sigma), rabbit anti-CK19 antibody (Abcam), rabbit anti-pancreatic polypeptide antibody (Abcam), rabbit anti-glucagon antibody (Cell Signaling), goat anti-somatostatin antibody (Santa Cruz), and/or rat anti-Ki67 antibody (Dako), rabbit anti-Ki67 antibody (Abcam), and mouse anti-PCNA (Abcam) and were subsequently developed by Alexa Fluor 488 goat anti-guinea pig IgG (H^+L), Alexa Fluor 488 donkey anti-rabbit IgG (H^+L), Alexa Fluor 488 donkey anti-goat IgG (H^+L), and/or Alexa Fluor 594 donkey anti-rat IgG (H^+L), Alexa Fluor 594 donkey anti-rabbit IgG (H^+L), and Alexa Fluor 594 donkey anti-mouse IgG (H^+L) (Invitrogen). For DAB staining of the pancreatic β cells, guinea pig anti-insulin antibody (Dako) and HRP-conjugated donkey anti-guinea pig antibody (Jackson ImmunoResearch) were used and developed by ImmPACT DAB kit (Vector Laboratories). For immunocytochemistry in Hepa1-6 cells and immunofluorescence of m/hbetatrophin-Myc in liver after hydrodynamic tail vein injection, cells or tissue cryosections were stained by rabbit anti-Myc tag antibody (Abcam) and developed by Alexa Fluor 594 donkey anti-rabbit IgG (H^+L). All cells and sections were mounted in

(C) Two representative low-magnification images of pancreatic sections from mice injected with plasmids encoding GFP or betatrophin. Immunofluorescence of replicating cells (Ki67) seen as white dots; the outline of the β cell area is based on insulin staining (not shown). Note the absence of significant replication in the exocrine tissue.

(D) Expansion of β cell area in mice expressing betatrophin compared to GFP controls, as shown by insulin immunohistochemistry (shown in brown).

(E) Quantification of β cell area/total pancreas area in mice injected with betatrophin (n = 7) or control plasmids (GFP, n = 5).

(F) Total pancreatic insulin content (normalized by total protein content) in GFP- or betatrophin-injected mice. n = 3 for the GFP group; n = 4 for the betatrophin group.

(G) The replication rates (fold over GFP control) in pancreatic β cells (Ins^+), non-β-cell endocrine cells (Gcg+Sst+Ppy), exocrine cells, and duct cells, as well as liver, white fat, and brown fat after betatrophin injection (n = 5) or GFP injection (n = 5).

*p < 0.05 and **p < 0.005 compared to control-injected animals. Data are represented as mean ± SEM. See also Figures S2, S3, and S5.

Vectorshield with DAPI (Vector Laboratories). Images were obtained using Zeiss LSM510 confocal microscope, Olympus IX51 microscope, or Leica MZ16FA microscope.

Quantifying Pancreatic β Cell Proliferation, Islet Size and Area, Non-β Cell Proliferation, and Average β Cell Size

Whole-mouse pancreata were fixed and cryosectioned throughout. A series of sections 300 μm apart were chosen for each set of experiments. For β cell proliferation assays, sections were immunostained with anti-insulin and anti-Ki67 antibody. For non-β cell pancreatic cell proliferation, sections were immunostained with anti-amylase, anti-CK19, anti-somatostatin, anti-glucagon, and anti-pancreatic polypeptide together with anti-Ki67 antibody. Liver, white fat, and brown fat sections were immunostained with anti-Ki67 antibody. All islets were imaged, and total β cell number was counted by counting nuclei surrounded by cytoplasmic insulin immunostaining, and the proliferating β cells were assessed by counting nuclei stained with Ki67 within cytoplasmic insulin immunostaining. For each mouse, about 10,000 β cells were counted, and the β cell proliferation ratio was calculated by dividing $Ki67^+$ cell number by total β cell number. The non-β-cell pancreatic cells and liver/fat cell proliferation rates were counted in a similar manner. For islet size and β cell area quantification, the whole area for all sections was imaged. The total pancreas area and insulin-positive area was selected using Adobe Photoshop for each image. The total insulin-positive (β cell) area and the average islet size (calculated from individual islet areas) were calculated from these data for each mouse. The average β cell size was calculated by dividing the total β cell area ($insulin^+$ area) by the total β cell number from all imaged islets.

Microarray and Real-Time PCR Analysis

β cells were purified from pancreata of Pdx1-GFP-transgenic mice after infusion and digestion by collagenase P (Roche) by FACS. Total RNA from various organs/tissues/cells was extracted using TRIzol (Invitrogen), and genomic DNA was removed using QIAGEN RNeasy kit. For microarray analysis, total RNA was amplified and biotin labeled using Illumina TotalPrep RNA Amplification kit (Ambion). The cRNA was analyzed by in-house Illumina BeadArray Reader and quantified using Illumina BeadStudio. For real-time PCR analysis, cDNA was synthesized using Superscript III First Strand cDNA synthesis kit (Invitrogen) and was analyzed using an ABI 7900 system. The Taqman gene expression assays used were mbetatrophin (Mm01175863_g1, Applied Biosystem), mActb (Mm00607939_s1, Applied Biosystem), and hbetatrophin (Hs00218820_m1, Applied Biosystem).

Cell-Cycle Genes and Transcript Profiling

Pancreatic islets were isolated after infusion and digestion of the pancreata by collagenase P (Roche). The total RNA was immediately isolated from islets using an RNeasy mini kit (QIAGEN). The cDNA was synthesized using RT^2 First Strand Kit (QIAGEN), and quantitative PCR reactions were performed using RT^2 Profiler PCR Array Mouse Cell Cycle (QIAGEN).

DNA Transfection and Western Blotting

293T cell were transfected with GFP or betatrophin expression plasmids using Lipofectamine 2000 Transfection Reagent (Invitrogen). Supernatants were collected, filtered through 0.22 μm membrane, and concentrated 10 times using Amicon Ultra Centrifugation Filter with 10 kDa cutoff (Millipore). The cell lysate was prepared using RIPA buffer (Santa Cruz) with protease inhibitors. The liver lysate was prepared by homogenization in RIPA buffer with protease inhibitors. For western blotting, the primary antibodies used include rabbit anti-Myc-HRP antibody (Abcam), chicken anti-GFP antibody (Aves Lab, Inc.), rabbit anti-GAPDH antibody (Abcam), and mouse anti-TD26 antibody (Novus). The secondary antibodies used include HRP-conjugated goat anti-rabbit IgG (H^+L) (Jackson ImmunoResearch), HRP-conjugated donkey anti-mouse IgG (H^+L) (Jackson ImmunoResearch), and HRP-conjugated donkey anti-chicken IgY (H^+L) (Jackson ImmunoResearch). The signal was developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Hydrodynamic Tail Vein Injection

Seven-week-old male ICR mice (Taconic) were used for the hydrodynamic tail vein injection for the ease of identifying the tail vein. The injections were performed according to published methods (Liu et al., 1999; Song et al., 2002; Yant et al., 2000; Zhang et al., 1999). 100 μg of GFP or betatrophin expression plasmid DNA, controlled by either the CAG promoter or the EF1α promoter in the sleeping beauty transposon backbone together with 4 μg of sleeping beauty transposase expression plasmid (pCMV-SB100), were diluted in sterile saline. The mice were anesthetized using avertin (250 mg/Kg) and were injected with 8% of body weight volumes (ml/g) of diluted plasmid DNA within 5 to 7 s through the lateral tail veins. Glucose tolerance tests or insulin tolerance test were performed 6 days after injection, and the mice were sacrificed 8 days after injection for pancreatic β cell analysis.

Glucose Tolerance Test and Insulin Tolerance Test

Mice were fasted for 6 hr and then injected with 1 mg/g body weight of D-glucose intraperitoneally. The blood glucose was measured from the tail tip using One Touch Ultra 2 blood glucose meter (LifeScan, Inc.) at 0, 15, 30, 60, and 90 min postinjection. The area under curve is calculated using standard methods. For insulin tolerance tests, mice were not fasted and were injected with 0.75 U/Kg of human insulin (Sigma) intraperitoneally. The blood glucose was measured from the tail tip using One Touch Ultra 2 blood glucose meter (LifeScan, Inc.) at 0, 30, and 60 min postinjection.

Insulin ELISA

Blood was collected after mice were sacrificed, and the plasma was separated according to standard protocols. Insulin ELISA assay was carried out with Mouse Ultrasensitive Insulin ELISA Jumbo (ALPCO).

Statistics

All of the p values were calculated by a standard Student's t test with two-tails distribution.

ACCESSION NUMBERS

Microarray data were deposited in the Gene Expression Omnibus Database of the National Centre for Biotechnology Information under the accession number GSE45694.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.04.008.

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