SOX6 Suppresses Cyclin D1 Promoter Activity by Interacting with β-Catenin and Histone Deacetylase 1, and Its Down-regulation Induces Pancreatic β-Cell Proliferation

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Sex-determining region Y-box (SOX) 6 negatively regulates glucose-stimulated insulin secretion from β-cells and is a down-regulated transcription factor in the pancreatic islet cells of hyperinsulinemic obese mice. To determine the contribution of SOX6 to insulin resistance, we analyzed the effects of SOX6 on cell proliferation. Small interfering RNA-mediated attenuation of SOX6 expression stimulated the proliferation of insulinoma INS-1E and NIH-3T3 cells, whereas retroviral overexpression of SOX6 to insulin resistance, we analyzed the effects of SOX6 on hyperinsulinemic obese mice. To determine the contribution of SOX6 to insulin resistance, we analyzed the effects of SOX6 on cell proliferation. Small interfering RNA-mediated attenuation of SOX6 expression stimulated the proliferation of insulinoma INS-1E and NIH-3T3 cells, whereas retroviral overexpression of SOX6 to insulin resistance, we analyzed the effects of SOX6 expression on cell proliferation. Small interfering RNA-mediated attenuation of SOX6 expression resulted in inhibition of cell growth. Quantitative real-time PCR analysis revealed that the levels of cyclin D1 transcripts were markedly decreased by SOX6 overexpression. Luciferase-reporter assay with β-catenin showed that SOX6 suppresses cyclin D1 promoter activities. In vitro binding experiments showed that the LZ/Q domain of SOX6 physically interacts with armadillo repeats 1–4 of β-catenin. Furthermore, chromatin immunoprecipitation assay revealed that increased SOX6 expression significantly reduced the levels of acetylated histones H3 and H4 at the cyclin D1 promoter. By using a histone deacetylase (HDAC) inhibitor and co-immunoprecipitation analysis, we showed that SOX6 suppressed cyclin D1 activities by interacting with β-catenin and HDAC1. The data presented suggest that SOX6 may be an important factor in obesity-related insulin resistance.

In obesity-related insulin resistance, pancreatic islets compensate for chronic insulin insensitivity by expanding β-cell mass and increasing insulin secretory capacity (1, 2). In a previous study, we identified the sex-determining region Y-box (SOX) 6 as a down-regulated transcription factor in obesity-related insulin-resistant animals. We also showed that SOX6 directly binds with pancreatic-duodenal homeobox factor 1 (PDX1) and negatively regulates glucose-stimulated insulin secretion (3). Based on the role of PDX1 in the development and proliferation of β-cells, insulin/insulin-like growth factor signaling pathways, and the onset of type 2 diabetes (4), we suggested that the attenuated expression of SOX6 may contribute to β-cell adaptation in obesity-related insulin resistance.

SOX6 is a member of the SOX family of transcription factors containing a DNA-binding high mobility group (HMG) domain that is highly conserved among species (5). SOX6 and closely related SOX5 are co-expressed during mouse chondrogenesis (6). Mice lacking both SOX6 and SOX5 exhibit impaired chondrogenesis and die in utero (7). In adult mice, relatively high levels of SOX6 protein are present in β-cells. The levels of SOX6 expression in the pancreatic islets are not regulated by fasting and subsequent refeeding but are reduced by feeding a high fat diet and by defects in the leptin gene. In addition to the suppression of PDX1, SOX6 also negatively regulates genes involved in ATP production in insulinoma MIN6 cells; these include NADH dehydrogenase complex subunit (complex I), the cytochrome c1 complex subunit (complex III), cytochrome c oxidase complex subunit (complex IV), and the ATP synthase subunit (complex V). Consistent with the suppression of genes involved in ATP production, overexpression of SOX6 in MIN6 cells suppresses the induction of ATP content and the ATP/ADP ratio by high glucose (3).

To further define the role of SOX6 in obesity-related insulin resistance, we analyzed the effects of SOX6 expression on cell proliferation. siRNA-mediated knockdown of SOX6 significantly stimulated cell proliferation, whereas induced SOX6 expression resulted in the inhibition of cell growth. In this paper, we demonstrate the mechanism by which SOX6 sup-

The abbreviations used are: SOX, sex-determining region Y-box; BrdUrd, bromodeoxyuridine; ChIP, chromatin immunoprecipitation; DME, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; GST, glutathione S-transferase; HDAC, histone deacetylase; HMG, high mobility group; Lef, lymphoid-enhancer factor; LZ/Q, leucine zipper and poly(Q); PDX1, pancreatic-duodenal homeobox factor 1; QRT, quantitative real time; siRNA, small interfering RNA; TCF, T-cell factor; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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presses cell proliferation. Our current studies reveal that SOX6 binds with β-catenin and recruits histone deacetylase 1 for suppression of cyclin D1 promoter activities induced by β-catenin. Together with the stimulation of glucose-stimulated insulin secretion, the induced cell proliferation by attenuation of SOX6 expression may account for the hyperinsulinemia and hyperplasia characteristic of insulin resistance.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit polyclonal anti-β-catenin (H-102) (sc-7199) and anti-HDAC1 (H51) (sc-7872) antibodies and peroxidase-conjugated affinity-purified donkey anti-rabbit and anti-mouse IgGs were obtained from Santa Cruz Biotechnology; monoclonal anti-FLAG M2 (F3165) was from Sigma; monoclonal anti-RGS-His (34610) was from Qiagen; a rabbit polyclonal anti-SOX6 (Ab-12054) and monoclonal anti-FLAG M2 (F3165) antibody was from Sigma; monoclonal IgGs were obtained from Santa Cruz Biotechnology; monoclonal anti-nucleoporin (610497) was from BD Transduction Laboratories; control rabbit IgG (I-1000) was from Vector Laboratories. MG132 (ZW8440) and Scriptaid (GR-326) were purchased from Biomol. β-Galactosidase staining kit was purchased from Mirus (Madison, WI). Other reagents were obtained from sources as described previously (3, 8–10).

Cell Culture—INS-1E cells, a clone of parental rat β-cell line INS-1 cells (a gift from Dr. Pierre Maechler), were cultured in RPMI 1640 containing 11.6 mM glucose, 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 5% fetal bovine serum at 37 °C in 5% CO2. NIH-3T3 cells, a mouse embryonic fibroblast cell line, were obtained from the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan), and 293 cells, a human embryonic kidney cell line (HEK293, CRL-1573), were obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO2. MIN6 cells, a mouse insulinoma cell line (a kind gift from Dr. Jun-Ichi Miyazaki), were cultured in DMEM containing 25 mM glucose, 5.5 μM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, supplemented with 15% fetal bovine serum at 37 °C in 5% CO2. NIH-3T3 cells, a mouse embryonic fibroblast cell line, were obtained from the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan), and 293 cells, a human embryonic kidney cell line (HEK293, CRL-1573), were obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO2. MIN6 cells, a mouse insulinoma cell line (a kind gift from Dr. Jun-Ichi Miyazaki), were cultured in DMEM containing 25 mM glucose, 5.5 μM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, supplemented with 15% fetal bovine serum at 37 °C in 5% CO2.

Small Interfering RNA—The duplexes of each small interfering RNA (siRNA), targeting SOX6 mRNA and negative control (nonsilencing siRNA) were purchased from Qiagen (Valencia, CA) and used as described (3). For cell growth curve analysis, INS-1E, NIH-3T3, MIN6, and HIT-T15 cells were cultured at a density of 5 × 10^5 cells/flask in T-25 flasks. On the following day, siRNA transfections to INS-1E and NIH-3T3 cells were accomplished with an HVJ envelope vector kit GenomONE (Ishihara Sangyo Kaisha) and those to MIN6 and HIT-T15 cells were carried out with Lipofectamine PLUS reagent. These cells were split and plated into 6-well plates at the densities described in the figure legends, and cell numbers were counted.

FIGURE 1. Effect of SOX6 expression on cell growth of INS-1E and NIH-3T3 cells. A and B, promotion of cell growth by depletion of cellular SOX6 through application of siRNA. One day prior to the cell growth assay, INS-1E (A) and NIH-3T3 cells (B) were transfected with either SOX6-specific siRNA (si-SOX6) or control siRNA (si-cont) as described under “Experimental Procedures.” After incubation for 24 h, INS-1E and NIH-3T3 cells were trypsinized and plated at densities of 1.8 × 10^5 and 0.6 × 10^5, respectively, into 6-well plates (day 0). Cell numbers were counted every 24 h (top panel). The cells on day 2 were harvested for cell extracts, and aliquots of protein were subjected to SDS-PAGE and immunoblotted with either anti-SOX6, anti-nucleoporin, or anti-β-actin antibody (bottom panel). C–F, inhibition of cell growth by constitutive overexpression of SOX6. INS-1E (C) and NIH-3T3 cells (D) were stably transduced with retroviruses encoding either SOX6 or GFP, and the cell proliferation assay was performed as described under “Experimental Procedures.” BrdUrd uptake assay as an index of DNA synthesis (C) and fluorescence-activated cell sorting analysis (F) were performed with NIH-3T3 cells overexpressing SOX6 in comparison with those overexpressing GFP, as described under “Experimental Procedures.” Results are expressed as the means ± S.D. of five independent experiments. *, p < 0.01 compared with control. At some points, the error bars are too small to show (A–D). NIH-3T3 cells overexpressing SOX6 had very similar morphologies to those overexpressing GFP (F, top panel) but had a markedly reduced number of cells in G2 (F, bottom panel).
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FIGURE 2. Expression of genes involved in the cell cycle in SOX6-transduced NIH-3T3 cells. On day 0, NIH-3T3 cells were prepared for experiments and infected with retrovirus encoding either GFP or SOX6, as described under "Experimental Procedures." At 12 h after serum addition, cells were harvested for isolation of total RNA and used for reverse transcription and QRT-PCR. Mouse cyclophilin mRNA was used as the invariant control. The mRNA values are depicted relative to mRNA in GFP-transduced NIH-3T3 cells, which are arbitrarily defined as 1. Each bar represents mean ± S.E. of triplicate experiments. *, p < 0.01 compared with control.

every 24 h for 3 or 4 days. SOX6 expression was determined by immunoblot. To deplete cellular human β-catenin, we obtained validated Stealth™ RNAi DuoPak (12938-123) (Invitrogen). As a control, we used Stealth™ RNAi Negative Control Med GC (12935-300). For transfection, 293 cells were cultured overnight at density of 5 × 10^5 cells/flask in T-25 flasks, and 200 pmol of siRNAs were transfected using Lipofectamine 2000 reagent. After 48 h, the cells were harvested, and the cell extracts were used for chromatin immunoprecipitation assay.

Retroviral Transduction, Cell Proliferation, BrdUrd Incorporation, and Cell Cycle Assays—INS-1E, MIN6, and NIH-3T3 cells were transduced with retroviruses that expressed either SOX6 or green fluorescent protein (GFP) as described previously (11, 12). One day later, transduced cells were selected with puromycin (5 µg/ml). The cells were selected for 6 more days before cell cycle analysis by FACS Calibur. The puromycin-selected cells were immediately subjected to growth curve and cell cycle analysis.

For growth curve analysis, the same batch of virally transduced cells was divided, and 0.3 × 10^5 cells were plated into 6-well plates. Cell numbers were counted every 24 h for 4 days. Mixed clones were used for these experiments to minimize possible effects caused by the sites of integration of the foreign genes. The proliferative activity of virally transduced cells was detected by BrdUrd incorporation, using a BrdUrd labeling kit (Roche Applied Science).

For cell cycle analysis, cells were arrested at G1 phase by changing cultured medium to DMEM containing 0.1% fetal bovine serum and subsequent incubation for 30 h. Cells were harvested and resuspended with 0.2% Triton X-100 in phosphate-buffered saline solution containing 0.1 mg/ml RNase A and 25 µg/ml propidium iodide (Sigma). The stained cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences) using CellQuest software, and the percentages of cells in the G1, S, and G2/M phases of the cell cycle were determined using ModFit LT software (Verity Software House, Topsham, ME).

Luciferase Reporter Assay—NIH-3T3 or 293 cells were cultured at a density of 1.0 × 10^5 cells/well in 24-well tissue culture plates overnight, and transfections were performed using FuGENE6™ (Roche Applied Science). The luciferase reporter plasmid, expression plasmids, and either β-galactosidase or Renilla luciferase reference gene plasmid, as internal control, were transfected in each well. The total amount of DNA was normalized by adding empty vector. After 20 h, the cells were harvested for firefly luciferase activities and normalized to either β-galactosidase activity or Renilla luciferase activity as described previously (3, 13). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

Quantitative Real Time-PCR (QRT-PCR)—The method for QRT-PCR has been described (14, 15).

Plasmids—Mammalian expression vectors for full-length and deletion mutant SOX6 that encode amino acids 1–617, 181–827, 263–827, 181–262, 617–827, 617–696, and an internal deletion (Δ181–262) were constructed in pcDNA3 (Invitrogen) as described previously (3). Mammalian expression plasmid for human TCF4 (pcDNA/myc-mTFC4) was a kind gift of Dr. Bert Vogelstein (The Johns Hopkins Oncology Center). Luciferase reporter plasmids of mouse cyclin D1 promoter and Tcf/LEF were generously provided by Dr. Johan Auwerx (CNRS/INSERM/Université Louis Pasteur).

The following plasmids were constructed by standard methods (16). The mammalian expression vector pCMV-β-catenin, which encodes full-length mouse β-catenin (amino acids 1–781) under the control of the cytomegalovirus promoter, was constructed by reverse transcription-PCR using total RNA from mouse liver as a template and by insertion of the full-length coding sequence into pcDNA3. pCMV-β-catenin(S33Y), which encodes a mutant β-catenin where serine 33 is mutated to tyrosine, was generated with a QuickChange™ site-directed mutagenesis kit (Stratagene). To create the deletion mutants encoding amino acids 1–468, 1–134, 135–300, and deletion mutant SOX6 that encode amino acids 1–617, 1–781, 1–135–387) of β-catenin, corresponding sequences were amplified by PCR with suitable restriction endonucleases and ligated into pcDNA3. pCMV-β-catenin-His, which encodes full-length mouse β-catenin followed by three tandem copies of the c-Myc epitope and six histidine residues, was constructed with pcDNA3.1/myc-His (Invitrogen) as the parent vector. pCMV-β-catenin(S33Y)-His is identical to pCMV-β-catenin-His except that serine 33 is changed to tyrosine. pCMV-FLAG-SOX6 and pCMV-FLAG-SOX9, which encode full-length mouse SOX6 (amino acids 1–827) and full-length human SOX9 (amino acids 1–509), respectively, containing FLAG-epitope at the N terminus, were constructed with pCMV-Tag2 (Stratagene) as the parent vector. pBIND-β-catenin, which encodes GAL4-β-catenin fusion protein, was constructed by inserting the full length of mouse β-catenin cDNA fragment into a poly-linker site of pBIND (Clontech), which contains the DNA binding domain of yeast GAL4 protein. For in vitro transcription and translation, full-length and
deletion sequences of HDAC1 (amino acids 1–482, 1–139, and 140–482) were generated by PCR amplification using pCIneo mouse HDAC1-myc (a kind gift from Dr. Christian Seiser, University of Vienna) as a template and inserted into pCR2.1 (Invitrogen). All the clones were confirmed by DNA sequencing.

**In Vitro Binding Assay**—This assay was carried out as described previously using the MagneGST™ pulldown system (Promega) (3). pcDNA3- and pCR2.1-based constructs, a TNT quick-coupled transcription/translation system (Promega), and L-[³⁵S]methionine (1000 Ci/mmol; Amersham Biosciences) were used for synthesizing [³⁵S]-labeled in vitro proteins. GST or GST fusion vectors for full-length mouse SOX6 (amino acids 1–827), mouse β-catenin (amino acids 1–781), and mouse HDAC1 (amino acids 1–482) were created in bacterial expression vector pGEX-4T-2 (Amersham Biosciences) and expressed in *Escherichia coli* BL21 (DE3) pLysS (Novagen).

For in vitro binding competition assay, the cDNAs encoding N-terminal histidine-tagged SOX6 and SOX9 fusion proteins were created in pET15b (Takara, Kyoto, Japan), and the proteins were expressed in *E. coli*, purified by Ni²⁺/H₁₁₀₀₁-nitrilotriacetic acid column (Qiagen), and used for the assays. The purity and size of the proteins were confirmed by SDS-PAGE.

**Immunoprecipitation**—For immunoprecipitation, cells were lysed in buffer A containing 20 mM HEPES, pH 7.9, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1/₁₁₀₀₀ g/ml aprotinin, followed by passing through a 22.5-gauge needle 15 times to shear cellular DNA as described previously (13). After the cell extracts were clarified by centrifugation at top speed in a microcentrifuge, the supernatant was subjected to immunoprecipitation as described (13), using the indicated antibodies described in the figure legends.

**Immunoblot Analysis**—SDS-8% polyacrylamide gels were calibrated with molecular weight markers (Bio-Rad), and polyclonal anti-SOX6, monoclonal anti-β-actin, monoclonal anti-nucleoporin, polyclonal anti-β-catenin, monoclonal anti-RGS His, monoclonal anti-FLAG, and polyclonal anti-HDAC1 antibodies (0.2/₁₁₀₀₀ g/ml) were used as primary antibodies. Two secondary antibodies (horseradish peroxidase-conjugated, affinity-purified donkey anti-mouse IgG, and anti-rabbit IgG) were each used at a dilution of 1:3,000. Bound antibodies were visualized by chemiluminescence using the ECL-Plus™ system (Amersham Biosciences), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film Inc.).

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP was performed using a kit from Upstate (Charlottesville, VA) as under “Experimental Procedures.” B, 293 cells were transfected with 0.1 μg of cyclin D1 promoter luciferase reporter plasmid and 0.01 μg of pRL-TK together with the indicated amounts of pCMV-SOX6 and pCMV-β-catenin (S33Y) as described previously (13). After the cell extracts were clarified by centrifugation at top speed in a microcentrifuge, the supernatant was subjected to immunoprecipitation as described (13), using the indicated antibodies described in the figure legends.
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A. GST-SOX6 Pulldown

| GST-fused SOX6 | 1 181 262 617 696 827 |
|----------------|-------------------------|
| GST           | LZ/Q HMG                |

in vitro translated β-catenin
1 132 300 468 781

Armadillo repeats 1-12
1 148-583-801

LZ/Q
1 181 262 617 696 827

B. GST-β-catenin Pulldown

| GST-fused β-catenin | 1 132 300 468 781 |
|---------------------|-------------------|
| GST                 | LZ/Q HMG          |

in vitro translated SOX6
1 181 262 617 696 827

Armadillo repeats 1-12
1 132 300 468 781

C. Luciferase Reporter Assay

| Normalized luciferase activity (Fold/Remits) | 1 2 3 4 5 6 |
|---------------------------------------------|-------------|

D. Immunoprecipitation

| Flag-SOX6 | Blot:Anti-Flag |
|-----------|---------------|
| IP        | IP pellet     |

| B-catenin | IP pellet     |

FIGURE 4. Functional interaction between Armadillo repeats 1–4 of β-catenin and leucine zipper and poly(Q) domains within SOX6. A and B, in vitro interaction of β-catenin and SOX6. GST-fused full-length SOX6 (A) or GST-fused full-length β-catenin protein (B) immobilized to glutathione beads were incubated with in vitro translated 35S-labeled β-catenin and its deletion mutants (A) or in vitro translated 35S-labeled SOX6 and its deletion mutants (B), respectively, at room temperature for 1 h. Purified GST was used as a negative control for nonspecific binding. After washing extensively with protein G beads, and 5% of the input proteins were resolved on SDS-PAGE and visualized by a FUJIX BAS2000 imaging system (Fuji Film, Tokyo, Japan).

Effects of SOX6 Expression on Cell Proliferation—In a previous study, we showed that SOX6 expression is down-regulated in hyperinsulinemic obese mice and that this attenuation led to increased insulin secretion from cultured β-cells (3). These data suggested that the attenuation of SOX6 may account for hyperinsulinemia in obese animals and type 2 diabetes. To further evaluate the role of SOX6 in insulin resistance and type 2 diabetes, we analyzed the effects of SOX6 expression on cell proliferation. As shown in Fig. 1, A and B, siRNA-mediated knockdown of SOX6 increased cell proliferation of insulinoma INS-1E and NIH-3T3 cells. Furthermore, in MIN6 and HIT-T15 cells, which are derived from β-cells, the application of siRNA specific for SOX6 stimulated cell growth (supplemental Fig. 1, A and B), suggesting that SOX6 attenuates cell proliferation of β-cells. In a complementary manner to the results of siRNA experiments, retroviral overexpression of SOX6 inhibited cell proliferation of INS-1E and NIH-3T3 cells (Fig. 1, C and D). Similarly, cell growth of MIN6 cells was suppressed by forced expression of SOX6 (supplemental Fig. 2). The expression level of SOX6 induced by retroviral transduction was 6–7-fold higher than endogenous SOX6 in the cells (data not shown). Consistent with the inhibition of cell proliferation, DNA synthesis, as determined by BrdUrd incorporation, was also significantly reduced in NIH-3T3 cells retrovirally transfected with SOX6 compared with those transduced with control virus encoding GFP (Fig. 1E). Although retroviral transfection of SOX6 inhibited cell proliferation and DNA synthesis, it did not induce morphological changes of NIH-3T3 cells (Fig. 1F, top panel). Quantitative analysis of cell cycle distribution showed that the percentage of G2 phase cells was largely reduced when SOX6 was retrovirally transfected into NIH-3T3 cells (Fig. 1F, bottom panel).

SOX6-mediated Suppression of Cyclin D1 Promoter Activity—Quantitative RT-PCR analysis of representative cell cycle-regulating proteins revealed that the levels of cyclin D1 transcripts were down-regulated in hyperinsulinemia, as described previously (3). Briefly, DNA was cross-linked to protein with formaldehyde. Cellular lysate was obtained by scraping, followed by pulse ultrasonication to shear cellular DNA. Immunoprecipitations were performed with the following antibodies (1.0 μg): anti-acetylated histone H3, anti-acetylated histone H4, anti-β-catenin, and anti-FLAG for epitope-tagged SOX6. Subsequently, cross-links were reversed, and bound DNA was purified by phenol:chloroform extraction. QRT-PCR was performed using the primers specific for human cyclin D1 promoter as follows: 5′-CCGACTGGTCAGGTAGGAA-3′ and 5′-CCAGGGGTATAACCTAAA-3′ corresponding to nucleotide positions −996 to −977 and −836 to −817 with respect to the first ATG codon of cyclin D1. QRT-PCR data are presented as a percentage of input DNA recovered.

RESULTS

Effects of SOX6 Expression on Cell Proliferation—In a previous study, we showed that SOX6 expression is down-regulated in hyperinsulinemic obese mice and that this attenuation led to increased insulin secretion from cultured β-cells (3). These data suggested that the attenuation of SOX6 may account for hyperinsulinemia in obese animals and type 2 diabetes. To further evaluate the role of SOX6 in insulin resistance and type 2 diabetes, we analyzed the effects of SOX6 expression on cell proliferation. As shown in Fig. 1, A and B, siRNA-mediated knockdown of SOX6 increased cell proliferation of insulinoma INS-1E and NIH-3T3 cells. Furthermore, in MIN6 and HIT-T15 cells, which are derived from β-cells, the application of siRNA specific for SOX6 stimulated cell growth (supplemental Fig. 1, A and B), suggesting that SOX6 attenuates cell proliferation of β-cells. In a complementary manner to the results of siRNA experiments, retroviral overexpression of SOX6 inhibited cell proliferation of INS-1E and NIH-3T3 cells (Fig. 1, C and D). Similarly, cell growth of MIN6 cells was suppressed by forced expression of SOX6 (supplemental Fig. 2). The expression level of SOX6 induced by retroviral transduction was 6–7-fold higher than endogenous SOX6 in the cells (data not shown). Consistent with the inhibition of cell proliferation, DNA synthesis, as determined by BrdUrd incorporation, was also significantly reduced in NIH-3T3 cells retrovirally transfected with SOX6 compared with those transduced with control virus encoding GFP (Fig. 1E). Although retroviral transfection of SOX6 inhibited cell proliferation and DNA synthesis, it did not induce morphological changes of NIH-3T3 cells (Fig. 1F, top panel). Quantitative analysis of cell cycle distribution showed that the percentage of G2 phase cells was largely reduced when SOX6 was retrovirally transfected into NIH-3T3 cells (Fig. 1F, bottom panel).
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In the absence of SOX6, TOPFLASH activity was completely blocked by SOX6 expression. Furthermore, SOX6 overexpression inhibited cyclin D1 promoter activity (Fig. 3B), suggesting that SOX6 inhibited transcriptional activation of the cyclin D1 promoter activated by β-catenin (Fig. 3, A and B). It also suggests that SOX6, like SOX9, binds to β-catenin (20). To confirm this possibility, we carried out co-transfection of SOX6 with β-catenin fused to GAL4 DNA-binding domain (GAL4-β-catenin). As shown in Fig. 3C, SOX6 co-transfection strongly suppressed the transcriptional activation by GAL4-β-catenin. These results indicate that SOX6 physically binds with β-catenin and thereby interferes with transcriptional activation mediated by β-catenin.

Leucine Zipper and Poly(Q) (LZ/Q) Domain of SOX6 Binds to Armadillo Repeats 1–4 of β-Catenin—To dissect the physical interaction between β-catenin and a mutant form S33Y, which serine 33 was substituted with tyrosine. This mutant β-catenin is not phosphorylated at Ser-33 by glycogen synthetase kinase 3β and is resistant to proteolytic degradation. As shown in Fig. 3A, normal and S33Y β-catenin activated TOPFLASH activity in the absence of SOX6. In contrast, β-catenin-induced TOPFLASH activity was completely blocked by SOX6 expression.

SOX6 and β-catenin, we performed an in vitro binding assay using two series of deletion mutants as schematically depicted in Fig. 4, A and B. A series of deletion mutants of β-catenin was translated in vitro with [35S]methionine and incubated with GST-fused SOX6 or GST alone, and the resulting complexes were precipitated with glutathione beads as described under "Experimental Procedures." As shown in Fig. 4A, deletion of armadillo repeats 1–4 completely abolished the interaction with SOX6 (Fig. 4A, panel v), and the armadillo repeats 1–4 alone are sufficient for binding with SOX6 (Fig. 4A, panel iv). The central core of β-catenin contains 12 armadillo repeats that form a highly ordered structural domain committed to interaction with a variety of proteins, including TCF, axin, APC, α-catenin, and E-cadherin (21). Using a similar method, the β-catenin binding domain of SOX6 was determined. As shown in Fig. 4B, deletion of the LZ/Q domain of SOX6 completely abolished the binding to β-catenin. Furthermore, the LZ/Q domain of SOX6 alone is sufficient for binding to β-catenin (Fig. 4B, panels iv and v).
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FIGURE 6. SOX6 localizes to the cyclin D1 promoter via binding to β-catenin. The diagram shows a ChIP assay for detecting a β-catenin-SOX6-TCF complex on the cyclin D1 promoter. A and B, ChIP assay with anti-FLAG and anti-β-catenin antibodies. On day 0, 293 cells were set up for the experiment as described under "Experimental Procedures." A, on day 1, 293 cells were transfected with 5 μg of either pCMV-FLAG-SOX6 or pCMV empty vector and harvested for ChIP with either anti-FLAG (anti-SOX6) (left panel) or anti-β-catenin antibody (right panel) as described under "Experimental Procedures." B, on day 1, 293 cells were transfected with either 200 pmol of β-catenin-specific siRNA (si-β-catenin, +) or control siRNA (−) along with 5 μg of pCMV-FLAG-SOX6. Three days after transfection, the cells were harvested and subjected to ChIP using anti-FLAG (anti-SOX6) (left panel) or anti-β-catenin antibody (right panel). A and B, recovery of cyclin D1 promoter fragment following ChIP was quantified by QRT-PCR. The presence of FLAG-tagged SOX6 (A) or β-catenin (B) in cell extracts before immunoprecipitation (Pre IP) and the immunoprecipitated samples (Post IP) was detected by immunoblot analysis (bottom panels). All data represent recovery, in percent, of each DNA fragment relative to total input DNA. Each bar represents mean ± S.E. of triplicate experiments. *, p < 0.01 compared with control n.s., not significant.

Consistent with the lack of β-catenin binding, the deletion mutant SOX6 lacking the LZ/Q domain did not suppress cyclin D1 promoter activity induced by S33Y β-catenin (Fig. 4C, top panel); the levels of full-length SOX6 and ΔLZ/Q expression were evaluated by immunoblotting (Fig. 4C, bottom panel).

To further delineate the interaction between β-catenin and SOX6 in vivo, we carried out co-immunoprecipitation analysis using N-terminal FLAG-tagged SOX6 (FLAG-SOX6) and endogenous β-catenin. Cells were transfected with FLAG-SOX6 or empty vectors, immunoprecipitated with either anti-β-catenin or anti-FLAG antibodies, and subjected to SDS-PAGE and immunoblotting with either anti-β-catenin or anti-FLAG antibodies. As shown in Fig. 4D, FLAG-SOX6 and endogenous β-catenin were co-precipitated with anti-β-catenin and anti-FLAG, respectively, indicating that the two proteins are present as a complex in vivo. Taken together, these results indicate that the LZ/Q domain of SOX6 physically interacts with armadillo repeats 1–4 of β-catenin.

Effects of SOX6 Expression on the Stability of β-Catenin—Akiyama et al. (20) showed that SOX9 physically interacts with β-catenin and competes with TCF/Lef for β-catenin binding. In contrast to SOX9, SOX6 had no effects on the binding of β-catenin to TCF in vitro (Fig. 5A). To further define the binding of SOX6 to β-catenin, we compared in vivo β-catenin binding to SOX6 or SOX9 in the absence or presence of MG132, a proteasome inhibitor. Although MG132 strongly stabilized the binding of SOX9 to β-catenin (Fig. 5B, compare lanes 6 and 12), it had no effects on the binding between SOX6 and β-catenin (Fig. 5B, compare lanes 4 and 10), suggesting that the SOX6-β-catenin complex does not undergo proteolytic degradation, whereas the SOX9-β-catenin complex is susceptible to proteolytic degradation, as described previously (20). Consistent with the stabilization of SOX9-β-catenin complex by MG132, the β-catenin(S33Y)-induced cyclin D1 promoter activity was not affected by SOX9 in the presence of MG132 (Fig. 5C, compare lanes 8 and 12). These data indicate that the SOX6-β-catenin complex is stable and thereby β-catenin binding to TCF/Lef was not inhibited, whereas the complex of SOX9-β-catenin is readily degraded in the proteasome, resulting in the reduction of cyclin D1 promoter activity. This difference between SOX6 and SOX9 may be explained by the difference in their binding sites for β-catenin; SOX6 binds to armadillo repeats 1–4, whereas SOX9 interacts with armadillo repeats 4–10 (20)
Association of SOX6, β-Catenin, and Acetylated Histones with the Cyclin D1 Promoter—To further define the role of SOX6 in the regulation of cyclin D1 promoter, we carried out a ChIP assay using 293 cells transfected with epitope-tagged SOX6. When SOX6 was expressed by transfection, the cyclin D1 promoter sequences were present at a high level in the SOX6 immunoprecipitate (Fig. 6A, top panel, 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 β-catenin to the cyclin D1 promoter (Fig. 6A, top panel, compare lanes 6 and 8), consistent with the data in Fig. 5 that SOX6 does not induce β-catenin degradation. As shown in Fig. 6B, siRNA-mediated knockdown of β-catenin significantly decreased the association of SOX6 with the cyclin D1 promoter. These data indicate that SOX6 has no effect on the association of β-catenin with the cyclin D1 promoter. In contrast, the association of SOX6 with the cyclin D1 promoter is dependent on the expression of β-catenin. The immunoblot analyses of epitope-tagged SOX6 and β-catenin revealed that these proteins were efficiently recovered during the immunoprecipitation procedures (Fig. 6, A and B, bottom panels). Together with the sequence-independent inactivation of β-catenin by SOX6 (Fig. 3C), these data indicate that SOX6 associates with cyclin D1 promoter via binding with β-catenin, although our results do not conclusively exclude the possibility that there are separate binding sites for β-catenin and SOX6 in the cyclin D1 promoter.

We next analyzed the effects of SOX6 expression on the acetylation levels of histones H3 and H4 in chromatin at the cyclin D1 promoter. As shown in Fig. 7A, the expression of SOX6 significantly reduced the levels of acetylated histones H3 and H4 at the cyclin D1 promoter. These inhibitory effects by SOX6 were almost completely blocked by the addition of Scriptaid, an HDAC inhibitor (Fig. 7A, compare lanes 4 and 6, 10 and 12). Consistent with the decreased levels of acetylated histones H3 and H4, the addition of Scriptaid also prevented the suppression of β-catenin-induced cyclin D1 promoter activities by SOX6 (Fig. 7B, compare lanes 2, 3 and 5). These data suggest that SOX6 recruits HDACs for the suppression of β-catenin-induced cyclin D1 promoter activities.

SOX6 Recruits HDAC1 as a Co-repressor for β-Catenin—HDACs remove acetyl groups from histones, following which the chromatin structure undergoes condensation, thereby suppressing the transcriptional activities of target genes (22, 23). To test whether SOX6 recruits HDACs, we carried out ChIP assays using anti-HDAC1 antibody. As shown in Fig. 8A, the levels of HDAC1 in chromatin at the cyclin D1 promoter were significantly induced by the expression of SOX6. In addition, both HDAC1 and β-catenin were immunoprecipitated with anti-FLAG antibody (against FLAG epitope-tagged SOX6) in 293 cells transfected with FLAG-SOX6 and β-catenin-His expression vectors (Fig. 8B, lane 1). Similarly, HDAC1 and SOX6 were co-immunoprecipitated with anti-His antibody (against His epitope-tagged β-catenin) (Fig. 8B, lane 3). In contrast, HDAC1 was not immunoprecipitated with anti-His antibody in the absence of FLAG-SOX6 (Fig. 8B, lane 2), indicating that SOX6 is necessary for the interaction between HDAC1 and β-catenin. These results suggest the formation of a heterotrimeric complex—SOX6-β-catenin-HDAC1, the basis for the HDAC inhibitory activity by SOX6 is reversed by HDAC inhibitor. The diagram shows the model for the β-catenin-SOX6-HDAC complex, the basis for the HDAC inhibitorScriptaid experiment. A, ChIP for acetylated histone H3 and H4 association with the cyclin D1 promoter. On day 0, 293 cells were set up for experiments as described under “Experimental Procedures.” On day 1, cells were transfected with either 5 μg of pCMV-FLAG-SOX6 or control pCMV empty vector. After transfection, cells were cultured in the presence or absence of 3 μM Scriptaid. After 16 h of incubation, the cells were harvested and subjected to ChIP using anti-acetylhistone H3 (AcH3, left panel) or anti-acetylhistone H4 (AcH4, right panel). Recovery of cyclin D1 promoter fragment following ChIP was quantified by QRT-PCR. All data represent recovery, in percent, of each DNA fragment relative to total input DNA. The transfection efficiency of 293 cells as evaluated by X-gal staining (β-galactosidase staining kit, Mirus, Madison, WI) was 70–80%. B, effect of HDAC inhibitor on SOX6-mediated inhibition of the cyclin D1 promoter activation by β-catenin. On day 1, 293 cells were transfected with 0.1 μg of cyclin D1 promoter luciferase plasmid and 0.01 μg of pRL-TK together with 0.1 μg of pCMV-SOX6 and 0.1 μg of pcDNA-β-catenin. Total amount of DNA was adjusted to 0.5 μg by the addition of pCDNA3. Twenty four hours after the transfection, cells were switched to medium containing the indicated concentration of Scriptaid and incubated for 16 h. The cells were then harvested, and firefly luciferase activity was measured and normalized to Renilla luciferase activity. Each bar represents mean ± S.E. of triplicate experiments. *, p < 0.01 compared with control.
SOX6 Acts as a Co-repressor for β-Catenin

**A. ChIP**

![Graph showing ChIP results with antibodies against HDAC1 and SOX6.](image)

**B. Immunoprecipitation**

![Diagram showing immunoprecipitation with HDAC1 and SOX6 antibodies.](image)

**C. GST-SOX6 Pulldown**

![Diagram showing GST-pulldown assay with HDAC1 and SOX6.](image)

**D. GST-HDAC1 Pulldown**

![Diagram showing GST-pulldown assay with HDAC1 and SOX6.](image)

**E. Schematic Diagram**

![Diagram showing the interaction of HDAC1 and SOX6.](image)

**FIGURE 8.** SOX6 directly interacts with histone deacetylase 1 and recruits it to cyclin D1 promoter in 293 cells. A and B, on day 0, 293 cells were set up for experiments at a density of 5 x 10^5 cells in a T-25 flask as described under the “Experimental Procedures.” A, ChIP assay using anti-HDAC1 antibody. On day 1, cells were transfected with 1 μg of either pCMV-FLAG-SOX6 (+) or pCMV empty vector (−). After incubation for 20 h, cells were harvested and subjected to ChIP using anti-FLAG antibody as described (top panel). The presence of epitope-tagged SOX6 and HDAC1 in cell extracts before immunoprecipitation (pre IP) and the immunoprecipitated samples (post IP) was detected by immunoblot analysis with the same antibodies used in the immunoprecipitation (bottom panel). Recovery of cyclin D1 promoter fragment following ChIP was quantified by QRT-PCR. All data represent recovery, in percent, of each DNA fragment relative to total input DNA. Each bar represents mean ± S.E. of triplicate experiments. *p < 0.01 compared with control.

**DISCUSSION**

In this study, we demonstrate that SOX6 inhibits cell proliferation and β-catenin/TCF transactivation of the cyclin D1 promoter by recruiting HDAC1. On the other hand, attenuated expression of SOX6 stimulates cell proliferation. We showed that the LZ/Q domain of SOX6 physically interacts with armadillo repeats 1–4 of β-catenin, as schematically depicted in Fig. 8E.

In a previous study, we showed that SOX6 is down-regulated in hyperinsulinemic obese animals and that this attenuation leads to stimulation of glucose-stimulated insulin secretion by reducing the suppression of PDX1 mediated by SOX6 (3). In this study, we demonstrate that SOX6 physically interacts with PDX1 and β-catenin on the promoters of insulin and cyclin D1, respectively, and recruits HDAC1 for the suppression of these genes. Under a down-regulated condition of SOX6, these genes are not suppressed, thereby stimulating insulin secretion and cell proliferation.

**FIGURE 9.** A model for hyperinsulinemia and hyperplasia by down-regulation of SOX6. SOX6 physically interacts with PDX1 and β-catenin on the promoters of insulin and cyclin D1, respectively, and recruits HDAC1 for the suppression of these genes. Under a down-regulated condition of SOX6, these genes are not suppressed, thereby stimulating insulin secretion and cell proliferation.

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els of SOX6 are expressed. In contrast, the levels of SOX6 are markedly decreased in diet-induced and genetically obese mice. This down-regulation of SOX6 well agrees with the increased insulin secretory capacity and increased β-cell mass in these mice (Fig. 9). Recent studies by Dor et al. (26) have demonstrated that adult pancreatic β-cells are generated by self-duplication rather than from stem cell differentiation. Although the factors regulating β-cell duplication are currently unknown, the involvement of SOX6 is highly possible.

Our current data indicate that SOX6 negatively regulates both cell proliferation and insulin secretion. In insulin-resistant conditions, the levels of SOX6 are markedly decreased, and this down-regulation eventually leads to the increased production of insulin and stimulation of β-cell proliferation. Although it is unlikely that these events occur simultaneously, SOX6 may be a double-faced transcription factor that plays a part both in the regulation of insulin synthesis and proliferation of β-cells.

Although further studies are required for elucidating the mechanism by which SOX6 is down-regulated, our current data provide a novel insight into β-cell function in obesity-related insulin resistance. SOX6 appears to be a promising therapeutic target for treating type 2 diabetes.

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