

# Transgenic Mice Overexpressing Nuclear SREBP-1c in Pancreatic $\beta$ -Cells

Akimitsu Takahashi,<sup>1</sup> Kaori Motomura,<sup>1</sup> Toyonori Kato,<sup>1</sup> Tomohiro Yoshikawa,<sup>1</sup> Yoshimi Nakagawa,<sup>1,2</sup> Naoya Yahagi,<sup>2</sup> Hirohito Sone,<sup>1</sup> Hiroaki Suzuki,<sup>1</sup> Hideo Toyoshima,<sup>1</sup> Nobuhiro Yamada,<sup>1</sup> and Hitoshi Shimano<sup>1,2</sup>

**Influx of excess fatty acids and the resultant accumulation of intracellular triglycerides are linked to impaired insulin secretion and action in the pathogenesis of type 2 diabetes. Sterol regulatory element-binding protein (SREBP)-1c is a transcription factor that controls cellular synthesis of fatty acids and triglycerides. SREBP-1c is highly expressed in high-energy and insulin-resistant states. To investigate effects of this synthetic lipid regulator on insulin secretion, we generated transgenic mice overexpressing nuclear SREBP-1c under the insulin promoter.  $\beta$ -Cell-specific expression of SREBP-1c caused reduction in islet mass and impaired glucose-stimulated insulin secretion and was associated with accumulation of triglycerides, suppression of pancreas duodenal homeobox-1, and upregulation of uncoupling protein 2 gene expression. The mice presented with impaired glucose tolerance that was exacerbated by a high-energy diet. Taken together with enhanced insulin secretion from SREBP-1-null islets, these data suggest that SREBP-1c and endogenous lipogenesis could be involved in  $\beta$ -cell dysfunction and diabetes. *Diabetes* 54:492–499, 2005**

**T**he main pathophysiological features of type 2 diabetes are defects in insulin secretion and action. The involvement of lipid accumulation in nonadipose tissue has been implicated in both pathologies, which is often referred to as “lipotoxicity,” although the molecular mechanism remains to be fully understood (1,2). Together with insulin resistance in insulin-sensitive peripheral tissues, the lipotoxicity in  $\beta$ -cells

leading to impaired insulin secretion is also thought to precipitate the development of type 2 diabetes.  $\beta$ -Cell lipotoxicity has been extensively explored in association with high-plasma free fatty acids (FFAs) often observed in obesity. In vitro, long-term exposure of  $\beta$ -cells to FFAs results in elevated basal insulin secretion with a concomitant suppression of glucose-stimulated insulin secretion (GSIS), elevated production of reactive oxygen species, inhibition of insulin biosynthesis, and potential induction of  $\beta$ -cell death both in vivo and in vitro (1,3,4). Similar changes associated with the impairment of  $\beta$ -cell function were observed in the Zucker diabetic fatty rat (5). Long-term exposure to fatty acids could also affect the coupling of glucose metabolism to insulin secretion. Uncoupling protein (UCP)-2 has been implicated as a causative factor in this process by dissociating glucose consumption and mitochondrial ATP production, resulting in a reduction of insulin release (6,7).

Sterol regulatory element-binding protein (SREBP)-1c is a member of the SREBP family, which has been established to possess transcription factors regulating transcription of genes involved in cholesterol and fatty acid synthesis (rev. in 8). Three isoforms of SREBP, SREBP-1a, -1c, and -2, are known. Whereas SREBP-2 plays a crucial role in the regulation of cholesterol synthesis, SREBP-1c controls gene expression of lipogenic enzymes (rev. in 9–11). In vivo studies demonstrated that SREBP-1 plays a crucial role in the dietary regulation of most hepatic lipogenic genes (12,13) and that SREBP-1c expression itself is nutritionally regulated (12,14–19).

In addition to plasma fatty acid levels, factors modifying cellular fatty acid metabolism, such as hormone-sensitive lipase, have been shown to affect insulin secretion in  $\beta$ -cells in vivo (20). However,  $\beta$ -cell lipotoxicity has never been tested in terms of endogenous fatty acid synthesis. In the current study, effects of overproduction of the active form of SREBP-1c were estimated in transgenic mice to investigate potential roles of endogenous lipogenesis in pancreatic  $\beta$ -cell function.

## RESEARCH DESIGN AND METHODS

The techniques used for generating transgenic mice were previously described (21). An expression plasmid-based vector containing a cDNA encoding nuclear human SREBP-1c (amino acids 1–436) (22), which is the constitutively active form under control of the rat insulin I promoter (RIP; bp –715 to 31), followed by a 3' polyadenylation signal from the human growth hormone (hGH) cDNA, was constructed and designated pRIP-SREBP1c436. The *NotI*-*XhoI* fragment (Fig. 1A) of pRIP-SREBP1c436 was microinjected into C57BL/6J  $\times$  SJL hybrid eggs. Founder mice were subjected to pancreatectomy and truncated human SREBP-1, as determined by RT-PCR, and two lines of

From the <sup>1</sup>Department of Internal Medicine (Metabolism and Endocrinology), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba, Ibaraki, Japan; and the <sup>2</sup>Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tennodai, Tsukuba, Ibaraki, Japan.

Address correspondence and reprint requests to Hitoshi Shimano, MD, PhD, Department of Internal Medicine, Institute of Clinical Medicine and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tennodai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: shimano-tky@umin.ac.jp.

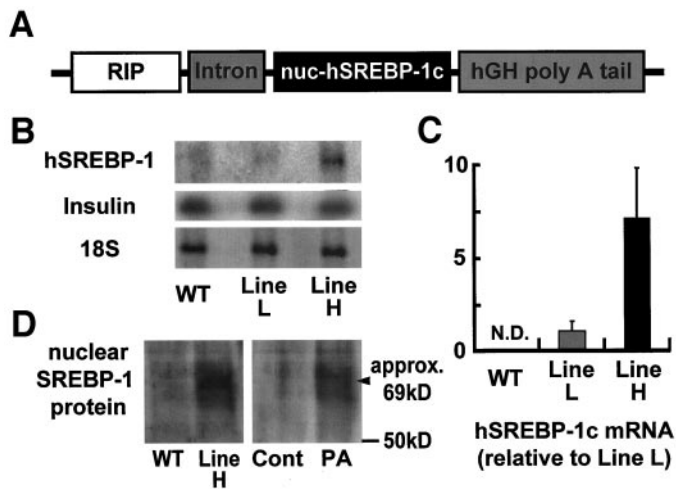
Received for publication 3 June 2004 and accepted in revised form 11 November 2004.

A.T., K.M., and T.K. contributed equally to this work.

CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; HFHS, high fat/high sucrose; IGT, impaired glucose tolerance; IRS, insulin receptor substrate; KRBB, Krebs-Ringer bicarbonate buffer with 10 mmol/l HEPES; Nkx6.1, NK6 transcription factor related, locus 1; Pdx, pancreas duodenal homeobox; RIP, rat insulin I promoter; SCD, stearyl CoA desaturase; SREBP, sterol regulatory element-binding protein; UCP, uncoupling protein.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.



**FIG. 1.** Specific overexpression of human SREBP-1c in pancreatic  $\beta$ -cells. **A:** A DNA construct for microinjection to generate transgenic mice for an active (nuclear) form of human SREBP-1c (nuc-hSREBP-1c, cDNA encoding amino acids 1–436) in pancreatic  $\beta$ -cells. The hybrid-adenovirus-immunoglobulin intron (Intron) and 3' poly A signal from the human growth hormone (hGH poly-A tail) were used. **B:** Expression of transgene (hSREBP-1) and insulin in islets from a high-expression line (line H) and a low-expression line (line L) of transgenic mice and wild-type (WT) controls, as estimated by Northern blot analysis. Loading controls were by 18S ribosomal RNA. **C:** hSREBP-1c mRNA levels in islets from the two transgenic lines and controls by real-time quantitative PCR. mRNA levels of cyclophilin were measured as an internal control. **D:** Nuclear SREBP-1c protein was detected in whole-cell extracts of islets by immunoblotting using SREBP-1 antibody. **Left panel:** Islets from wild type (WT) and high-expression line (line H). **Right panel:** Islets from C57Bl6 mice incubated in the presence (PA) or the absence (Cont) of palmitate (200  $\mu$ mol/l) for 48 h. All values are means  $\pm$  SE.

SREBP-1c transgenic mice under the control of rat insulin promoter (TgRIP-SREBP1c) were established. SREBP-1 knockout mice used for islet investigations were as described (23).

The mice were fed a normal rodent diet (MF; Oriental yeast, Tokyo, Japan) or a synthetic high-fat/high-sucrose (HFHS) diet containing 30% (wt/wt) fat and 20% (wt/wt) sucrose (24) and were housed in colony cages and maintained on a 12-h light/12-h dark cycle. The Animal Care Committee of the University of Tsukuba approved animal care and procedure.

**Intravenous glucose tolerance tests.** Glucose (1 g/kg body wt) was administered intravenously through tail vein after an overnight (12-h) fast. Blood samples were obtained from the retro-orbital plexus before (0 min) and 5, 15, and 30 min after glucose injection for plasma preparation by centrifugation.

**Isolation of mouse pancreatic islets.** After sacrifice by cervical dislocation, pancreatic islets were isolated by collagenase digestion of the total pancreas as previously described (25). A freshly separated batch of islets was used for content of triglyceride determination, and the remaining islets were put in culture for 2 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and in regular RPMI-1640 medium supplemented with 10% FCS.

**Preparation of islet RNA and estimation of gene expression.** Total RNA extraction using the TRIzol reagent (Invitrogen, Carlsbad, CA) and DNase-I treatment using the RNeasy Micro kit (Qiagen, Hilden, Germany) were performed according to the manufacturer's instructions. Northern blot analyses were performed with the indicated cDNA probes using Rapid-hyb buffer (Amersham Biosciences).

cDNA was synthesized with ThermoScript (Invitrogen) and real-time fluorescent detection PCR or TaqMan (Applied Biosystems, Foster City, CA) quantitative PCR (50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) was performed with the ABI Prism 7000 PCR instrument (Applied Biosystems) to amplify samples for the human SREBP-1c (transgene), mouse SREBP-1c, fatty acid synthase (FAS), stearyl CoA desaturase (SCD)-1, carnitine palmitoyltransferase (CPT)-I, UCP-2, pancreas duodenal homeobox (Pdx)-1, Nk6 transcription factor related, locus 1 (Nkx6.1), and cyclophilin genes. Primers and probes were as follows: 5'-GGTGTAGTGGCGGAACCATC-3' and 5'-GAGCCGGTTGATAGGCAGC-3' with fluorescent detection for human SREBP-1c, 5'-CGGCGCGGAAGCTGT-3' and 5'-TGCAATCCATGGCTCCGT-3' with 5'-CGTCTGCACGCGCTA-3' TAQMAN probe for mouse SREBP-1c, 5'-ATCCTGGAACGAGACACCATCTGGT-3' and 5'-AAGTCCAGGAGTGACAGTCTCT-3' with 5'-GGAGCGTATAT

GTGAACAGCGCA-3' probe for FAS, 5'-AGATCTCCAGTTCTTACACGACCA C-3' and 5'-CTTTCATTTCAGGACGGATGTCT-3' with 5'-TCTTCACCTTCTCGTTCATTTCGGGA-3' probe for SCD-1, 5'-CCTGGGCATGATTGCAAAG-3' and 5'-GGAGCCACTCAGCATGTT-3' with 5'-ACCCTAGACACCACTGGCCGCATGT-3' probe for CPT-I, 5'-GACCTCATCAAAGATACTCTCCTGAA-3' and 5'-ATCTCGTCTTGACCACATCAACAG-3' with 5'-TGACAGATGACCTCCCTGCCACTTCA-3' probe for UCP-2, 5'-AGTCCCACATCGGGCTTGAAG-3' and 5'-GGTCTGCACGGATGACCTTAG-3' with 5'-CCTTCAAGTCAGCCAGCCCC TG-3' probe for insulin receptor substrate (IRS)-2, 5'-TCCCTGTAAGGCAG AAGACAT-3' and 5'-ATTGCCACCACATCCATCTCA-3' with 5'-CTCTTGATAG CATCTCGGAGAAGTCCCA-3' probe for GK, 5'-GAAGAGCCCAACCGCGT-3' and 5'-TTGTTTTCTCGGGTTCGG-3' with 5'-CTCCTGCCACTGGCCTTCC A-3' probe (antisense) for Pdx-1, 5'-TTCGGAAATGAGGAGGATGA-3' and 5'-AAAAGCACAATCGAGCGGT-3' with 5'-ACAACCTCTGGAGCGAACTC TGACG-3' probe for Nkx6.1, and 5'-TGGCTCACAGTCTTTCATAACCA-3' and 5'-ATGACATCTCAGTGGCTGTGTC-3' with 5'-TCCATGCCCTCTAGAAGTTT GCCGAA-3' probe for cyclophilin.

**Western blot analysis of islets.** Isolated islets were dissolved in Triton-X lysis buffer (25 mmol/l HEPES, 50 mmol/l KCl, 6% glycerol, 5 mmol/l EDTA, 5 mmol/l EGTA, 0.5% Triton-X100, 50  $\mu$ mol/l NaF, 40 mmol/l glycerophosphate, and 25 mmol/l sodiumpyrophosphate with proteinase inhibitors) with needles passing. Total protein was measured (BCA protein assay; Pierce, Rockford, IL), and 15  $\mu$ g protein were fractionated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA). The primary antibody used was rabbit anti-SREBP-1 (human, mouse) antibody (H-160; Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G prepared in goat (Amersham Biosciences). Detection was performed using an ECL advance Western blotting detection kit and Hyperfilm ECL (Amersham Biosciences).

**Insulin secretion experiments of islets.** A group of 10 islets were preincubated in Krebs-Ringer bicarbonate buffer with 10 mmol/l HEPES (KRBH; pH 7.4) containing 0.5% BSA at 2.8 mmol/l glucose and submitted to three consecutive 30-min incubations in 1 ml KRBH with 0.5% BSA at 2.8 mmol/l glucose (low glucose), 20 mmol/l glucose (high glucose), and 2.8 mmol/l glucose with 20 mmol/l KCl. At the end of the incubation period, media were withdrawn for insulin determination followed by total islet insulin extraction by 0.5 ml cold acid ethanol mixture (75% ethanol with 0.2 mol/l HCl). Hoechst-33258 staining to sonicated islets was performed to determine the islet DNA content.

**Determination of ATP and triglyceride contents of islets.** A batch of 10 islets was resuspended in 500  $\mu$ l of 5% trichloroacetic acid and neutralized by NaOH. ATP was measured by a CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI). Triglycerides were measured by extracting lipids with the Folch's method followed by triglyceride determination with the GPO-trinder kit (Sigma, St. Louis, MO).

**Glucose oxidation and glucose incorporation into lipids in islets.** Glucose oxidation was determined as production of <sup>14</sup>CO<sub>2</sub> after incubation of the islets with [U-<sup>14</sup>C]glucose (20). After terminating the glucose oxidizing reaction, inactivated islets were washed with PBS and then the lipids were extracted from islets by Folch's method and the radioactivity was counted.

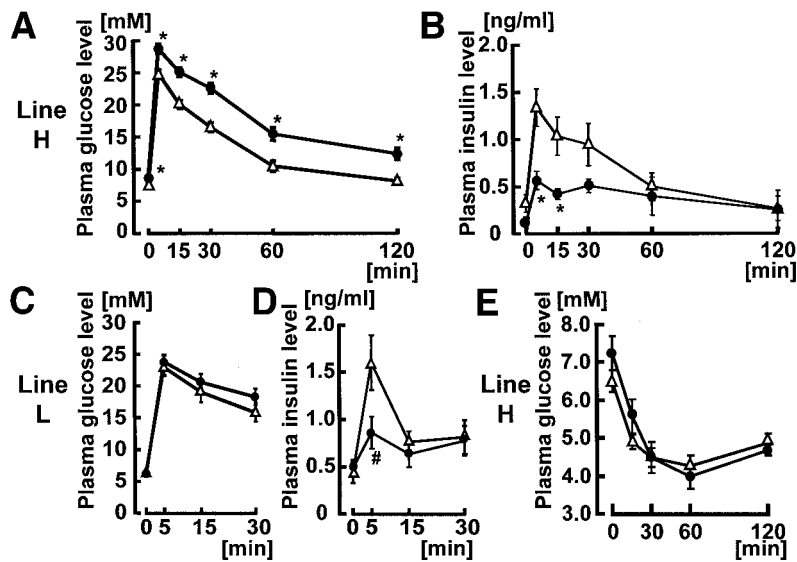
**The size of pancreatic islets from transgenic mice and controls.** The freshly isolated pancreatic islets were visualized under a light microscope at 40 $\times$  magnification using a charge-coupled device camera (CoolSNAP; Roper Scientific, Trenton, NJ). The areas of islets ( $n = 100$  from each group) were measured by numbers of pixels using the Adobe Photoshop software package (Adobe Systems, San José, CA). The islet volume was estimated as a mean of (pixel numbers of each islet)<sup>3/2</sup>.

In the animals that were fed a HFHS diet for 3 months, the islet size was determined by microscopic examination of multiple histological sections of whole pancreas after HE staining. The areas of islets ( $n = 100$ ) and nonislet region in multiple slides were measured by numbers of pixels, as described above.

**Immunohistochemistry.** Immunohistochemistry with anti-insulin antibody was performed as previously described (26).

## RESULTS

**Transgene expression in islets of TgRIP-SREBP1c mice.** Two transgene-expressing lines have been established following microinjection of the construct, as depicted in Fig. 1A, to create pancreatic  $\beta$ -cell-specific transgenic mice overexpressing nuclear human SREBP-1c under the control of RIP. These were designated as TgRIP-SREBP1c. The higher expresser (line H) exhibited



**FIG. 2.** Glucose (A–D) and insulin (E) tolerance tests on the TgRIP-SREBP1c mice and controls. Plasma glucose (A and C) and plasma insulin (B and D) levels of high expressors (line H, A and B,  $n = 20$ ) and low expressors (line L, C and D,  $n = 15$ ) of TgRIP-SREBP1c (●) and respective nontransgenic littermate controls (△,  $n = 20$  in controls for line H and  $n = 15$  for line L littermates) were measured before (0 min) and at indicated time points (in minutes) after intravenous administration of glucose (1g/kg body wt) following an overnight fast. E: Plasma glucose levels after intraperitoneal injection of human insulin (0.5 units/kg) to five line H mice (●) and littermate controls (△). All values are means  $\pm$  SE. # $P < 0.05$  and \* $P < 0.01$  vs. littermate controls.

detectable transgene mRNA levels by Northern blot analysis of isolated islets and was roughly seven times higher than that in low expressors (line L), as estimated by RT-PCR (Fig. 1B and C). Insulin gene expression was similar among these transgenic and nontransgenic littermate control mice (Fig. 1B), suggesting that use of RIP did not markedly compromise functions of mouse endogenous insulin promoters. The human SREBP-1c protein from the transgene was highly induced in islets of line H of TgRIP-SREBP-1c at the level comparable to that of endogenous SREBP-1c induced by incubation with a saturated fatty acid (Fig. 1D).

**Impaired glucose tolerance in TgRIP-SREBP1c mice.**

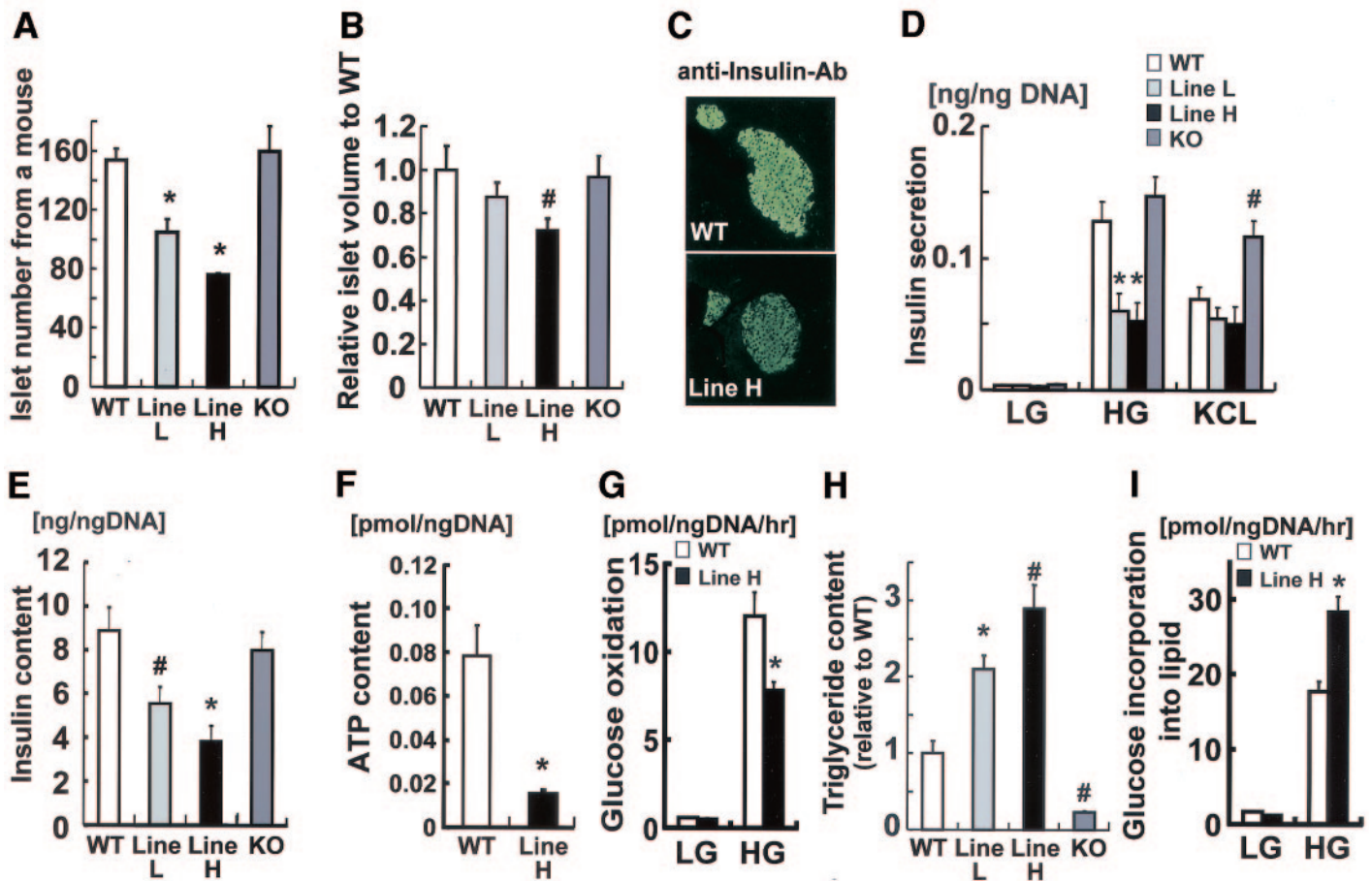
When transgenic mice were fed a normal diet, they exhibited impaired glucose tolerance (IGT). Line H showed a slightly higher plasma glucose level at fasting, followed by significantly higher elevations after intravenous glucose loading compared with wild-type controls (Fig. 2A). A rapid increment in plasma insulin levels observed in wild type after glucose injection was markedly impaired in high expressors of TgRIP-SREBP1c, indicating impaired insulin secretion (Fig. 2B). Glucose tolerance testing in the lower expression line (line L) revealed lower insulin levels only at 5 min, with no significant difference in plasma glucose levels at other time points from littermate controls. This impairment in the early response of insulin secretion to glucose in the lower line mimics the oral glucose tolerance test pattern observed in patients with IGT or those at an early stage of type 2 diabetes. These data demonstrate that SREBP-1c overexpression in β-cells causes IGT in a dose-dependent manner. These transgenic mice did not show any difference in body weights and plasma cholesterol, triglycerides, and FFAs levels from control mice (data not shown). Insulin tolerance tests showed no significant difference between line H mice and wild-type littermates, indicating no signs of peripheral insulin resistance in the transgenic mice (Fig. 2E).

**Reduction in the number and size of islets from TgRIP-SREBP1c mouse.**

In isolation of islets from whole pancreas, the numbers of islets from TgRIP-SREBP1c were significantly decreased compared with control animals (Fig. 3A). In addition, islets from line H were considerably smaller than controls, even when taking into account intrinsic variability in islet sizes. Microscopic examination of isolated islets indicated that volume of islets was also significantly lower in transgenic animals (Fig. 3B). The reduction of islet mass was related to the levels of transgene expression and could partially explain the impaired insulin response to glucose that was observed in vivo. We also compared the size of islets from SREBP-1-null mice and did not observe a significant difference from that of wild-type mice. Insulin staining of microscopic sections demonstrated that line H showed a similar pattern to that of wild type, indicating that there was not a marked reduction in the number of β-cells per islet, although there was a slight decrease in intensity of insulin immunoreactivity (Fig. 3C).

**Disturbed GSIS from isolated islets of TgRIP-SREBP1c mouse.**

Insulin secretion was estimated from isolated islets of similar sizes. Basal insulin secretion in low-glucose medium (2.8 mmol/l) was slightly decreased in line L, but not in line H, as compared with wild-type islets. GSIS in high-glucose medium (20 mmol/l) was significantly impaired in both line H and L (Fig. 3D). Conversely, islets from SREBP-1c-null mice showed a trend to a higher insulin secretion in glucose response. In contrast to reduced GSIS, insulin secretion stimulated by KCl was not impaired in islets from TgRIP-SREBP1c. Insulin content was decreased in islets from TgRIP-SREBP1c, which also partially explains the GSIS suppression (Fig. 3E). Consistent with disturbances in GSIS, ATP content was decreased in SREBP-1c-expressing β-cells (Fig. 3F). The glucose oxidation in islets from TgRIP-SREBP1c was also reduced, supporting the impaired ATP



**FIG. 3.** Analyses on islets isolated from TgRIP-SREBP-1c and wild-type controls. *A*: Islets were isolated from six mice of each line at age 26 weeks, and the number isolated was counted. *B*: In some other sets of animals, isolated islets were prepared and relative islet volume to wild type (WT) was estimated from a digital microscopic image of 100 islets from each group, as described in RESEARCH DESIGN AND METHODS. *C*: Islets were immunostained by anti-insulin antibody and visualized using fluorescein isothiocyanate-labeled secondary antibody. *D*: Glucose- or KCl-stimulated insulin secretion per DNA contents of islet. Ten isolated islets with similar sizes from each group (six batches in each genotype) were sequentially incubated in 1 ml KRBH containing 2.8 mmol/l glucose (low glucose [LG]), 20 mmol/l glucose (high glucose [HG]), and 20 mmol/l KCl for 30 min at 37°C, with preincubation at each interval in 2.8-mmol/l glucose buffer for 30 min. Insulin contents in the buffer were determined by enzyme-linked immunosorbent assay and corrected by DNA contents of islets. *E*: Insulin was extracted by the acid-ethanol method from six batches in each genotype and measured by enzyme-linked immunosorbent assay. *F*: ATP contents from 10 isolated islets were measured and corrected by DNA contents ( $n = 6$ ). *G*: Glucose oxidation of islets was detected by radioactivity of  $^{14}\text{CO}_2$  oxidized from  $[\text{U-}^{14}\text{C}]\text{glucose}$  ( $n = 6$ ). *H*: The amount of triglyceride was measured from lipid extraction from 100 isolated islets (three mice in each genotype) and corrected by estimated volume of islets. *I*: Incorporation of  $[\text{U-}^{14}\text{C}]\text{glucose}$  into lipids was measured ( $n = 6$ ). All values are means  $\pm$  SE. # $P < 0.05$  and \* $P < 0.01$  vs. wild type.

production (Fig. 3G). Reflecting forced expression of human nuclear SREBP-1c in  $\beta$ -cells, contents of triglycerides from pancreatic islets, corrected for by relative islet volume, were increased in TgRIP-SREBP1c in a transgene expression-dependent manner (Fig. 3H). In contrast, islets from SREBP-1-null mice contained markedly less triglycerides than those from wild type. Glucose incorporation into lipid was increased in the islets from TgRIP-SREBP1c mice (Fig. 3I). These data suggest that SREBP-1c could regulate islet triglycerides content through regulation of endogenous synthesis and play a negative role in GSIS in  $\beta$ -cells. The disturbance was located prior the ATP-sensitive  $\text{K}^+$  channel level.

**UCP-2 is upregulated and Pdx-1 is downregulated in SREBP-1c-overexpressing islets.** Expression of various genes in islets was estimated by RT-PCR to elucidate the molecular mechanism for insulin secretion disturbance in TgRIP-SREBP1c (Fig. 4). The endogenous mouse SREBP-1c mRNA level did not show significant changes in wild type and the two transgenic lines. Consistent with

increased triglyceride contents, mRNA levels of FAS and SCD-1, key enzymes for synthesis of fatty acids and major transcriptional target genes of SREBP-1c, were also markedly elevated in TgRIP-SREBP1c islets in a transgene expression-dependent manner. UCP-2, recently established as a regulator for ATP production and a marker for mitochondrial stress, has been reported to negatively regulate insulin secretion in  $\beta$ -cells (6,27). Gene expression of UCP-2 was dose dependently upregulated by SREBP-1c overexpression, explaining ATP depletion in TgRIP-SREBP1c islets. It was also found that Pdx-1, a homeodomain product that plays an important role in pancreatic islet genesis and insulin gene regulation (28–30), was dose dependently downregulated by SREBP-1c. Nkx6.1, a transcription repressor that is also important for  $\beta$ -cell differentiation (31), was upregulated by SREBP-1c overexpression. Conversely, these genes are regulated in opposite directions by SREBP-1 depletion. Expression of IRS-2, an important insulin signaling mediator, was sup-

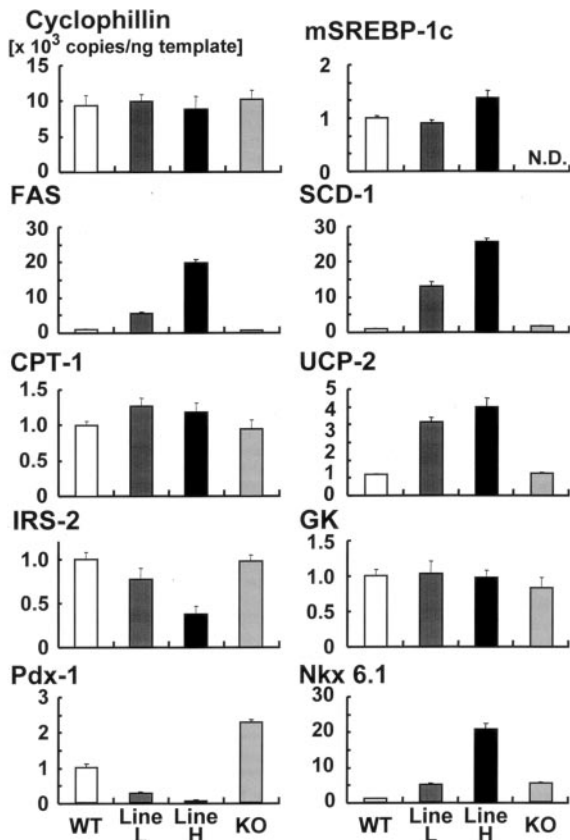


FIG. 4. Gene expression profile in islets from TgRIP-SREBP1c and SREBP-1 null mice. mRNA amounts of indicated genes in islets were estimated by RT-PCR. Cyclophilin mRNA level was expressed as amounts of molecular copies estimated from the quantitative PCR signal and used as an internal control for each gene expression. Relative expression ratio to wild-type control is shown. All values are means  $\pm$  SE.

pressed in TgRIP-SREBP-1c islets, consistent with our previous observation in livers (32).

**Suppression of hyperinsulinemia and exacerbated IGT in TgRIP-SREBP1c mice by a high-energy diet.** To overload requirement for insulin secretion, TgRIP-SREBP1c mice were put on a HFHS diet. This high-energy diet induced an insulin-resistant state with obesity and hyperinsulinemia, as evidenced by gradual increases in body weight, fasting plasma glucose, and insulin levels in wild-type mice (Fig. 5A, B, and C). Whereas line H of TgRIP-SREBP1c mice also exhibited a similar time course change in body weight, hyperinsulinemia was markedly suppressed, highlighting an insulin secretion defect in these mice. An intravenous glucose tolerance test was performed at 16 weeks after the HFHS diet, at the time point when increased blood glucose level was significant. TgRIP-SREBP1c became more diabetic, showing evidently higher plasma glucose levels than wild type (Fig. 6A). In the transgenic mice on HFHS diet, plasma insulin levels did not respond to glucose loading and instead were decreased (Fig. 6B). Consistent with exacerbation in IGT, islets from the transgenic mice on a high-fat diet essentially lost glucose response to insulin secretion, whereas in normal mice, insulin secretion on HFHS diet was enhanced (Fig. 6C). Triglyceride contents in islets were increased in both wild type and TgRIP-SREBP1c by HFHS

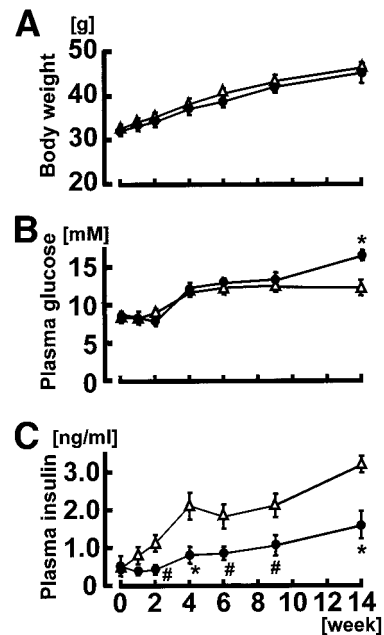


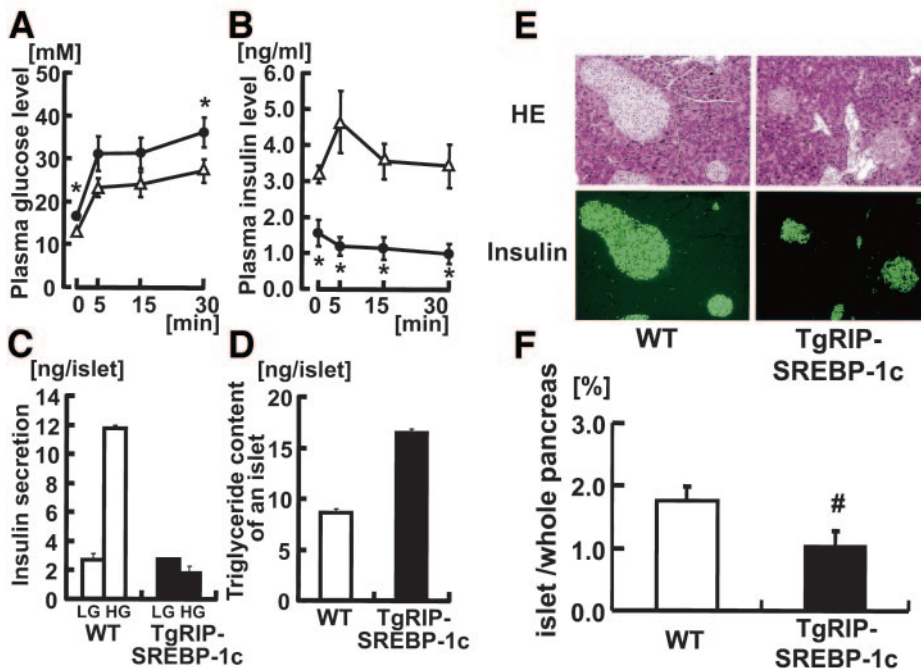
FIG. 5. Changes of body weight (A), plasma glucose (B), and plasma insulin (C) levels of TgRIP-SREBP1c on a high-energy diet. Male line H of transgenic ( $\bullet$ ,  $n = 6$ ) and nontransgenic littermate control ( $\Delta$ ,  $n = 10$ ) mice were put on a HFHS diet, and the parameters were measured at indicated weeks after an overnight fast. All values are means  $\pm$  SE. # $P < 0.05$  and \* $P < 0.01$  vs. littermate controls.

diet, and the difference was still appreciable (Fig. 6D). HFHS-induced obesity and insulin resistance was followed by hyperinsulinemia from enlarged islets in wild-type mice (Fig. 6E). Consistent with insulin secretion defect, this islet enlargement was markedly suppressed in TgRIP-SREBP1c on HFHS diet. The number and size of islets were appreciably reduced on hematoxylin-eosin staining of microscopic examination (Fig. 6E and F), implicating that SREBP-1c could play a negative role in  $\beta$ -cell growth.

## DISCUSSION

Current studies clearly demonstrate that activation of SREBP-1c in  $\beta$ -cells caused the onset of IGT or mild diabetes due to impaired insulin secretion. Reduction in  $\beta$ -cell mass, indicated by decreases in both the number and size of TgRIP-SREBP1c islets, at least partially contributes to these phenotypes. Retarded growth of islets by SREBP-1c was more obvious with marked impairment in insulin response to glucose when the high-energy diet evoked obesity-induced peripheral insulin resistance, enhancing insulin requirement for these mice. Meanwhile, in comparison with islets of similar size, GSIS from TgRIP-SREBP1c was significantly decreased compared with that from control mice. Suppression of GSIS by SREBP-1c is consistent with previous data on  $\beta$ -cell lines (33). Thus, impaired insulin secretion of TgRIP-SREBP-1c in vivo is featured by two abnormalities: islet volume loss and dysfunction of  $\beta$ -cells.

The amount of SREBP-1c expression was positively associated with triglyceride content of islets and negatively with GSIS in a wide range from SREBP-1c-null mice to high expressers. As established in livers and adipose tissue, SREBP-1c activates expression of lipogenic genes, such as FAS and SCD-1 in  $\beta$ -cells, whereas expression of



**FIG. 6.** Investigations on TgRIP-SREBP1c mice fed a high-energy diet. Six male TgRIP-SREBP1c mice (line H, ●) and nontransgenic littermate controls (○) were fed a HFHS diet for 14 weeks. Plasma glucose (A) and insulin (B) levels were measured before (0 min) and at indicated time points (in minutes) after intravenous administration of glucose (1g/kg body wt) following an overnight fast ( $n = 6$ ). C: Glucose-stimulated insulin secretion from isolated islets obtained from indicated HFHS-fed mice ( $n = 3$ ). D: Triglyceride content per islet from TgRIP-SREBP1c and their wild-type (WT) littermates ( $n = 3$ ). E: Light microscopic findings of hematoxylin-eosin stain (HE; upper panels) and immunostaining with insulin antibody detected by fluorescein isothiocyanate (insulin; lower panels). F: Islet volume estimated by area ratio of islets to whole pancreas sections from wild-type mice and line H of TgRIP-SREBP1c mice fed a HFHS diet. All values are means  $\pm$  SE. # $P < 0.05$  and \* $P < 0.01$  vs. littermate controls.

CPT-1 and PPAR $\alpha$  was slightly increased (Fig. 4 and data not shown), suggesting that triglyceride accumulation in  $\beta$ -cells from TgRIP-SREBP1c is due to enhanced synthesis rather than decreased degradation of fatty acids. Moreover, the glucose incorporation into lipid was accelerated in islets from TgRIP-SREBP1c. These data suggest that SREBP-1c led to impaired insulin secretion through lipotoxicity by activation of fatty acid and triglyceride synthesis. Lipotoxicity in  $\beta$ -cells was originally proposed to be a result of influx of high-plasma fatty acids often associated with obesity. TgRIP-SREBP1c mice were not obese, and plasma FFA levels were not elevated. Thus, these mice provide a new model for  $\beta$ -cell lipotoxicity, the etiology of which is extended to increased endogenous lipogenesis. Accelerated lipogenesis by SREBP-1c is associated with consumption of acetyl-CoA and glucose, which could lead to diminished ATP production.

UCP-2 upregulation in TgRIP-SREBP1c islets could play an important role in molecular mechanisms, by which SREBP-1c causes  $\beta$ -cell dysfunction, as already noticed in cultured cells (34–36). It has been recently demonstrated that UCP-2 negatively regulates insulin secretion (6,27). Activation of UCP-2 by fatty acids was suggested to play an important role in obesity-induced  $\beta$ -cell dysfunction induced by exogenous fatty acid or triglyceride loading as the inducer of lipotoxicity (20,27). In addition to fatty acid-mediated induction, SREBP was reported to directly activate UCP-2 transcription (37,38). Considering that we saw no change in UCP-2 expression in SREBP-1 knockout islets, SREBP-1c may not be involved in physiological expression of UCP-2, but impaired insulin secretion by activated SREBP-1c in  $\beta$ -cells might be mediated through UCP-2 activation.

Downregulation of Pdx-1 could explain the reduction in islet volume and possibly the disturbed insulin secretion in TgRIP-SREBP1c (39). Expression of Pdx-1 was inversely correlated to that of SREBP-1c in a range from SREBP-1c knockout to transgenic mice, suggesting that SREBP-1c

could regulate Pdx-1, although the molecular mechanism is still under investigation. Nkx6.1 activation in the transgenic islets could be a compensatory response to Pdx-1 suppression. Gene expression profile of  $\beta$ -cells from TgRIP-SREBP-1c is essentially consistent with the previous report on short-term overexpression of SREBP-1c in INS-1 cells by the Tet-On inducible system. Overall, SREBP-1c could function as a toxic gene for  $\beta$ -cells by increasing intracellular fatty acids (lipotoxicity), and by directly modulating transcription of key genes such as Pdx-1 and UCP-2, which could provide a new role for SREBP-1c in  $\beta$ -cells. In addition, we recently reported that SREBP-1c suppresses insulin signaling in liver by repression of IRS-2 gene expression (32). Suppression of IRS-2 expression was also observed in TgRIP-SREBP1c islets, which could lead to impaired insulin secretion, as already suggested in  $\beta$ -cell-specific insulin receptor knockout or IRS-2 knockout mice (33,40).

The potential for endogenous SREBP-1c to be involved in the pathophysiology of  $\beta$ -cells is highly relevant and needs to be studied to verify the significance in the current data of our ectopic expression experiments. In several insulin-resistant animals, such as Zucker fatty rats and *ob/ob* mice, SREBP-1c is highly expressed in both liver and islets (41). Also, it has been reported that endogenous nuclear SREBP-1c was upregulated by exposure to a high-glucose medium in hepatocytes and  $\beta$ -cell lines (19,42). Hyperglycemia, as observed in STZ-induced diabetic, insulin-depleted mice, could stimulate SREBP-1c expression in the liver (26).  $\beta$ -Cells and hepatocytes take up glucose through insulin-independent GLUT2, which prompts us to speculate that endogenous SREBP-1c in  $\beta$ -cells could be activated and involved in glucolipotoxicity in diabetic states with severe hyperglycemia. An opposite phenotype in islets of SREBP-1 knockout mice compared with TgRIP-SREBP1c islets strongly supports the involvement of SREBP-1c in  $\beta$ -cell lipotoxicity. Thus, these mice are not only a good model for IGT and type 2 diabetes but

also a useful tool to investigate a novel mechanism of insulin secretion defects. Further studies are needed to investigate the more precise molecular mechanism by which SREBP-1c could cause  $\beta$ -cell dysfunction and growth impairment and to seek potential roles for SREBP-1c in clinical relevance to some clinical categories of type 2 diabetes, such as nonobese Japanese patients (43).

#### ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the Ministry of Science, Education, Culture, and Technology of Japan.

The authors are grateful to Dr. A.H. Hasty for critical reading of this manuscript and to Drs. S. Ishibashi and J. Osuga for helpful discussion.

#### REFERENCES

- Unger RH: Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes* 44:863–870, 1995
- Boden G, Shulman GI: Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *Eur J Clin Invest* 32 (Suppl. 3):14–23, 2002
- McGarry JD: What if Minkowski had been ageusic? An alternative angle on diabetes. *Science* 258:766–770, 1992
- Prentki M, Corkey BE: Are the  $\beta$ -cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* 45:273–283, 1996
- Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH: Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships. *Proc Natl Acad Sci U S A* 91:10878–10882, 1994
- Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F, Tsushima RG, Pennefather PS, Salapatek AM, Wheeler MB: Increased uncoupling protein-2 levels in  $\beta$ -cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. *Diabetes* 50:1302–1310, 2001
- Lameloise N, Muzzin P, Prentki M, Assimacopoulos-Jeannet F: Uncoupling protein 2: a possible link between fatty acid excess and impaired glucose-induced insulin secretion? *Diabetes* 50:803–809, 2001
- Brown MS, Goldstein JL: The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331–340, 1997
- Shimano H: Sterol regulatory element-binding protein-1 as a dominant transcription factor for gene regulation of lipogenic enzymes in the liver. *Trends Cardiovasc Med* 10:275–278, 2000
- Shimano H: Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res* 40:439–452, 2001
- Horton JD, Goldstein JL, Brown MS: SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109:1125–1131, 2002
- Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, Yamada N: Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem* 274:35832–35839, 1999
- Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, Brown MS: Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J Biol Chem* 277:9520–9528, 2002
- Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H: Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* 101:2331–2339, 1998
- Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM: Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101:1–9, 1998
- Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL: Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:13656–13661, 1999
- Foretz M, Guichard C, Ferre P, Foufelle F: Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc Natl Acad Sci U S A* 96:12737–12742, 1999
- Foretz M, Pacot C, Dugail I, Lemarchand P, Guichard C, Le Liepvre X, Berthelie-Lubrano C, Spiegelman B, Kim JB, Ferre P, Foufelle F: ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol Cell Biol* 19:3760–3768, 1999
- Hasty AH, Shimano H, Yahagi N, Amemiya-Kudo M, Perrey S, Yoshikawa T, Osuga J, Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, Harada K, Gotoda T, Nagai R, Ishibashi S, Yamada N: Sterol regulatory element-binding protein-1 is regulated by glucose at the transcriptional level. *J Biol Chem* 275:31069–31077, 2000
- Winzell MS, Svansson H, Enerback S, Ravnskjaer K, Mandrup S, Esser V, Arner P, Alves-Guerra MC, Miroux B, Sundler F, Ahren B, Holm C: Pancreatic beta-cell lipotoxicity induced by overexpression of hormone-sensitive lipase. *Diabetes* 52:2057–2065, 2003
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL: Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* 98:1575–1584, 1996
- Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL: Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J Clin Invest* 99:846–854, 1997
- Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD: Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. *J Clin Invest* 100:2115–2124, 1997
- Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y: Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8:731–737, 2002
- Sekiya M, Osuga J, Okazaki H, Yahagi N, Harada K, Shen WJ, Tamura Y, Tomita S, Iizuka Y, Ohashi K, Okazaki M, Sata M, Nagai R, Fujita T, Shimano H, Kraemer FB, Yamada N, Ishibashi S: Absence of hormone-sensitive lipase inhibits obesity and adipogenesis in Lep ob/ob mice. *J Biol Chem* 279:15084–15090, 2004
- Matsuzaka T, Shimano H, Yahagi N, Amemiya-Kudo M, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Tomita S, Sekiya M, Hasty A, Nakagawa Y, Sone H, Toyoshima H, Ishibashi S, Osuga J, Yamada N: Insulin-independent induction of sterol regulatory element-binding protein-1c expression in the livers of streptozotocin-treated mice. *Diabetes* 53:560–569, 2004
- Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grubic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB: Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* 105:745–755, 2001
- Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
- Ohlsson H, Karlsson K, Edlund T: IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J* 12:4251–4259, 1993
- Petersen HV, Serup P, Leonard J, Michelsen BK, Madsen OD, Jonsson J, Carlsson L, Edlund T, Edlund H: Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through the CT boxes Insulin-promoter-factor 1 is required for pancreas development in mice. *Proc Natl Acad Sci U S A* 91:10465–10469, 1994
- Jensen J, Serup P, Karlsen C, Nielsen TF, Madsen OD: mRNA profiling of rat islet tumors reveals Nkx 6.1 as a beta-cell-specific homeodomain transcription factor. *J Biol Chem* 271:18749–18758, 1996
- Ide T, Shimano H, Yahagi N, Matsuzaka T, Nakakuki M, Yamamoto T, Nakagawa Y, Takahashi A, Suzuki H, Sone H, Toyoshima H, Fukamizu A, Yamada N: SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nat Cell Biol* 6:351–357, 2004
- Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339, 1999
- Andreolas C, da Silva Xavier G, Diraison F, Zhao C, Varadi A, Lopez-Casillas F, Ferre P, Foufelle F, Rutter GA: Stimulation of acetyl-CoA carboxylase gene expression by glucose requires insulin release and sterol regulatory element binding protein 1c in pancreatic MIN6  $\beta$ -cells. *Diabetes* 51:2536–2545, 2002
- Diraison F, Parton L, Ferre P, Foufelle F, Briscoe CP, Leclerc I, Rutter GA: Over-expression of sterol regulatory element binding protein-1c in rat

- pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminoimidazole-4-carboxamide ribonucleoside. *Biochem J* 378:769–778, 2004
36. Yamashita T, Eto K, Okazaki Y, Yamashita S, Yamauchi T, Sekine N, Nagai R, Noda M, Kadowaki T: Role of uncoupling protein-2 up-regulation and triglyceride accumulation in impaired glucose-stimulated insulin secretion in a beta-cell lipotoxicity model overexpressing sterol regulatory element-binding protein-1c. *Endocrinology* 145:3566–3577, 2004
37. Medvedev AV, Robidoux J, Bai X, Cao W, Floering LM, Daniel KW, Collins S: Regulation of the uncoupling protein-2 gene in INS-1 beta-cells by oleic acid. *J Biol Chem* 277:42639–42644, 2002
38. Medvedev AV, Snedden SK, Raimbault S, Ricquier D, Collins S: Transcriptional regulation of the mouse uncoupling protein-2 gene: double E-box motif is required for peroxisome proliferator-activated receptor-gamma-dependent activation. *J Biol Chem* 276:10817–10823, 2001
39. Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H: Beta-cell-specific inactivation of the mouse *Ipf1/Pdx1* gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev* 12:1763–1768, 1998
40. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of *IRS-2* causes type 2 diabetes in mice. *Nature* 391:900–904, 1998
41. Kakuma T, Lee Y, Higa M, Wang Z, Pan W, Shimomura I, Unger RH: Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. *Proc Natl Acad Sci U S A* 97:8536–8541, 2000
42. Wang H, Maechler P, Antinozzi PA, Herrero L, Hagenfeldt-Johansson KA, Bjorklund A, Wollheim CB: The transcription factor SREBP-1c is instrumental in the development of beta-cell dysfunction. *J Biol Chem* 278:16622–16629, 2003
43. Sone H, Yoshimura Y, Ito H, Ohashi Y, Yamada N: Energy intake and obesity in Japanese patients with type 2 diabetes. *Lancet* 363:248–249, 2004