Identification of an INSM1-binding site in the insulin promoter: negative regulation of the insulin gene transcription

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Abstract

In this study, an insulinoma-associated antigen-1 (INSM1)-binding site in the proximal promoter sequence of the insulin gene was identified. The co-transfection of INSM1 with rat insulin I/II promoter-driven reporter genes exhibited a 40–50% inhibitory effect on the reporter activity. Mutational experiments were performed by introducing a substitution, GG to AT, into the INSM1 core binding site of the rat insulin I/II promoters. The mutated insulin promoter exhibited a three- to 20-fold increase in the promoter activity over the wild-type promoter in several insulinoma cell lines. Moreover, INSM1 overexpression exhibited no inhibitory effect on the mutated insulin promoter. Chromatin immunoprecipitation assays using β TC-1, mouse fetal pancreas, and Ad-INSM1-transduced

human islets demonstrated that INSM1 occupies the endogenous insulin promoter sequence containing the INSM1-binding site *in vivo*. The binding of the INSM1 to the insulin promoter could suppress $\sim$ 50% of insulin message in human islets. The mechanism for transcriptional repression of the insulin gene by INSM1 is mediated through the recruitment of cyclin D1 and histone deacetylase-3 to the insulin promoter. Anti-INSM1 or anti-cyclin D1 morpholino treatment of fetal mouse pancreas enhances the insulin promoter activity. These data strongly support the view that INSM1 is a new zinc-finger transcription factor that modulates insulin gene transcription during early pancreas development.

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Introduction

Insulin gene transcription is regulated by both the ubiquitous and the β -cell-specific transcription factors (Melloul *et al.* 2002, Brink 2003). In β -cells, multiple regulatory elements in the basal insulin promoter control insulin gene expression. These regulatory elements include E-box elements, A elements, the RIPE3b region, the C1 element, G elements, and some consensus binding sites such as Sp-1, CCAAT, and the cAMP regulatory element (CRE). A handful of transcription factors that govern β -cell differentiation (Pdx-1, NeuroD/ β 2, Pax4/6, and Nkx2.2) have been shown to interact directly with the insulin promoter's regulatory elements. A majority of these factors not only bind to specific binding sites but also form multiprotein transcriptional complexes situated on the insulin promoter (Ohneda *et al.* 2000a, Qiu *et al.* 2002). The recruitment of the co-activator p300, along with the binding of Pdx-1 and bHLH factors, E47 and NeuroD/ β 2, in a complex with a high mobility group I protein on the E-A mini-enhancer, results in a synergistic activation of insulin gene expression (Ohneda *et al.* 2000b, Qiu *et al.* 2002, 2004, Mosley *et al.* 2004,

Stanojevic *et al.* 2004). Another A3/A4-binding homeoprotein, *lmx1.1*, activates transcription by acting synergistically, via its LIM domain, with E47 (German *et al.* 1992). Rat insulin promoter element 3 (RIPE3), which spans bases -125 to -86 , was shown to contain cell-specific and ubiquitous factors that confer enhancer activity (Shieh & Tsai 1991). The C1/RIPE3b1 ($-118/-107$ bp) binding factor regulates both pancreatic β -cell-specific and glucose-regulated transcription of the insulin gene, which includes members of the large Maf transcription factor family (Kataoka *et al.* 2002, Olbrot *et al.* 2002, Matsuoka *et al.* 2003, Zhao *et al.* 2005). By investigating novel regulatory elements on the rat insulin promoter, a conserved sequence spanning between -61 to -50 bp and resembling the binding site of insulinoma-associated antigen-1 (INSM1, formerly IA-1) was identified (Breslin *et al.* 2002).

The INSM1 gene encodes a 58 kDa protein that contains five zinc-finger DNA-binding motifs, originally isolated from a human insulinoma subtraction library (Goto *et al.* 1992). The expression pattern is restricted to the fetal pancreas, fetal nervous system, and in tumors of neuroendocrine origin (Xie *et al.* 2002, Zhu *et al.* 2002, Breslin *et al.* 2003).

Functional studies have revealed that the amino terminus of the INSM1 protein possesses repressor activity and that the zinc-finger motifs recognize the conserved target sequence, T^G/T^C/T^C/T^T/_AGGGG^G/_TC^G/_A, which is located in the promoter regions of both the NeuroD/β2 and INSM1 genes (Breslin *et al.* 2002, Liu *et al.* 2006). *In vitro* induction of AR42J amphicrine cells and normal human ductal epithelial cells induction into insulin-producing cells suggested that INSM1's gene expression is closely associated with the expression of islet-specific transcription factors and it was shown to be an immediate downstream the target gene of neurogenin3 (Ngn3; Zhu *et al.* 2002, Mellitzer *et al.* 2006, Breslin *et al.* 2007). Global INSM1 mutant mice revealed that INSM1 plays an essential role for the development of both pancreatic β-cells and intestinal endocrine cells (Gierl *et al.* 2006).

An INSM1-binding site in the insulin promoter region was identified in this study, suggesting that insulin could be a target gene of the INSM1. This INSM1-binding site is highly conserved among humans, rats, and mice. EMSA and reporter gene transfection analyses of rat and mouse insulin promoters (MIP) support that the INSM1 protein binds and suppresses insulin promoter activity. The occupancy of the INSM1 transcription factor on the insulin promoter sequence was confirmed by the chromatin immunoprecipitation (ChIP) assay. It was also shown that overexpression of INSM1 in human islets could directly downregulate the insulin gene message, whereas INSM1 anti-sense treatment of embryonic mouse pancreas increased insulin promoter activity. These results strongly support its negative regulatory role in insulin gene transcription. Furthermore, the mechanism for transcriptional repression of the insulin gene by INSM1 is mediated through the recruitment of cyclin D1 and histone deacetylase-3 (HDAC-3) to the insulin promoter.

Materials and Methods

Cell culture and reagents

Insulinoma (MIN6, HIT, and RIN) and HeLa cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained as described by the protocol. βTC-1 cells were kindly provided by Dr E H Leiter (Jackson Laboratory, Bar Harbor, ME, USA). The mouse or rabbit anti-cyclin D1, anti-HDAC-3, and anti-Flag antisera were obtained from Biosource (Camarillo, CA, USA), Upstate (Lake Placid, NY, USA), and Sigma respectively. A mouse anti-INSM1 monoclonal antibody (6-1-1) was generated against the carboxyl-terminal peptide of the INSM1 protein. Recombinant adenoviral vectors were constructed as described (Liu *et al.* 2006).

EMSA

EMSA was performed using two copies of double-strand oligo spanning the -64 to -47 bp region of the rat insulin I promoter (containing INSM1-binding site),

5'-AAAGTCCAGGGGGCAGAG-3', and the complementary strand. The double-stranded oligo was end-labeled using [γ -³²P]ATP (3000 Ci/mmol, Perkin Elmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). The INSM1 carboxyl-terminal peptide (Cpep, aa 257-510) was synthesized using a pGBKT7-Cpep vector and the TNT coupled rabbit reticulocyte lysate kit (Promega). The synthesis of Cpep was confirmed by western blot analysis using an anti-Cpep antibody (6-1-1). Nuclear extracts from cultured cell lines were prepared as described (Dignam *et al.* 1983). The EMSA reaction condition was described previously (Liu *et al.* 2006). Competitive EMSA was performed using unlabeled cold probe, random probe, or mutant probe.

Construction of rat insulin I and II promoter mutants

The rat insulin I (-410 to +50 bp; accession number, J00747) and insulin II promoters (-792 to -4 bp; accession number, J00748) were PCR amplified from rat genomic DNA. The amplified sequences were confirmed by sequence analysis and cloned into a pGL3-Basic vector (Promega) for promoter-driven luciferase assay. The INSM1-binding site on the rat insulin I/II promoters, GTCCAGGGGG/TCA, was mutated into GTCCAGATGG/TCA using PCR primers, PInI-1, GATCGGTACCTGAGCTAAGAATCCA; PInI-2, GATCAGATCTACATACCTGCTTGCT; PInI-3, CGGCAAAGTCCAGATGGCAGAGAGGA; PInI-4, TCCTCTCTCTGCCATCTGGACTTTGCCG; PInII-1, GATCGGTACCCCCAACCACTCCAAGTGG; PInII-2, GATCCTCGAGGGTTACTGAATCCAC; PInII-3, AAACAGCAAAGTCCAGATGTCAGGGG; and PInII-4, CCCCTGACTACTGGACTTTGCTGTTT. The mutant rat insulin I and II promoters were confirmed by sequence analysis and subcloned into the pGL3-Basic vector for promoter-driven luciferase assays. Three copies of the INSM1-binding site derived from the insulin promoter were subcloned in front of the E1bTATA basic promoter and the luciferase gene.

Transient transfection and insulin promoter assays

The rat insulin promoter driven luciferase vector (1.0 μg), rInIp or rInIip (I: -410/+50 bp; II: -792/-4 bp), was transfected into a 70% confluent rat insulinoma cell line, RIN, in the presence or absence of CMV-INSM1 (pcDNA3-INSM1) (0.5 μg). An empty pcDNA3 vector was also included to ensure that each transfection had an equal amount of DNA. A CMV-β-galactosidase construct (0.5 μg) was used as an internal control to normalize transfection efficiency. Twenty-four hours post-transfection, the cells were washed with PBS and collected for luciferase and β-galactosidase assays (Promega). Each experiment was performed at least six times, and the results represent the average of six times ± s.e.m. For rat insulin I/II promoter mutants (rInIpm and rInIipm), three insulinoma cell lines, MIN (mouse), RIN (rat), and HIT (hamster), and HeLa

cells were used. The wild-type rat insulin I/II promoter activities were designated as 100% and the mutant promoter activities were expressed as fold relative to wild-type activity. Each experiment was performed at least six times, and the results represent the average of six times $\pm$ S.E.M.

Co-immunoprecipitation and western blot analysis

Insulinoma cell line, β TC-1, was transduced with Flag-tag Ad-INSM1 (m.o.i. 50:1). The transduced cells were harvested 48 h post-transduction. Cell lysates were prepared with a protein-binding buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). For co-immunoprecipitation, 300 μ g cell lysates were pre-cleared with rProtein G-Agarose, and incubated for 2 h at 4 °C with anti-cyclin D1, anti-Flag, or normal control IgG antibody, followed by incubation for 1 h at 4 °C with rProtein G-Agarose beads. The beads were washed 5 $\times$ with a binding buffer, separated on 10% SDS-PAGE, and transblotted onto a nitrocellulose membrane (Invitrogen). INSM1, cyclin D1, and HDAC-3 were detected by western blotting (1:1000) with each antibody. The horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (1:4000) and chemiluminescent substrate (Pierce, Rockford, IL, USA) was used for detection.

ChIP assay

ChIP assays were performed in β TC-1, mouse fetal pancreas, and Ad-INSM1 transduced human islets. A ChIP assay kit (Upstate) was used and the procedures were followed as described (Liu *et al.* 2006). A mouse monoclonal antibody (6-1-1) to INSM1, anti-HDAC-3 (Biosource), anti-Flag (Sigma), and anti-acetyl-H3/H4 antibodies (Upstate) were used in ChIP assays. The PCR primers used to detect target sequences were as follows: mouse insulin II (forward) 5'-ACCTAGCACCAGGGAA-GTGT-TT-3'; (reverse) 5'-ATAGACCAAAGCACCTCC-TCT-3', (132 bp); human insulin (forward) 5'-TGAGGAA-GAGGTGCTGACGA-3'; (reverse) 5'-CCTCTTCTGAT-GCAGCCTGT-3' (225 bp); and phosphoenolpyruvate carboxykinase (PCK) (forward) 5'-GAGTGACACCTCA-CAGCTGTGG-3'; (reverse) 5'-GGCAGGCCTTTGGAT-CATAGCC-3' (339 bp) used as a control.

Northern blot and real-time RT-PCR analysis of Ad-INSM1-transduced human islets

Fresh human islets (5000 IEQs, 95% purity) obtained from Islet Cell Resources (City of Hope SC-ICR Center, Duarte, CA, USA) was transduced with either Ad-INSM1 (100:1 m.o.i.) or Ad-LacZ (100:1 m.o.i.) for 48 h. Total RNA was isolated using Trizol reagent (Invitrogen). Total RNA (20 μ g) was separated on a formaldehyde denaturing gel, transblotted, and subjected to northern blot analysis. INSM1 and glyceraldehyde-3-phosphate dehydrogenase probes were random primer labeled using

α -³²P-dCTP (Perkin-Elmer Life Sciences, Waltham, MA, USA). The same blot was stripped and re-hybridized with each probe. The insulin message was determined by quantitative real-time RT-PCR. First-strand cDNA was prepared from 5 μ g total RNA with the SuperScript II RT kit (Invitrogen). One-tenth of the cDNA was used as a template with IQ SYBRgreen supermix (Bio-Rad) for PCR. The expression level of the insulin gene was corrected for that of the β -actin housekeeping gene. The human insulin primers are as follows: (forward) 5'-CCTTGTGAACCAACACCTG-3', (reverse) 5'-GTA-GAAGAAGCCTCGTTCCC-3' (80 bp) and human β -actin primers are (forward) 5'-CTGTACGCCAACACAGT GCT-3', (reverse) 5'-GCTCAGGAGGAGCAATGATC-3' (127 bp). Three batches of human islets were received from ICR for these studies.

Morpholino treatment of MIP-enhanced green fluorescence protein (MIP-EGFP) fetal pancreas

Fetal pancreata were harvested from E16 day MIP-EGFP transgenic mice obtained from the Jackson Laboratory (Hara *et al.* 2003). Each fetal pancreas was cultured in Costar 96-well black opaque plate. The culture was incubated in DMEM (10% FBS and penicillin/streptomycin) supplemented with INSM1 anti-sense (5'-GCATGTTGGCGC-GGTGAAAAGGGCG-3'), cyclin D1 anti-sense (5'-GCA-GCACAGGAGCTGGTGTTCATG-3'), or inverted INSM1 control (5'-GCGGGAAAAGTGGCGCGGTTG-TACG-3') morpholino oligonucleotides (25 μ M) (Gene Tools, LLC) and partial medium changes were performed after 24 h. The fluorescence signal was read at different time points using a BIO-TEK fluorimeter at excitation 485/20 and emission 528/20. The experiments were repeated three times using embryos from different pregnant mice.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test for unpaired comparisons. Data are presented as means $\pm$ S.E.M.; *P* < 0.05 was considered significant.

Results

INSM1 functions as a transcriptional repressor of the insulin gene

The INSM1 protein consists of 510 amino acid residues with five zinc-finger DNA-binding motifs at the carboxyl terminus (Goto *et al.* 1992). A selected and amplified binding site selection strategy was used to identify the INSM1 consensus DNA-binding site, T^G/T^C/T^C/T^T/A^GGGGG^G/T^CG/A (Fig. 1) (Breslin *et al.* 2002). By searching the eukaryotic promoter GenBank database, a potential INSM1-binding site was found at the proximal region of the insulin gene promoter. This potential INSM1-binding site is highly conserved in human, rat, and mouse insulin genes (Fig. 1). In order to determine whether the

Figure 1 EMSA. The INSM1 consensus binding site sequence was derived from our previous study (Breslin *et al.* 2002). The conserved INSM1-binding sequence in the insulin gene is located at the proximal promoter region of humans, mice, and rats. A modified schematic of the rat insulin I gene promoter with major regulatory elements and a subset of binding factors was derived from a previously published chart (Ohneda *et al.* 2000a). A tandem repeated double-strand oligo spanning the -64 to -47 bp region of the rat insulin I promoter was end-labeled as a probe. The carboxyl terminus (Cpep) of INSM1 was *in vitro* transcribed and translated in a TNT coupled rabbit reticulocyte lysate system. (A) The probe alone (lane 1), and probe with control lysate (lane 2), Cpep lysate (lane 3), Cpep lysate plus anti-Cpep antibody (lane 4) and Cpep lysate plus mouse IgG control (lane 5) are shown in the left panel. The right-hand panel shows different concentrations of cold competitor oligos (0–200 $\times$). (B) The same probe was gel-shifted with nuclear extracts (10 μ g) derived from β -TC1 and NIH3T3 cells (lane 1), with anti-Cpep antibody (lane 2), and with control mouse IgG (lane 3). Arrows indicated gel-shift bands and super-shift (SS) bands in both translated and INSM1-positive nuclear extracts.

INSM1 protein can bind to the INSM1-binding site within the insulin gene promoter, we used an *in vitro* synthesized carboxyl terminus of INSM1 (Cpep, 257–510 amino acid residues) protein or nuclear extracts isolated from insulinoma cells (INSM1-positive) or NIH3T3 (INSM1-negative), and a probe ($-47/-64$ bp) spanning the rat insulin I promoter region in an EMSA study. As shown in Fig. 1A, a band shift was seen with the Cpep lysate. This band was super-shifted by an INSM1 antibody, but not with the normal IgG control demonstrating a specific protein–DNA interaction. Competition experiments demonstrated that the binding of the Cpep lysate and insulin promoter probe can be abolished by increasing the unlabeled cold probe concentration. A similar EMSA using the nuclear extracts isolated from insulinomas and NIH3T3 cells indicated that a unique shifted band and super-shifted band can

also be seen with the nuclear extracts from the INSM1-positive cell line β TC-1, but not with the INSM1-negative NIH3T3 cell line (Fig. 1B). Taken together, our data support that INSM1 protein is capable of binding to the insulin promoter. To further determine whether INSM1 functions as a transcriptional repressor of the insulin gene, rat insulin promoters I ($-410/+50$ bp) and II ($-792/-4$ bp) were subcloned into a pGL3-Basic vector. These insulin promoter-driven luciferase vectors were transfected into RIN cells with or without an INSM1 expression vector. As shown in Fig. 2A, INSM1 exhibits a 40–50% repression effect on either rat insulin I or II promoter activity. To further investigate whether this inhibitory effect was mediated through the INSM1-binding site within the insulin promoter, three copies of the INSM1-binding site sequence derived from rat insulin I or mouse insulin I/II

Figure 2 INSM1 suppresses mouse and rat insulin promoter activity. (A) Rat insulin I (rInIp: -410 to +50 bp) and insulin II promoters (rInIIP: -792 to -4 bp) were subcloned into a pGL3-Basic vector for luciferase activity assay. Insulin promoter activity was shown by co-transfection of reporter construct with INSM1 expression vector or control pcDNA3 vector into RIN cells. (B) The same experimental procedures were used for the (In)₃-E1bTATA reporter construct to measure suppressor activity. Three copies of INSM1-binding site-E1bTATA reporter vector were compared with E1bTATA-reporter or pGL3-Basic vector. The experiments were repeated at least six times and normalized with β -gal.

promoters were subcloned into an E1bTATA-luciferase vector. The transfection of these vectors into RIN cells with or without an INSM1 expressing vector resulted in a 40–50% repression (Fig. 2B). While comparing three-copy binding site-E1bTATA with E1bTATA alone showed slight decreases in their reporter activities suggesting that endogenous INSM1 imposed a suppression effect but no other activators affecting the same binding sequence. These results demonstrated that the INSM1-binding site is involved in the INSM1-mediated repression activity.

Our previous study showed that mutation analysis of any two G nucleotides in the GGGG core consensus binding site abolished INSM1 binding, suggesting that the core GGGG nucleotides are critical for INSM1-binding activity (Breslin *et al.* 2002). In the present study, the middle GG bases within the core GGGG of the rat insulin I/II INSM1-binding site were mutated into AT. The wild-type promoter luciferase or

the mutated promoter luciferase vector was transfected into different insulinoma cell lines or HeLa cells. In each cell line, when compared with the wild-type rat insulin I promoter activity, the mutated rat insulin I promoter activity increased eight- to 20-fold in insulinoma cell lines, but not in HeLa cells (Fig. 3A). Similarly, the mutated rat insulin II promoter activity increased two- to threefold in insulinoma cell lines with no change observed in the HeLa cells (Fig. 3A). The drastic differences of wild-type rat insulin promoter I and II versus mutant promoters could be due to the additional sequence present in both the promoters. Further co-transfection studies revealed that overexpressed INSM1 lost its suppressor effect on the mutated rat insulin I promoter in RIN cells (Fig. 3B), indicating that the core 'GGGG' within the INSM1-binding site is critical for transcriptional repression activity. An EMSA competitive study using either random probe or mutant probe also demonstrated no

Figure 3 The INSM1-binding site in the insulin promoter is a negative regulatory element. The INSM1-binding site in the rat insulin I/II promoter, GTCCAGGGG/TCA, was mutated into GTCCAGATGG/TCA. The mutant rat insulin I and II promoters (rInIpm and rInIpm) were confirmed by sequence analysis and subcloned into the pGL3-Basic vector for promoter driven luciferase assay. (A) Three insulinoma cell lines and a HeLa cell line were tested for wild-type and mutant promoter activities. The mutant promoter activities were compared with wild-type promoter activities in each individual cell line. (B) The mutated rat insulin I promoter activity was examined for its response to the INSM1 suppression effect. INSM1 suppresses wild-type rat insulin I promoter ($P < 0.001$), but not the mutant rat insulin I promoter in RIN cells. Transfections were normalized with β -gal. Each cell line was repeated at least six times. An EMSA study using cold probe (CP), random probe (RP), or mutant probe (MP) to compete with gel-shift band (arrow) show that mutant probe is no longer capable of competing with wild-type binding sequence.

competition for the shift band (Fig. 3B). Collectively, these results strongly support that INSM1 is a transcriptional repressor of the insulin gene.

INSM1 downregulates endogenous insulin gene expression

ChIP assay is designed to study protein–DNA interactions *in vivo*. In the previous section, we demonstrated *in vitro* that INSM1 binds and represses the rat insulin I/II promoter

region containing an INSM1-binding site (Fig. 2). Here, ChIP assays were performed to investigate the occupancy of the mouse insulin gene promoter region by endogenous INSM1 in β TC-1 cells. β TC-1 cells were cross-linked with formaldehyde, sheared, and precipitated with anti-INSM1. As shown in Fig. 4, the mouse insulin II promoter sequence (–152/–21 bp) was selectively amplified by PCR from chromatin precipitated by anti-INSM1 antibody, but not from the chromatin precipitated by normal mouse IgG. A PCK promoter sequence was included to show the lack of INSM1 binding as a negative control. These experiments confirmed that endogenous INSM1 occupies the endogenous mouse insulin gene promoter region spanning the INSM1-binding site in β TC-1 cells. Since INSM1 is not expressed in the human adult islets, we used the Ad-INSM1-transduced human islets to show that the INSM1 protein is capable of binding to the human insulin gene promoter by ChIP assay (Fig. 4B). To demonstrate that INSM1 is actually capable of suppressing insulin gene expression, the suppression effect of INSM1 on endogenous insulin gene expression was tested by using an adenovirus overexpression system in normal human islets. Fresh human islets were transduced with Ad-LacZ or Ad-INSM1 for 48 h. Northern blot analysis showed a strong expression of the Ad-INSM1 message in contrast to the absence of endogenous INSM1. Quantitative real-time RT-PCR analyses revealed that the insulin message was suppressed by $\sim 50\%$ in the INSM1 overexpressing islets (Fig. 4C). The suppression effect seen in the human islets is consistent with the transient transfection study using the rat insulin promoter. The suppression effect of INSM1 should change the acetylation pattern of chromatin on the insulin promoter. We further performed the ChIP assay on the Ad-INSM1 or Ad-LacZ-transduced human islets using anti-acetyl H3/H4. As shown in Fig. 4D, acetyl-H4 on the insulin promoter decreased in Ad-INSM1-transduced islets suggesting that a suppression event could occur. Since INSM1 functions as a developmental transcription factor in fetal pancreas, we performed a ChIP assay using E16 day fetal pancreas and included fetal liver as a negative control. Figure 6A shows that fetal pancreas expressing INSM1 occupies the insulin promoter in contrast to the fetal liver control.

INSM1 transcriptional repression activity is mediated through the recruitment of cyclin D1 and HDAC-3

INSM1 was shown to form a transcriptional complex with cyclin D1 and HDAC-3 (Liu *et al.* 2006). The molecular mechanisms of INSM1 repressor activity on the insulin gene promoter were investigated by transient transfection and co-immunoprecipitation of INSM1 and cyclin D1 in β TC-1 insulinoma cells. Figure 5A shows that the overexpression of cyclin D1 could enhance the INSM1 repression effect on the three copies of the INSM1-binding site derived from the rat insulin I promoter linked to the E1bTATA basic promoter. However, only cyclin D1 or HDAC-3 with the reporter

Figure 4 INSM1 binds to the endogenous insulin promoter and downregulates insulin gene transcription. (A) ChIP assay in β-Tc1. Formaldehyde cross-linked chromatin from β-Tc1 cells was incubated with an antibody to INSM1 (lane 2). Immunoprecipitated DNA was analyzed by PCR with primers specific to the transcriptional regulatory sequences of the mouse insulin II promoter, containing the INSM1-binding site and a control promoter sequence derived from PCK. As controls, PCRs were performed with input DNA (lane 1) and DNA immunoprecipitated by normal IgG (lane 3). (B) ChIP assay in human islets. Fresh human islets obtained from ICR were transduced with Ad-LacZ (5000 IEQs) or Ad-Flag-INSM1 (5000 IEQs) for 48 h. A ChIP assay similar to that above was performed using anti-Flag antibody (lane 2) or normal control IgG (lane 3). The human insulin promoter sequence was amplified from both input DNA (lane 1) and Ad-Flag-INSM1-transduced islets. No signal was detected in the control IgG or Ad-LacZ transduced islets. (C) Northern analysis and real-time RT-PCR. Fresh human islets obtained from ICR were transduced with Ad-LacZ (5000 IEQs) or Ad-INSM1 (5000 IEQs) for 48 h. Total RNA was isolated using Trizol reagent. Twenty micrograms of RNA were separated on a denaturing agarose gel and hybridized with 32 P-labeled INSM1 and GAPDH probes sequentially. In the right-hand panel, quantitative real-time RT-PCR was performed with β-actin as an internal control. The graph displays the insulin message after normalization with β-actin from three different experiments. (D) ChIP analysis was performed with the Ad-LacZ- or Ad-INSM1-transduced human islets with anti-acetyl H3 or H4 antibody. Each ChIP experiment was repeated to ensure its reproducibility.

vector showed a minor suppression effect probably due to the endogenous INSM1. The enhanced suppression effect by the overexpression of INSM1 and cyclin D1 is statistically significant, but the low magnitude of enhancement could be explained by the presence of endogenous cyclin D1. We also compared the effects of INSM1, cyclin D1, and HDAC-3 in (In)₃-E1bTATA and mutant (In)₃-E1bTATA reporter vectors (Fig. 5B). The wild-type promoter showed a more than 60% suppression by the INSM1–cyclin D1–HDAC-3 complex in contrast to that of the mutant promoter, which was unaffected. Figure 5C shows that overexpressed INSM1 and endogenous cyclin D1 or histone deacetylase (HDAC-3) are capable of interacting with each other using the co-immunoprecipitation method. Furthermore, anti-cyclin D1 is capable of co-precipitating HDAC-3 directly. In ChIP experiment, anti-HDAC-3 antibody successfully precipitated the endogenous HDAC-3–insulin II promoter complex, suggesting that HDAC-3 could bind to the INSM1 transcription factor to regulate insulin promoter activity (Fig. 5D). These data illustrate that INSM1, cyclin D1, and HDAC-3 constitute three important components for the transcriptional repression of the insulin gene.

INSM1 regulates insulin promoter in the embryonic mouse pancreas

To further clarify that INSM1 is truly involved in regulating insulin promoter activity during pancreas development, we selected a MIP-EGFP transgenic model (Hara *et al.* 2003). This animal model expresses the EGFP reporter under the control of mouse insulin promoter. Day E16 fetal pancreata were isolated from mice expressing MIP-EGFP. Explants were incubated with INSM1 or cyclin D1-specific anti-sense morpholino oligos to reduce INSM1 or cyclin D1 expression. An invert INSM1 oligo was used as a negative control. The anti-sense oligonucleotide technology to knock down specific gene expression in embryonic pancreas *in vitro* has been reported previously (Prasadan *et al.* 2002, Li *et al.* 2004, Mellitzer *et al.* 2006). Our study revealed that during the first 14 h of incubation, all three groups of fetal pancreas, control, anti-INSM1, and anti-cyclin D1 exhibited a burst of fluorescence signals (Fig. 6B). The control morpholino treatment showed no further increase at 24 and 38 h. By contrast, anti-INSM1 treatment showed a similar burst of fluorescence at 14 h and continued to increase steadily through the 38 h incubation. The gradual increase in the insulin promoter activity suggested that anti-INSM1 morpholino treatment alleviates the INSM1 suppression effect to the insulin promoter. Anti-cyclin D1 treatment presented a lower burst of fluorescence signal at 14 h incubation. However, a steady increase in the fluorescence signal was observed through 38 h. It is speculated that anti-cyclin D1 could not only affect the insulin promoter, but also affect the pancreas growth in general. Therefore, these results demonstrated that INSM1 and cyclin D1 are involved in insulin promoter suppression during pancreas development.

Figure 5 Cyclin D1-mediated HDAC-3 recruitment contributes to insulin transcriptional repression. (A) Cyclin D1 cooperates with INSM1 to suppress the rat INSM1-binding site-E1bTATA reporter vector. Cyclin D1 or HDAC-3 alone exhibits minor suppression effect probably due to the endogenous INSM1 in βTC-1 cells. (B) (In)₃-E1bTATA and mutant (In)₃-E1bTATA reporter vectors were co-transfected with INSM1, cyclin D1, and HDAC-3. The wild-type promoter showed a greater than 60% of suppression by INSM1-cyclin D1-HDAC-3 complex in contrast to that of the mutant promoter, which showed little suppression effect. (C) Co-immunoprecipitation of INSM1, cyclin D1, and HDAC-3. βTC-1 cells were transduced with Ad-INSM1 for 48 h. Cell lysates were immunoprecipitated with anti-Flag-INSM1, anti-cyclin D1, or normal IgG control antiserum. Co-precipitated HDAC-3 was revealed by western blot analysis with an anti-HDAC-3 antibody. The precipitates were also blotted for cyclin D1 or Flag-INSM1 to ensure their presence in the immunoprecipitated complex. (D) HDAC-3 is associated with the insulin gene promoter sequence *in vivo* by ChIP assay. Formaldehyde cross-linked chromatin isolated from Ad-INSM1-transduced β-TC1 cells were incubated with rabbit antiserum to HDAC-3 (lane 3) or control normal IgG (lane 4). Immunoprecipitated DNA was analyzed by PCR with primers specific for the transcriptional regulatory sequence of the mouse insulin II promoter (the same primers used in Fig. 4A) and the PCK promoter sequence. As controls, PCRs were performed with no DNA (lane 1), input DNA (lane 2), DNA immunoprecipitated by normal IgG (lane 4), and DNA that was immunoprecipitated in the absence of antiserum (lane 5). The ChIP assay was repeated in two separate experiments.

Discussion

β-Cell-specific expression of the insulin gene is regulated by a complex network consisting of cis-acting elements and trans-regulators. The most extensively studied enhancer/promoter region in the insulin gene is within 350 bp upstream from the transcriptional start site (Ohneda *et al.* 2000a, Melloul *et al.* 2002). This region is highly conserved in humans and rodents, suggesting that they share common regulatory mechanisms to regulate insulin gene transcription. Among these elements, A, C, and E are considered to be the major determinants of β cell-specific expression of the insulin gene. Transcription factors Pdx1, NeuroD/β2/E47, and MafA bind to the A–E minienhancer/promoter cooperatively with the co-activator p300, and activate insulin gene transcription

(Qiu *et al.* 2002, 2004, Mosley *et al.* 2004, Stanojevic *et al.* 2004, Zhao *et al.* 2005). In contrast to the positive regulation of insulin gene expression, negative regulation was not as frequently reported. Nkx2.2 is an islet-enriched transcription factor belonging to the NK2 family. Gene targeting experiments have shown that Nkx2.2 is important for islet cell development and function, especially for the maturation of β-cells (Sussel *et al.* 1998). A recent study demonstrated that Nkx2.2 bound to the rat insulin II promoter region (–140/–119 bp) both *in vitro* and *in vivo*, and inhibited insulin promoter activity (Cissell *et al.* 2003). Another transcription factor, Pax-4, was shown to downregulate the human insulin promoter through a C2 element located between –253 and –244 bp (Campbell *et al.* 1999). In the present study, an INSM1 zinc-finger transcription factor

Figure 6 INSM1 interacts with insulin promoter in fetal pancreas. (A) Fetal pancreas ChIP assay was performed using E16 day fetal pancreas or fetal liver. Due to the small amount of DNA sample, we increased the PCR cycle number to 45. The rest of the procedures were followed as above. Lower band labeled as dimer represents primer dimer from PCR. (B) E16 day fetal pancreas was isolated from MIP-EGFP transgenic mouse. Fetal pancreata incubated in a 96-well plate was supplemented with either anti-INSM1 (close diamond), anti-cyclin D1 (open square), or control oligo (open triangle) morpholinos. At different time points, fluorescence signals were measured using BIO-TEK fluorimeter. Relative fluorescence signal was calculated against the 0-h time point. The results were shown as the average of three different experiments.

binding site was identified at the proximal promoter region (−61/−50 bp) of the rat insulin gene, which is highly conserved between the human, rat, and mouse species. It is located between the A1 and G1 elements and is in very close proximity to the TATA box-binding site. Gel mobility-shift analysis and transient transfection assays determined the physical interaction and repression by INSM1. The mutation of GGGG to GATG within the INSM1-binding site greatly increased rat insulin I promoter activity and modestly increased the rat insulin II promoter activity. The same mutation resulted in the loss of INSM1 transcriptional repressor activity in transfected cells, supporting the notion that INSM1 binds to the predicted INSM1 sequence. In addition to the *in vitro* studies, ChIP assays support the finding that the endogenous INSM1 protein is capable of binding to the mouse insulin II promoter sequence. The overexpression of Ad-INSM1 in normal human islets represses the insulin message by ~50%. To further address the question whether the INSM1 suppression effect to the insulin promoter is physiologically significant, we demonstrated the occupancy of INSM1 on the insulin promoter in E16 day fetal pancreas. We also employed anti-sense experiments to an MIP-EGFP-tagged embryonic mouse pancreas. The knock down of INSM1 showed a steady increase in green fluorescence

signals. Thus, INSM1 is truly involved in insulin promoter suppression during pancreas development.

The transient expression patterns of INSM1 in the fetal pancreas were reported in both humans and mice (Zhu *et al.* 2002, Mellitzer *et al.* 2006). Since the INSM1 gene is silenced in the adult stage of the pancreas, it suggests that INSM1 plays a minimum role in the insulin gene regulation in mature β -cells. However, due to its close association with *ngn3*, *NeuroD*/ β 2, and insulin genes, its biological function cannot be ignored during the early stages of embryonic pancreatic endocrine cell development. Recently, INSM1 has been shown to be essential for pancreatic endocrine development (Gierl *et al.* 2006). During pancreas development, we and others have shown that INSM1 is an immediate downstream target gene of *ngn3* (Mellitzer *et al.* 2006, Breslin *et al.* 2007). The differentiation of adult ductal epithelial cells by *ngn3* clearly indicated that *ngn3* first activates the INSM1 gene and subsequently activates *NeuroD*/ β 2. Our previous studies have shown that the *NeuroD*/ β 2 and E47 heterodimer activate the INSM1 gene (Breslin *et al.* 2003). Conversely, INSM1 can suppress *NeuroD*/ β 2 gene expression (Liu *et al.* 2006). *NeuroD*/ β 2 is a basic helix-loop-helix transcription factor that plays an essential role in β -cell development and insulin gene activation (Naya *et al.* 1995, 1997). These studies situate INSM1 in between *ngn3* and *NeuroD*/ β 2 in pancreatic endocrine cell differentiation, generating a scenario in which *ngn3* initiates the transcription regulatory cascade by first activating INSM1 gene expression, and subsequently activating the *NeuroD*/ β 2 gene (Huang *et al.* 2000, Mellitzer *et al.* 2006). When *ngn3* expression becomes silenced, *NeuroD*/ β 2 continues to activate INSM1 gene expression contrary to its feedback inhibitory mechanism (Breslin *et al.* 2003). Therefore, INSM1 might play a pivotal role in the control of *NeuroD*/ β 2 and insulin gene expression at an early stage of endocrine cell development. Although it is unclear whether insulin gene expression during pancreas development contributes to endocrine cell differentiation, it is speculated that in the presence of INSM1 in the early stages of endocrine cell differentiation, the suppression of insulin gene transcription in premature islets could be an important event in islet maturation. Mellitzer *et al.* (2006) observed that knock down of the INSM1 gene in E12.5 dorsal pancreatic epithelia blocks islet cell differentiation, and results in a 40% decrease in α - and β -cells, whereas the overexpression of *ngn3* and INSM1 in pancreatic duct cells can enhance *NeuroD*/ β 2 and *Pax-4* gene expression as compared with *ngn3* alone. The discrepancy between decreasing the α - and β -cells versus increasing the insulin promoter activity through morpholino treatment could be explained by the fact that our study is directly measuring insulin gene expression instead of α - and β -cell differentiation. It is most likely that either reduction or enhancement of INSM1 gene expression could disturb normal pancreatic endocrine cell differentiation, which subsequently affects mature islet cell formation. Consistently, INSM1 gene ablation results in a dramatic decrease in pancreatic β -cells (Gierl *et al.* 2006). All of these observations suggest that INSM1 should

contribute greatly to pancreatic endocrine cell development. Overall, INSM1 not only plays a role as a transient negative regulator of NeuroD/ β 2, insulin, and its own gene expression, but is also required for endocrine cell differentiation.

The primary function of cyclin D1 is to initiate cell cycle progression by association with cyclin-dependent kinase 4 (CDK4) resulting in the phosphorylation of the Rb protein. Phosphorylation of the Rb protein disrupts its association with E2F, allowing transcriptional activation of S-phase genes. Additionally, cyclin D1 displays CDK4-independent roles in transcriptional regulation (Coqueret 2002). Cyclin D1 inhibits the transcriptional activation of multiple transcription factors and nuclear receptors (Skaper *et al.* 1996, Ganter *et al.* 1998, Inoue & Sherr 1998, Adnane *et al.* 1999, Knudsen *et al.* 1999, Bienvenu *et al.* 2001, Lin *et al.* 2002, Ratineau *et al.* 2002, Fu *et al.* 2005a). The co-repressor activities of cyclin D1 were shown by us and others to be closely associated with the recruitment of HDACs (Lin *et al.* 2002, Fu *et al.* 2005a, Liu *et al.* 2006). By contrast, cyclin D1 could also display inhibition to transcriptional activation by association with the histone acetyltransferase p300 (Ratineau *et al.* 2002, Fu *et al.* 2005b). In our previous studies, we have demonstrated that INSM1 interacts with cyclin D1 to recruit HDACs to form a transcriptional repressor complex on the NeuroD/ β 2 gene (Liu *et al.* 2006). It is conceivable that INSM1 exercises its repression effect upon the insulin gene through a similar mechanism. We performed transient transfection, co-immunoprecipitation, and ChIP assays in insulinoma cells to demonstrate that cyclin D1 cooperates with INSM1 to enhance its repression effect, and that INSM1 is capable of immunoprecipitating endogenous cyclin D1 and HDAC-3. Furthermore, anti-HDAC-3 antibody precipitates the transcriptional complex with the mouse insulin II promoter sequence in β TC-1 cells. A transcriptional complex of INSM1–cyclin D1–HDAC-3 is situated on the insulin gene promoter to confer the repression effect.

In summary, a unique INSM1-binding site in the insulin promoter that downregulated insulin gene transcription, was identified. The repression effect of INSM1 is mediated through the recruitment of cyclin D1 and HDAC-3. These studies demonstrate that INSM1 could be a negative regulator for insulin gene during early endocrine pancreas development.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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