SOX6 Attenuates Glucose-stimulated Insulin Secretion by Repressing PDX1 Transcriptional Activity and Is Down-regulated in Hyperinsulinemic Obese Mice

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In obesity-related insulin resistance, pancreatic islets compensate for insulin resistance by increasing secretory capacity. Here, we report the identification of sex-determining region Y-box 6 (SOX6), a member of the high mobility group box superfamily of transcription factors, as a co-repressor for pancreatic-duodenal homeobox factor-1 (PDX1). SOX6 mRNA levels were profoundly reduced by both a long term high fat feeding protocol in normal mice and in genetically obese ob/ob mice on a normal chow diet. Interestingly, we show that SOX6 is expressed in adult pancreatic insulin-producing β-cells and that overexpression of SOX6 decreased glucose-stimulated insulin secretion, which was accompanied by decreased ATP/ADP ratio, Ca2+ mobilization, proinsulin content, and insulin gene expression. In a complementary fashion, depletion of SOX6 by small interfering RNAs augmented glucose-stimulated insulin secretion in insulinoma mouse MIN6 and rat INS-1E cells. These effects can be explained by our mechanistic studies that show SOX6 acts to suppress PDX1 stimulation of the insulin II promoter through a direct protein/protein interaction. Furthermore, SOX6 retroviral expression decreased acetylation of histones H3 and H4 in chromatin from the promoter for the insulin II gene, suggesting that SOX6 may decrease PDX1 stimulation through changes in chromatin structure at specific promoters. These results suggest that perturbations in transcriptional regulation that are coordinated through SOX6 and PDX1 in β-cells may contribute to the β-cell adaptation in obesity-related insulin resistance.

Insulin resistance is tissue insensitivity to the regulatory effects of insulin and is the leading cause of type 2 diabetes (1, 2). Most affected individuals with insulin resistance do not directly develop diabetes but rather adapt to chronic insulin resistance by expanding pancreatic β-cell mass and/or insulin secretory capacity. To provide the required amount of insulin to maintain normal glucose levels, β-cell mass increases by islet neogenesis, β-cell replication, and β-cell hypertrophy. Pancreatic β-cells eventually fail to compensate for the increased insulin demand created by insulin resistance, leading to type 2 diabetes (1–6).

Pancreatic-duodenal homeobox factor-1 (PDX1), a homeodomain transcription factor, and the insulin/insulin-like growth factor signaling pathway are critical for β-cell replication and the compensatory response to insulin resistance (7). PDX1 is expressed in β-cells of the islets of Langerhans and is involved in regulating the expression of a number of key β-cell genes. It plays a pivotal role in the development of the pancreas and islet cell ontogeny (8). In a mouse model, inactivation of both pdx1 alleles results in pancreas agenesis, whereas heterozygous pdx1+/- mice or animals carrying a β-cell-specific mutation of the gene exhibit glucose intolerance (9–11). Mutations in the human PDX1 gene are associated with maturity onset diabetes of the young (MODY4) and predispose to late onset type II diabetes (12–14). Although these results show that PDX1 plays a key role in the development of the pancreas and glucose-stimulated insulin secretion (GSIS) from β-cells, its functional role in the β-cell adaptation seen in chronic insulin resistance is poorly understood.

PDX1 is a 284-amino acid protein consisting of 1) an NH2-terminal transactivation domain of 144 amino acids, 2) a homeodomain of 60 amino acids, and 3) a COOH-terminal domain of 80 amino acids. PDX1 binds through its homeodomain to target sequences called A-boxes (A/T-rich elements) of the insulin gene promoter (15). The NH2-terminal activation domain of PDX1 recruits the coactivator p300 and stimulates insulin gene expression synergistically with E12 and E47, which bind to E-boxes that are also located in the insulin gene promoter.
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19). Interestingly, p300 is recruited to the insulin gene promoter only when cells are cultured in high glucose media (20).

To identify additional factors that may contribute to the β-cell adaptation in insulin resistance, we have been characterizing genes that are selectively regulated in the islets of mice fed a high fat diet (HFD) using microarray analysis. Through the evaluation of transcriptional changes by microarray and quantitative real time PCR analyses, we found that one of the sex-determining region Y-box (SOX) transcription factors, SOX6, is markedly down-regulated in the islets of HFD-fed mice and normal chow fed ob/ob mice. Functional analyses with pancreatic β-cell line MIN6 cells revealed that SOX6 reduces GSIS by inhibiting PDX1 transcriptional activity, and our evidence indicates this occurs through a direct interaction between SOX6 and PDX1 proteins. We further show that overexpression of SOX6 results in decreased expression of genes involved in mitochondrial metabolism, including the NADH dehydrogenase complex of the mitochondrial respiratory chain, ATP synthase, and a subunit of cytochrome c oxidase. Taken together, the current data suggest that SOX6 is a key protein in the regulation of GSIS and that, together with PDX1, it contributes to the adaptive compensation of β-cells during the progression of obesity-related insulin resistance.

EXPERIMENTAL PROCEDURES

Materials—The luciferase reporter assay system and pGL3-basic (Promega) were used as the source of the luciferase gene in all constructs and for luciferase assay components. The RNaseasy kit was purchased from Qiagen. Acetyl-histone H3 and H4 immunoprecipitation assay kits were purchased from Upstate Biotechnology, Inc. Other reagents were obtained from sources as described previously (21–24). Antibodies were obtained from the following sources: a goat polyclonal anti-PDX1 (sc-14664) and anti-SOX6 (sc-17332), rabbit polyclonal anti-SOX5 (sc-20091) and anti-SOX9 (sc-20095), and peroxidase-conjugated affinity-purified donkey anti-rabbit and anti-goat IgG from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti-SOX6 (ab-12054) (directed against amino acids 349–354 of human SOX6) and anti-β-actin (ab-8226) from Abcam Ltd.; rabbit polyclonal anti-PDX1 (KR059) from Transgenic Inc. (Hyogo, Japan); Alexa Fluor 488 anti-guinea pig and anti-rabbit IgG and Zenon Alexa Fluor 594 anti-rabbit IgG labeling kit from Molecular Probes, Inc. (Eugene, OR); guinea pig polyclonal antibody to pig insulin from Nichirei (Tokyo, Japan); chicken polyclonal anti-SOX15 (AB-9180) from Chemicon International, Inc.; and control rabbit IgG (1:1000) from Vector Laboratories.

Animals, Diets, and Pancreatic Islet Preparation—10-Week male C57BL/6J mice and ob/ob mice were purchased from Charles River and housed in a temperature- and humidity-controlled (26.5 °C and 35%) facility with a 12-h light/dark cycle (09:00 to 21:00 h). Mice were fed with a normal chow diet (NCD) (CE-2; CLEA, Osaka, Japan); chicken polyclonal anti-SOX15 (AB-9180) from Chemicon International, Inc.; and control rabbit IgG (1:1000) from Vector Laboratories.

Quantitative Real Time PCR (QRT-PCR) and Affymetrix Oligonucleotide Microarray—The methods for microarray and QRT-PCR have been described (23, 24). We used Affymetrix Genechip MOE430-A and -B arrays that contain probe sets for >30,000 mouse genes. All primer sequences used in this article are available by request.

Immunohistochemistry—For light microscopy of paraffin-embedded sections, mouse pancreatic tissues were fixed with 10% (w/v) formalin at room temperature for 20 h. The samples were dehydrated with an alcohol series and embedded in paraffin. Antigen retrieval was performed by heating the sections in an autoclave at 121 °C for 15 min. The sections were incubated with anti-SOX6 antibody (ab-12054) (1:2000 dilution) for 16 h at 4 °C. Bound antibody was detected with the Simple Stain MAX-PO (Multi) reagent (Nichirei), an amino acid polymer-coated goat anti-rabbit IgG (Fab’2) and peroxidase using 3,3’-diaminobenzidine (Djingido, Kumamoto, Japan) as a substrate, and a hematoxylin counterstain was applied. For double immunofluorescence of adult mouse pancreas, fixed frozen tissues were permeabilized with 0.2% Triton X-100 for 20 min at 4 °C and stained with an anti-SOX6 antibody (ab-12054) (1:2000 dilution) labeled with Zenon Alexa Fluor 594 labeling kit and an anti-insulin or an anti-PDX1 antibody (KR059) (1:2000 dilution). For the detection of PDX1 and insulin, Alexa Fluor 488 anti-rabbit IgG (1:2000 dilution) and Alexa Fluor 488 anti-guinea pig IgG were used as a secondary antibody, respectively. Control experiments were carried out by omitting the primary antibody. Immunofluorescence was captured with a confocal laser scanning microscope (Fluoview FV500, Olympus, Japan).

Expression Plasmids—Retroviral expression vectors encoding mouse SOX6 and other SOX genes were generated by PCR and insertion of the cDNAs into the pMX, a cytomegalovirus (CMV) promoter-driven retroviral expression vector (provided by Dr. Tosho Kitamura at University of Tokyo) (27). pCMV-PDX1, a pcDNA3-based plasmid encoding mouse PDX1, was obtained from Dr. Kazuya Yamagata at Osaka University (28), and to create pcDNA3-based plasmids encoding mutant PDX1, the deletion sequences of PDX1 (amino acids 1–205, 1–144, and 145–284) were amplified by PCR and ligated into pcDNA3 (Invitrogen). To create pcDNA3-based plasmids encoding full-length and mutant SOX6, the full-length and deletion sequences of SOX6 (amino acids 181–827, 263–827, 617–827, 697–827, and 617–696) were generated by PCR amplification and ligated into pcDNA3. pCMV-ΔHMG-SOX6 encodes an internal deletion mutant form of SOX6 in which a 265-amino acid region containing the high mobility group (HMG) domain (amino acids 563–827) was deleted. This was constructed by digestion of pCMV-SOX6 with Apal to remove the Apal-Apal 0.8-kbp fragment containing sequences for the HMG domain, and the plasmid was subsequently religated. A GAL4-PDX1 fusion construct, pBIND-PDX1, and a GAL4-E47 fusion construct, pBIND-E47, were constructed by inserting each cDNA fragment into a polylinker site of pBIND plasmid (Promega), which contains the DNA binding domain of the yeast GAL4 protein.

Reporter Plasmids—pINS(-872)-luc is the rat insulin II gene promoter-luciferase reporter construct that spans −872 to −176 relative to the translation initiation site. pINS(-552)-luc and pINS(-413)-luc are 5′-deletion mutants of pINS(-872)-luc, and each contains a deletion with the 5′-end denoted in parentheses and the same 3′-end point at −176. pINS(-413mut)-luc is identical to pINS(-413)-luc except that the potential SOX binding site (nucleotides −248 to −242) is deleted. pINS(-370mut)-luc was constructed in an identical manner to pINS(-413mut)-luc, but starting from position −370. The 5′-flanking region of the rat insulin II gene (−872 to −176) (29) was amplified by PCR using a forward primer starting from −872 (5′-TATAGGTACCCCAACACTCCTCA-3′) and a reverse primer OlI(-176R) (5′-TATACCGGTTTACTGAATCC-3′) and cloned into pGL3-basic. pINS(-552)-luc and pINS(-413)-luc were constructed in a similar manner to pINS(-872)-luc using the respective forward primers starting from the positions −552 (5′-TATAGGTACCTGTAAGACCAACATGTTCAGG-3′) and −413 (5′-TATAGGTACCTCATCAGGCCACCAGGAG-3′) and coupled with a common reverse primer OlI(-176R).
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p(\mu E5 + \mu E2 + \mu E3)luc is a luciferase reporter plasmid driven by a promoter consisting of four tandem copies of the E47-responsive element (5'-ACACCTGACAGCAGCTGGCAGGAAGCAGTCATGTGGCA-3') from the mouse IgH promoter (30). It was constructed by annealing the oligonucleotides for the top and bottom strands and subsequent ligation into the MluI and BglII sites of pGL3-basic. All plasmid constructs were verified by restriction endonuclease mapping and DNA sequencing. pGSlac is a luciferase reporter construct driven by a promoter consisting of five copies of GAL4 binding sites plus the adenovirus E1B TATA box (Promega).

Cell Culture and Retroviral Infection—MIN6 cells (a line of mouse pancreatic \( \beta \)-cells) (31) and INS-1E cells (a clone of parental rat \( \beta \)-cell line INS-1E cells (32) selected for insulin content and adequate proliferation (33)) were kind gifts from Dr. Jun-Ichi Miyazaki (Osaka University) and Dr. Pierre Maechler (University Medical Center at Switzerland), respectively. MIN6 cells were grown in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 5.5 mM \( \mu \)M-mercaptoethanol, 100 units/ml penicillin, and 100 \( \mu \)g/ml of streptomycin sulphate, supplemented with 15% fetal bovine serum at 37°C in 5% CO\(_2\). INS-1E cells were cultured in RPMI1640 containing 11.6 mM glucose, 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 50 \( \mu \)g/ml \( \mu \)-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 \( \mu \)g/ml of streptomycin sulphate, supplemented with 5% fetal bovine serum at 37°C in 5% CO\(_2\). Retroviral infection to MIN6 cells was performed as previously described (23) using pMX plasmids (27, 34). Human embryonic kidney 293 cells and BHK21 cells (a line of hamster kidney cells) were obtained from the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan) and maintained in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 \( \mu \)g/ml of streptomycin sulphate, supplemented with 10% fetal bovine serum at 37°C in 5% CO\(_2\).

Transient Transfection Assays—MIN6, HEK293, or BHK21 cells were plated on day 0 at a density of 5 \times 10^4 cells/24-well plates. On day 1, cells were transfected with luciferase reporter plasmid, expression plasmids, and pCMVβ (Stratagene), a \( \beta \)-galactosidase reference gene, using Lipofectamine PLUS reagent (Invitrogen) as previously described (35, 36–37). The total amount of DNA in each transfection was adjusted to 0.2–0.7 \( \mu \)g/well. On day 2, the cells were harvested and assayed for firefly luciferase activity and normalized to \( \beta \)-galactosidase activity using kits from Promega and BD Biosciences, respectively.

siRNA Experiments—The duplexes of each small interfering RNA (siRNA), targeting SOX6 mRNA (target sequences of 5'-CGACCA-CACCAUCACCCAdTdT-3' and 5'-UGAGGAUGUUGUG-GUCGdTdT-3') and negative control (siCONTROL nontargeting siRNA) 2) were purchased from Dharmacon Inc. (Lafayette, CO). PDX1 siRNA (identification number 155849, target sequences of 5'-GGUCUGAGGCUUGCUUUADdTdT-3' and 5'-UAAAGCAGCAAGGCUA-GACCDdTdT-3') was purchased from Ambion (Austin, TX). The siRNAs were transfected by using Lipofectamine PLUS as described (35, 36, 38). Cells were harvested for RNA as well as for protein. SOX6 expression was confirmed by QRT-PCR and immunoblot analysis.

Insulin Secretion, Content, and Adenine Nucleotide Determinations and an Intracellular Ca\(^{2+}\) Assay—The secretory responses to glucose and other secretagogues were tested in MIN6 cells and INS-1E cells between passages 16–35 and 54–95, respectively (31, 39). Before the experiments, MIN6 or INS-1E cells were washed twice with phosphate-buffered saline and preincubated for 30 min at 37°C in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) of the following composition: 129 mM NaCl, 4.7 mM KCl, 5.0 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2.0 mM CaCl\(_2\), and 10 mM HEPES, pH 7.4. Bovine serum albumin (0.1%) was added as an insulin carrier. Next, cells were washed once with glucose-free KRBH and then incubated for 1 h in KRBH and stimulants as indicated. Incubation was stopped by putting the plates on ice, and the supernatants were collected for insulin secretion. Cellular insulin was extracted with acid–ethanol (0.4 M HCl in 74% ethanol) overnight at 4°C as described previously (22). Insulin secretion and content were determined by a rat insulin enzyme-linked immunosorbent assay kit (Shibayagi Co., Shibukawa, Japan). ATP and ADP content in MIN6 cells were determined using the ATP assay system (Toyobo-Net, Tokyo, Japan) as previously described (22, 40). To determine the intracellular Ca\(^{2+}\) levels, MIN6 cells were loaded with 2 \( \mu \)M fura-2/AM (Dingdoj) in KRBH containing 10 mM glucose at room temperature for 1 h as described (26). The loading solution was removed and then applied to a Functional Drug Screening System 6000 (Hamamatsu Photonics, Shizuoka, Japan). Intracellular Ca\(^{2+}\) concentration was measured by the ratio of emission fluorescence of 510 nm by excitation at 340 and 380 nm.

Chromatin Immunoprecipitation (ChIP) Assay—A commercially available assay kit (Upstate Biotechnologies, Charlottesville, VA) was used for ChIP studies according to the manufacturer’s protocol. Approximately 2 \times 10^6 MIN6 cells were cross-linked for 15 min at 37°C with formaldehyde (1% final concentration) in Dulbecco’s modified Eagle’s medium, subsequently washed twice with phosphate-buffered saline containing proteinase inhibitors. Cells were scraped, centrifuged, and resuspended in 0.5 ml of lysis buffer (50 mM Tris-HCl at pH 8.1, 10 mM EDTA, 1% SDS). The cells were then sonicated on ice 10 times using 30-s pulses using a Sonifier cell disrupter model Micro-150 (GENEQ Inc., Montreal, Canada), and then the debris was removed by centrifugation. Supernatants were collected and used for immunoprecipitation. To determine the nonspecific binding, samples were preincubated with protein A-Sepharose and sonicated salmon sperm DNA for 1 h at 4°C. After centrifugation, the supernatant was incubated with specific antibodies (anti-PDX1 (sc-14664), anti-SOX6 (sc-17332), anti-acetyl-histone H3 and H4), or control IgG overnight at 4°C followed by incubation with Protein A-Sepharose for 1 h. After centrifugation and washing, the immunocomplexes were eluted twice with 250 \( \mu \)l of elution buffer (1% SDS and 0.1 M NaHCO\(_3\)) for 15 min at room temperature. The cross-linking was then reversed by adding 20 \( \mu \)l of 5 M NaCl and 1 \( \mu \)l of 10 mg/ml RNase A and by incubating at 65°C for 6 h. After treating with 1.5 \( \mu \)l of proteinase K (10 \( \mu \)g/\( \mu \)l), the DNA was extracted with phenol/chloroform, subsequently ethanol-precipitated using 20 \( \mu \)g of glycogen carrier, and dissolved in 50 \( \mu \)l of distilled water. The amount of DNA recovered from immunoprecipitates with specific antibodies or control IgG was quantified by QRT-PCR, which was performed in triplicate. Data were represented as -fold change over DNA input. The primers used to amplify the insulin II promoter sequences were 5'-GGACATGTGAAA-CAGTCGAAGG-3' and 5'-CCCTGGATAATTTGCTTTG-3'.

GST Pull-down Assay—Glutathione S-transferase (GST) fusion constructs containing full-length PDX1 (amino acids 1–284) and full-length SOX6 (amino acids 1–827) were created in the bacterial expression vector pGEX-4T-2 (Amersham Biosciences), expressed in BL21 bacteria, and purified as previously described (21, 23). pcDNA3 constructs, a TnT quick coupled transcription/translation system (Promega), and \( ^{[35]} \)S-methionine (1000 Ci/mmol; Amersham Biosciences) were used for synthesizing \(^{35}\)S-labeled in vitro proteins. Purified GST or GST fusion proteins were incubated with \(^{35}\)S-labeled proteins for 2 h at 4°C and washed five times before SDS-PAGE was carried out.

Immunoblot Analysis—Aliquots of proteins were subjected to SDS-PAGE followed by immunoblot analysis using anti-SOX5, anti-SOX6 (ab-12054), anti-SOX9, and anti-SOX15 (1:1000 dilution); anti-PDX1 (sc-14664) (1:500 dilution); or anti-\( \beta \)-actin antibodies (1:5000 dilution).
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Immunoblots were visualized using the ECL-Plus® system (Amersham Biosciences) as previously described (36, 38, 41) and measured by LuminoVisionPRO (TAITEC, Osaka, Japan).

Statistical Analysis—For unpaired comparisons of two groups, Student’s t test was used. For multiple comparisons, one-way analysis of variance, followed by Tukey’s honestly significant difference test was used.

RESULTS

Identification of SOX6 as a Down-regulated Transcription Factor in Hyperinsulinemic Animals—In an attempt to uncover the mechanism underlying hyperinsulinemia and the β-cell adaptation phenotype in obesity-induced insulin resistance, we examined the gene expression profile in pancreatic islets of hyperinsulinenic obese mice that were fed an HFD. Using the Affymetrix mouse gene chip MOE430 and QRT-PCR of 59 transcription factors from the data base of the Beta Cell Biology Consortium (on the World Wide Web at www.cbil.upenn.edu/EPConDB/index.shtml), we identified 17 transcription factors whose expression levels were altered either more than 2 or less than 0.5 in response to HFD (supplemental Table 1) and subsequently examined their properties in insulinoma MIN6 cells (supplemental Fig. 1). Through these processes, we identified the unique SOX transcription factor, SOX6, as a specifically down-regulated gene in these HFD-fed mice, which also has the ability to modulate GSIS (supplemental Fig. 1). As shown in Fig. 1A, the islet expression of SOX6 mRNA was severalfold lower in both the HFD-induced obese mouse and genetically obese ob/ob mice than in normal mice. This result suggested that the levels of SOX6 mRNA might be negatively regulated by insulin or glucose. To test this hypothesis, we carried out fasting/refeeding experiments where insulin levels change to regulate blood glucose. As shown in Fig. 1B, the pancreatic levels of SOX6 RNA were essentially unchanged either by fasting or subsequent refeeding after 12 h of fasting, whereas expression of stearoyl-CoA desaturase 2, a well characterized insulin-regulated gene, was down- and up-regulated by fasting and refeeding, respectively. These results indicate that the expression of SOX6 may not be simply regulated by insulin or blood glucose levels but instead by the prolonged hyperglycemia, hyperinsulinemia, and/or insulin resistance generated in the course of developing obesity.

Next, we examined whether SOX6 protein was expressed in the pancreatic islet. Nuclear extracts were prepared from mouse pancreatic islets and insulinoma MIN6 cells and immunoblotted with an antibody raised against SOX6. A strong signal was detected at the predicted molecular weight in the nuclear extract from mouse islets and MIN6 cells (Fig. 1C). This signal was diminished by the application of siRNA for SOX6 (see Fig. 2D), indicating that this antibody specifically recog-
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The SOX gene family encodes a group of transcription factors defined by the conserved HMG DNA-binding domain and consists of more than 20 individual members (43). SOX proteins are classified into eight subgroups, A–H. SOX6 and its highly related proteins, SOX5 and SOX13, belong to group D, which is unique, because all three contain an additional specifically positioned leucine zipper motif. To evaluate the effects of these and other SOX proteins on GSIS, we selected SOX5, -9, -13, and -15 plus several other randomly selected SOX proteins and expressed each through retrovirus transduction in MIN6 cells. The mRNA expression of each SOX gene was confirmed by either QRT-PCR or immunoblotting or both. As shown in Fig. 2B, similar to SOX6, expression of the highly related SOX5 and SOX13 also attenuated GSIS, whereas expression of the other more distally related SOX proteins (SOX9 and SOX15) had minimal effects. The expression of SOX5, SOX9, and SOX15 was confirmed by immunoblotting (Fig. 2B, inset). In experiments not shown, we also determined that similar expression of six other more distantly related SOX proteins had minimal effects on GSIS. These studies demonstrate that SOX6 and its closest relatives all can potentially inhibit GSIS. However, unlike SOX6, the levels of the SOX5 and SOX13 transcripts are not down-regulated by the HFD or in ob/ob obese mice (data not shown), suggesting that SOX6 is the major SOX protein involved in modulating GSIS in obesity-induced pancreatic islets.

To complement the results obtained by retroviral expression, we next employed an RNA interference approach to knock down the expression of the endogenous SOX6 in mouse insulinoma MIN6 cells and rat insu-
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...incidence. Insulin secretion from transduced MIN6 cells in response to 10 mM [α-KIC]-KIC, tolbutamide, and KCl was significantly decreased (by 25–30%) in SOX6-expressing cells as compared with control ALP-transduced cells (Fig. 3E). Furthermore, glucose-induced intracellular Ca²⁺ ([Ca²⁺]i) transients (as determined by changes in the 340/380-nm fluorescence ratio), the eventual trigger for the exocytosis of insulin-containing vesicle (Fig. 3C), and total cellular insulin content (80% of ALP control cells) were significantly decreased in SOX6-expressing cells (Fig. 3D).

SOX6 Negatively Regulates Transcripts of Genes for Insulin as Well as ATP Production in Mitochondria—To further examine the molecular mechanism for the reduction in ATP generation and cellular insulin content, we examined gene expression in MIN6 cells transduced with SOX6. Consistent with reduced levels of ATP production, genes for the NADH dehydrogenase complex subunit (complex I), the cytochrome bc1 complex subunit (complex III), cytochrome c oxidase complex subunit (complex IV), and the ATP synthase subunit (complex V) were reduced by 40–60% compared with control ALP-expressing MIN6 cells (TABLE ONE). Importantly, consistent with the reduction of total cellular insulin content, the insulin I and II gene transcript levels in SOX6-expressing cells were markedly decreased, as quantified by QRT-PCR (to 60% of ALP control cells) (Fig. 3D).

SOX6 Acts as a Co-repressor for PDX1 on the Insulin Promoter—Based on the reduced insulin I and II gene mRNA in SOX6-overexpressing cells, we examined the effects of SOX6 expression on insulin gene promoter activity using the promoter region of the insulin II gene. A DNA fragment extending from nucleotide -872 to -176 of the rat insulin II gene was subcloned into the promoterless luciferase reporter gene, pGL3-basic, to create the promoter reporter construct pINS(-872)-luc. This promoter reporter construct was transiently transfected into MIN6 cells along with increasing amounts of pCMV-....

SOX6. As shown in Fig. 4A, the normalized luciferase activities in MIN6 cells were decreased in proportion to the amounts of co-transfected pCMV-SOX6.
There are three potential SOX elements in the rat insulin II gene (Fig. 4B). To analyze whether SOX6-mediated suppression of the insulin promoter is mediated by the direct binding of these SOX-like elements by SOX6, we constructed a series of deletion mutants, each lacking one of the three SOX-like elements (Fig. 4B). Co-transfection of these mutants with SOX6 revealed that deletion of any of these SOX elements did not affect the suppressive effects of SOX6. These results suggest that the suppression of the insulin promoter by SOX6 is either dependent on SOX6 binding to nonconsensus sites or independent of SOX6 binding to DNA. Consistent with previously reported observations (45, 46), deletion of nucleotides −413 to −370 of the insulin II promoter abolished all of the promoter activity (Fig. 4B).

We next examined the effects of SOX6 on the insulin II promoter activation by either PDX1, E47 (an E2A gene product), or both. As shown in Fig. 4C and consistent with a previous report (17), PDX1 together with E47 synergistically activated the insulin II promoter, and increasing amounts of the SOX6 expression plasmid resulted in a dose-dependent inhibition. Fig. 4D shows that SOX6 had almost no effect on the E47-mediated transactivation of a highly E47-responsive IgH enhancer reporter, p(µE5 + µE2 + µE3)-Luc, suggesting that SOX6 does not specifically repress E47 function.

To evaluate whether SOX6 might inhibit activation mediated by PDX1, we fused the coding sequence of PDX1 and E47 to the GAL4 DNA-binding domain, and the transactivation potential of the resulting GAL4 fusions was examined in the absence and presence of SOX6. As shown in Fig. 4E, SOX6 strongly suppressed GAL4-PDX1 transactivation, whereas it had almost no effects on GAL4-E47.

These results suggest that SOX6 and PDX1 proteins may directly interact together to inhibit the insulin II promoter. To evaluate this possibility, we carried out GST pull-down assays using a GST-SOX6 fusion protein and consistent with a previous report (17), PDX1 and E47 showed that SOX6 had almost no effect on GAL4-E47.

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These results suggest that SOX6 and PDX1 proteins may directly interact together to inhibit the insulin II promoter. To evaluate this possibility, we carried out GST pull-down assays using a GST-SOX6 fusion protein and in vitro-translated full-length PDX1, and to map the interaction domain we also evaluated a series of deletion mutants. As shown in Fig. 5A, i and ii, the full-length protein and the mutant lacking the COOH terminus (residues 206–284) were co-precipitated with GST-SOX6, whereas the mutant lacking the homeodomain and the COOH terminus had weak binding to GST-SOX6 (Fig. 5A, iii), and deletion of the NH2-terminal 144 amino acids abolished the binding to GST-SOX6 (Fig. 5A, iv). These data indicate...

**TABLE ONE**

The relative amounts of mRNAs for mitochondrial oxidative phosphorylation complexes in SOX6-transduced MIN6 cells

| Gene | Accession no. | SOX6/ALP Expt. 1 | SOX6/ALP Expt. 2 |
|------|---------------|------------------|------------------|
| Complex I of mitochondrial OXPHOS | | | |
| NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 | NM_025523 | 0.45 ± 0.014 | 0.62 ± 0.015 |
| NADH dehydrogenase (ubiquinone) 1 α subcomplex, 3 | NM_025348 | 0.64 ± 0.007 | 0.59 ± 0.023 |
| NADH dehydrogenase (ubiquinone) 1 β subcomplex, 1, 7 kDa | AW_060701 | 0.62 ± 0.014 | 0.63 ± 0.014 |
| NADH dehydrogenase (ubiquinone) 1 β subcomplex, 2 | NM_026612 | 0.64 ± 0.030 | 0.67 ± 0.010 |
| NADH dehydrogenase (ubiquinone) 1 β subcomplex 4 | NM_026610 | 0.59 ± 0.007 | 0.63 ± 0.010 |
| NADH dehydrogenase (ubiquinone) 1 β subcomplex 6, 17 kDa | XM_131359 | 0.62 ± 0.013 | 0.52 ± 0.028 |
| NADH dehydrogenase (ubiquinone) Fe-S protein 2 | NM_153064 | 0.57 ± 0.013 | 0.44 ± 0.014 |
| NADH dehydrogenase (ubiquinone) Fe-S protein 7 | NM_029272 | 0.50 ± 0.006 | 0.48 ± 0.011 |
| NADH dehydrogenase (ubiquinone) Fe-S protein 8 | NM_144870 | 0.59 ± 0.014 | 0.53 ± 0.010 |
| Complex III of mitochondrial OXPHOS | | | |
| Cytochrome c | NM_025567 | 0.46 ± 0.007 | 0.46 ± 0.022 |
| Ubiquinol-cytochrome c reductase (6.4 kDa) subunit | NM_025650 | 0.62 ± 0.024 | 0.64 ± 0.013 |
| Complex IV of mitochondrial OXPHOS | | | |
| Cytochrome c oxidase, subunit Vb | NM_009942 | 0.66 ± 0.004 | 0.66 ± 0.023 |
| Cytochrome c oxidase, subunit Vb | NM_025628 | 0.55 ± 0.005 | 0.64 ± 0.013 |
| Complex V of mitochondrial OXPHOS | | | |
| ATP synthase, H+ -transporting, mitochondrial F0 complex, subunit c (subunit 9), isofrom 1 | NM_007506 | 0.66 ± 0.011 | 0.49 ± 0.008 |
| ATP synthase, H+ -transporting, mitochondrial F0 complex, subunit f, isofrom 2 | NM_020582 | 0.44 ± 0.008 | 0.65 ± 0.010 |
| ATP synthase, H+ -transporting, mitochondrial F1 complex, ε subunit, isofrom 1 | NM_025983 | 0.66 ± 0.013 | 0.47 ± 0.009 |
| ATP synthase, H+ -transporting, mitochondrial F1 complex, O subunit | NM_138597 | 0.45 ± 0.004 | 0.43 ± 0.015 |
| ATP synthase, H+ -transporting, mitochondrial F1F0 complex, subunit ε | NM_007507 | 0.61 ± 0.007 | 0.53 ± 0.014 |
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that the NH₂-terminal 144 amino acids of PDX1 were critical for the interaction with SOX6.

As a complement to the GST-SOX6 pull-down experiment, we constructed a GST-PDX1 fusion protein and evaluated it for interaction with full-length SOX6 or a series of deletion mutants of SOX6. As shown in Fig. 5B, it is clear that the minimum PDX1 binding site of SOX6 is a region containing the functionally important HMG box of SOX6. Together with the GST-SOX6 pull-down assay findings, these data indicate that the HMG box of SOX6 interacts physically with the NH₂-terminal 144 amino acids of PDX1, as schematically depicted in Fig. 5C.

In Fig. 5D, top, we show that elimination of the HMG domain from SOX6 resulted in a protein that was unable to attenuate GSIS. The protein expression of mutant SOX6 lacking the HMG domain (ΔHMG-SOX6) was confirmed by immunoblotting with an anti-SOX6 antibody (Fig. 5D, bottom). Furthermore, the deletion of the HMG domain of SOX6 abolished the SOX6 suppressive effect on the insulin II gene promoter (Fig. 5E), whereas expression of the NH₂ terminus (residues 1–144) of PDX1 reversed the SOX6-mediated inhibition (Fig. 5F). Taken together with the GST interaction studies, these data demonstrate that the SOX6 HMG domain suppresses the insulin II gene promoter by interacting physically with the NH₂ terminus of PDX1, the key transactivator protein for positive regulation of insulin gene transcription.

siRNA-mediated Knockdown of PDX1 Decreased SOX6 Occupancy of the Insulin II Promoter—To further determine the mechanism underlying insulin II promoter inhibition by SOX6, we examined the association of PDX1 and SOX6 with the insulin II promoter in SOX6 or control ALP retrovirus and PDX1-specific siRNA-treated MIN6 cells by ChIP assays. We used QRT-PCR to evaluate the results of the ChIP, and the specific primers were designed as schematically depicted in Fig. 6.

When SOX6 was expressed by retroviral transduction, the insulin promoter sequence was present at a higher level in the SOX6 immunoprecipitate (Fig. 6A, top, compare lanes 2 and 4), suggesting an increased association of SOX6 with the promoter under these conditions. In contrast, SOX6 expression did not alter the DNA binding of PDX1 to the insulin II promoter (Fig. 6A, top, compare lanes 6 and 8), indicating that SOX6 does not simply interfere with the DNA binding of PDX1. The immunoblot in the bottom panel shows that SOX6 protein levels were elevated (6.5-fold, as evaluated by LumiVisionPRO) in the SOX6 retroviral transduced cells (Pre) and that SOX6 was efficiently collected by the immunoprecipitation procedure with anti-SOX6 antibody (Post) in proportion to the overall levels (Fig. 6A, bottom; compare the gels labeled Pre for direct immunoblot with those labeled Post).

It has been previously demonstrated that PDX1 specifically binds the proximal insulin promoter of MIN6 cells by a ChIP assay (47). 48 h following PDX1-siRNA treatment, we observed an 80% reduction in PDX1 occupancy at the insulin II promoter in MIN6 cells (Fig. 6B, top, compare lanes 2 and 4). Interestingly, a decrease in PDX1 at the insulin II promoter in MIN6 cells was also accompanied by a 60% reduction in the binding of SOX6 to the proximal insulin promoter (Fig. 6B, top, compare lanes 6 and 8). Taken together with the GST pull-down assays,
these results indicate that endogenous SOX6 protein in eukaryotic cells interacts with DNA-bound PDX1 at the insulin promoter in vivo.

**SOX6 Decreased Acetylation of Chromatin-associated Histones H3 and H4**—PDX1 transcriptional activity is reportedly regulated by the recruitment of p300, a coactivator protein that possesses intrinsic histone acetylase activity (20, 48), and similar to SOX6, p300 interacts directly with the NH2-terminal region of PDX1. We therefore evaluated the effects of SOX6 on the acetylation levels of histones H3 and H4 in chromatin at the insulin II promoter, using antibodies directed at the acetylated forms of either histone H3 or H4. Consistent with previous papers (20, 48), cultivation of MIN6 cells with a high glucose concentration (30 mM) leads to a significant increase in acetylation of histone H4 at the insulin II promoter (Fig. 6D, compare lanes 2 and 4), whereas there was no effect of glucose on the degree of H3 acetylation (Fig. 6C, compare lanes 2 and 4). In cells where SOX6 was expressed by retroviral transduction, the levels of both acetylated histones H3 and H4 at the insulin II promoter were profoundly reduced (Fig. 6, C, compare lanes 2 and 6, lanes 4 and 8, and D, compare lanes 4 and 8), indicating that SOX6 leads to a reduction in acetylation of both H3 and H4 in the chromatin on the insulin II promoter. Because p300 has histone acetylase activity, this suggests that SOX6 may interfere with the recruitment of p300 to PDX1 bound at the insulin II promoter.
DISCUSSION

In the current study, we demonstrated that SOX6 protein is expressed in adult pancreatic islets, where its expression is dramatically down-regulated both by HFD feeding and in genetically predisposed obese model (ob/ob) mice. In both cases, the mice are hyperinsulinemic with insulin resistance, and they have an increase in pancreatic islet mass. In contrast, SOX6 mRNA levels were not altered by fasting and refeeding experiments, suggesting that pancreatic expression of SOX6 is not regulated by acute changes of blood glucose or insulin levels. Based on the attenuation of GSIS by SOX6 and its down-regulation in hyperinsulinemic mice, we propose that changes in the ratio of SOX6 to PDX1 may be responsible for the compensatory islet hyperplasia that occurs in response to insulin resistance.

The SOX family of transcription factors contains a DNA binding HMG box, which is highly conserved across species. SOX proteins are involved in a number of developmental processes, including tissue spec-
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The decrease in insulin secretion by elevated SOX6 is likely to be mediated in part by the decreased insulin gene expression. We also showed that SOX6 overexpression resulted in a decrease in the ratio of ATP/ADP that was accompanied by a significant decrease in expression for several genes of oxidative metabolism (TABLE ONE). Because a decreased ATP/ADP ratio is also associated with decreased GSIS, our results are additionally consistent with a model where SOX6 represses GSIS through decreasing expression of genes involved in ATP production. Whether these genes are direct targets of PDX1 or whether SOX6 may decrease gene expression through other mechanisms requires further study. Interestingly, however, expression of a dominant negative form of PDX1 through adenovirus transduction in vitro reduced the expression of mitochondrial genes and also resulted in severe consequences in β-cell mitochondrial function (54).

Although SOX genes of the same group tend to be co-expressed at the same developmental stage and exhibit functional redundancy in development, it is intuitive that redundancy may not extend to key transcriptional regulatory decisions in adult tissues. SOX5, -6, and -13 are all detected in adult islets (49) (present work). SOX13 has been reported to be expressed in β-cells and functions as autotigent in type 1 diabetes (42). Our data show that they are all capable of suppressing GSIS when overexpressed; however, only SOX6 gene expression was reduced by the HFD or in ob/ob mice. Since all three are expressed in islets, it is not immediately clear why down-regulation of SOX6 alone by the HFD could result in an increase in GSIS. It is possible that the levels of endogenous SOX5 and SOX13 proteins are relatively low, so a decrease in SOX6 alone would be sufficient to initiate a response, or additional proteins that are also aberrantly expressed by the HFD augment the SOX6 effect specifically.

Experiments are in progress to address these and related issues.

It is conceivable that SOX genes in adult tissues are regulated in response to the circulating nutrients or hormones, such as glucose, insulin, or other nutrients. Thus, SOX6 may contribute to the insulin gene regulation in pathophysiological states where PDX1 function is compromised as observed in insulin resistance and diabetes. Therefore, the modulation of PDX1 function by SOX6 may provide a promising new therapeutic target for treatment of type II diabetes.

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