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Negative Regulation of c-Myc Transcription by Pancreas Duodenum Homeobox-1

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The pancreatic and duodenal homeobox factor-1 (Pdx1) is essential for pancreatic development and insulin gene transcription, whereas c-Myc has a deleterious effect on islet function. However, the relationship between c-Myc and Pdx1 is poorly concerned. Here we demonstrated that Pdx1 could suppress c-Myc promoter activity, which relied on T cell factor (Tcf) binding elements harbored in c-Myc promoter. Furthermore, the transcription activity of β-catenin/Tcf was markedly decreased on Pdx1 expression, but cotransfection of Pdx1 short hairpin RNA abrogated this effect. Pdx1 expression did not induce β-catenin degradation nor did it alter their subcellular distribution. The mutation analysis showed that the amino acids (1–209) of Pdx1 harboring an inhibitory domain, which might lead to the reduction of β-catenin/Tcf/p300 complex levels and attenuate their binding activity with c-Myc promoter sequences. Moreover, adenovirus-mediated Pdx1 interference caused cell proliferation and cytokine-induced apoptosis via the dysregulation of c-Myc transcription. These results indicated that the Pdx1 functioned as a key regulator for maintenance of β-cell function, at least in part, through controlling c-Myc expression and the loss of its regulatory function may be an alternative mechanism for β-cell neogenesis and apoptosis found in diabetes. (*Endocrinology* 148: 2168–2180, 2007)

PANCREATIC β-CELLS HAVE a remarkable capacity to maintain glucose levels within a narrow range. The development of diabetes is usually associated with β-cell failure, which is correlated with dysfunction of some important insulin release-related genes. Pancreatic-duodenal homeobox-1 (Pdx1; also called, IPF-1, IDX-1, STF-1, and GSF) is a homeodomain transcription factor essential for embryonic development of pancreas and differentiated pancreatic β-cell function (1–3). Homozygous and point mutations of Pdx1 gene result in pancreas agenesis in some diabetic patients and animal models (3–7). Pdx1 expression is first detected at embryonic d 8.5 in mice embryo (2) and predominantly found in islet β-cells during adulthood, in which it functions as a master regulator of a number of genes, including insulin, glucokinase, and islet amyloid polypeptide (2, 7, 8). A TAAT consensus site (A element) harboring the promoter region of these genes is recognized by N terminus of Pdx1 (9, 10) and evolutionarily conserved among different species, including hamster, rat, mouse, and frog (2, 7, 8, 11).

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Abbreviations: aa, Amino acids; CCK, cholecystokinin; ChIP, chromatin immunoprecipitation; DAPI, 4′,6′-diamino-2-phenylindole; EGFP, enhanced GFP; GFP, green fluorescent protein; GSK, glycogen synthase kinase; MOI, multiplicity of infection; Pdx1, pancreatic and duodenal homeobox factor-1; pEF, elongation factor-1 promoter; RNAi, RNA interference; siRNA, small interfering RNA; TBE, Tcf binding element; Tcf, T cell factor.

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c-Myc is a transcription factor of the basic helix-loop-helix leucine zipper that has been extensively studied as a protooncogene but is also essential for normal cell cycle progression. In some non-β-cell tissues, c-Myc promotes cell growth and proliferation, whereas in others it induces or sensitizes cells to apoptosis. Normal adult islets have low c-Myc expression (12, 13), which is then consistent with a low replication rate (14). Aberrant expression of c-Myc in β-cell markedly perturbs islet development, reduces insulin gene expression, promotes uniform β-cell proliferation, and also induces β-cell apoptosis (15–17), indicating that keeping c-Myc at a relatively low level in β-cells is crucial for normal islet function. However, the molecular mechanisms of c-Myc turnover have not been clearly elucidated. It has been identified that c-Myc is a target gene of Wnt/β-catenin pathway (18), which is activated during pancreas development (19). β-Catenin is identified as the component of the E-cadherin-mediated cell-cell adhesion system (20) and a key effecter of the Wnt signaling pathway, which plays a critical role in growth, division, and cell fate at early and late developmental stage (21–23). In respond to Wnt signals, β-catenin complexes with T cell factor (Tcf)/lymphoid-enhancing factors and p300 to induce transcription of target genes known to be important for normal cell proliferation, such as c-Myc (18) and cyclin D1 (24).

Here we showed that Pdx1 can suppress β-catenin/Tcf-induced c-Myc transcription by inhibiting the level of β-catenin/Tcf and p300 complex formation and reducing their binding capacities to endogenous c-Myc promoter region. Moreover, the dysfunction of Pdx1 in pancreatic cells resulted in β-cell failure accompanied by aberrant cell proliferation and apopto-

sis, which was mediated by the deregulation of c-Myc expression. In brief, we presented evidence that Pdx1 acts as a key regulator for maintenance of β-cell function, at least in part, through controlling c-Myc expression.

Materials and Methods

Plasmids

Expression vectors for β-catenin (wt), β-catenin (S37A), Tcf4, and Tcf (ΔC) were kindly provided by Dr. Axel Ulrich (Max-Planck-Institute of Biochemistry, Germany); human elongation factor promoter, the human c-Myc reporter Del1, Del2, Del3, Del4, and pGL-OT/OF were kindly provided by Daru Lu (Fudan University) and Kenneth-W. Kinzler (Johns Hopkins University, Baltimore, MD) (18).

Cell culture, islet isolation, transfection, and luciferase assay

HEK293, HeLa, HepG2, and HCT116 cells were maintained in DMEM supplemented with 10% fetal calf serum. Stable transfection was performed as described (25). Established clones were isolated and screened by a fluorescence microscopy and Western blot analysis. Islets from 6- to 8-wk-old male C57BJ/6 mice were isolated by collagenase digestion (26). All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by National Institutes of Health (publication 86–23, revised 1985).

In luciferase assays, cells were transfected with a reporter construct (pGL-OT/OF or Del 1, 2, 3, 4), an internal control (pRL-TK; Promega, Madison, WI), and the indicated plasmids in 48-well plates. Luciferase activity was measured at an indicated time after transfection. The histograms are presented as the average ± SD from at least three independent experiments after normalization against *Renilla* luciferase activities.

Northern blot analysis

Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA). RNA (40 ng) was separated and transferred to a nitrocellulose membrane and probe using β-catenin cDNA labeled by random oligonucleotide priming (Promega).

Cell fractionation, immunoblotting, immunoprecipitation, and chromatin immunoprecipitation (ChIP)

The anti-β-catenin and anti-histone H3 monoclonal together with anti-p300 and anti-Pdx1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-green fluorescent protein (GFP) and anti-c-Myc antibodies were from NeoMarkers (Fremont, CA). Cytoplasm and nuclear fractions were prepared by using NE-PER nuclear and cytoplasm extraction reagents (Pierce, Rockford, IL). Protein concentration was determined by the BCA method (Pierce). Immunoprecipitation and ChIP assay were preformed as previous described (27).

Pharmacological inhibitors administration

ALLN (Calbiochem, La Jolla, CA) and expoxomicine (Calbiochem) were used to inhibit protein degradation at 10 μM and 100 nM. Eighteen hours after transfection, inhibitor were added. After 6 h on incubation with the inhibitors, cells were lysed for luciferase assay or immunoblot analysis. Three hours after transfection, LiCl was added.

RNA interference

Oligonucleotides specifically targeted the middle region of murine Pdx1 gene were cloned into the *Bgl*II and *Hin*dIII sites of the pSuper-vector to generate pSuper-Pdx-i. The synthesized oligonucleotides were as follows: pSuper-Pdx i forward, 5′-***GATCCCC***CCCCGAGGAAAACAAGAGGA***TTCAAGAGA***ATCCTCTTGTTTTCCTCGGG***TTTTTA***-3′, reverse, 5′-***AGCTT***AAAAACCCGAGGAAAACAAGAGGAT***TCTCTTGAA***TCCTCTTGTTTTCCTCGGG***GGG***-3′.

Stealth small interfering RNA (siRNA) duplex oligoribonucleotides (Invitrogen) for mouse c-Myc gene were resuspended with diethylpyrocarbonate-treated water to make a 20-μM solution. The sequences were as followed: R1, 5′-AAU CGG ACG AGG UAC AGG AUU UGG G-3′; R2, 5′-UAG UCG AGG UCA UAG UUC CUG UUG G-3′; R3, 5′-UCA CCA UGU CUC CUC CAA GUA ACU C-3′.

Recombinant adenovirus preparation

Pdx1-ABC gene was cloned in *Eco*RI and *Sal*I sites of the shuttle plasmid (pEZ-AV) to generate pEZ-Pdx1-ABC. Pdx-i was cloned into *Nhe*I and *Hin*dIII sites of pEZ-AV together with H1 promoter. The recombinant adenovirus was made in HEK293A cells by homologous recombination between prepared shuttle plasmids (pEZ-Pdx1-ABC or pEZ-Pdx i) and the backbone plasmid (pBHG). Production of recombinant adenovirus was performed as previously described.

Cell proliferation, 4′,6′-diamino-2-phenylindole (DAPI) staining, and apoptosis assay

The cells (ASPC-1, 5×10^3 cells/well; β-TC 6, 2×10^4 cells/well) together with recombinant adenoviruses at indicated multiplicity of infection (MOI) were seeded in quintuplicate in 96-well flat-bottomed plates. MOCK (without adenovirus), Ad-Blank, and Ad-EGFP were used as controls. Each day 1:10 volume of cholecystokinin (CCK)-8 solution (Dojion Laboratories, Osaka, Japan) was added to each well and incubated for 1.5 h at 37 C. The plates were read in a microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA) at 570 nm. Each experiment was repeated twice. To visualize the nuclei of ASPC-1 cells and normal islet cells, DNA was stained with DAPI (1 μg/ml) and determined using fluorescence microscopy (IX70; Olympus, Tokyo, Japan). The numbers of apoptotic nuclei were counted in three random fields (>100 cells) each well, and percent apoptosis was determined based on the total number of cells. The apoptotic ratio of β-TC 6 cells was measured with Annexin V-based apoptosis detection kit (Oncogene, Boston, MA) by flow cytometry.

Results

Pdx1 reduced c-Myc promoter activity

To evaluate whether Pdx1 is physiologically relevant to the level of c-Myc expression, HEK293 cells were applied to exogenously express Pdx1 or the control vector for their high transfection efficiency. As shown in Fig. 1A, Pdx1 expression significantly reduced c-Myc expression level in HEK293 cells up to 50%. Consistently, c-Myc promoter-reporter activity was reduced by approximately 2-fold on cotransfection with Pdx1 expression vector (Fig. 1B). These results suggested that Pdx1 could exert inhibitory effect on c-Myc transcription. Given the critical role of β-catenin in regulating c-Myc expression (18), we hypothesized that Pdx1 might suppress the c-Myc gene expression by inhibiting β-catenin signaling. To test this proposal, we cotransfected Pdx1 with three deletion forms of c-Myc promoter reporter plasmids (Del2, Del3, or Del4) into 293 cells, respectively (18). Similarly, the luciferase activities of the smaller c-Myc promoter deletion constructs (Del2 and Del3) were attenuated in the presence of Pdx1 (Fig. 1D). Interestingly, the luciferase activity of the smallest c-Myc promoter deletion construct [Del4, which lacks the whole Tcf binding element (TBE)] was not affected after transfection with Pdx1, indicating that the TBEs within the c-Myc promoter are essential for the Pdx1-induced repression of c-Myc promoter activity. Taken together, these data suggested that Pdx1 expression evidently reduced c-Myc (Del 1, Del2, and Del3) promoter activities by inhibiting β-catenin-dependent transcription.

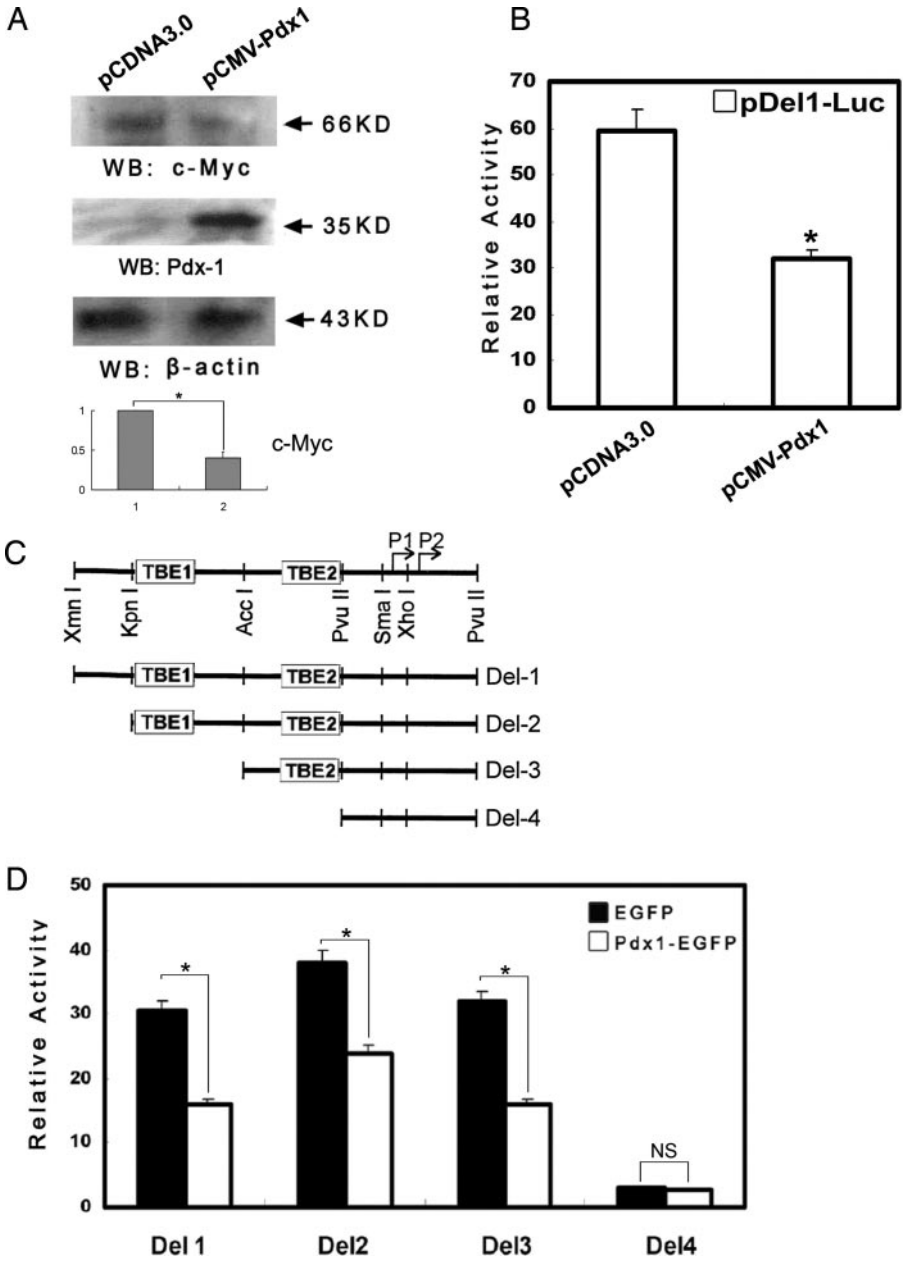

FIG. 1. Pdx1 inhibited c-Myc transcription. A, c-Myc protein was revealed by Western blot analysis (WB) on a 10% SDS-PAGE (*upper panel*). Pdx1 expression was determined with antibody specific for Pdx1 (*middle panel*). The same blot was also probed with antibody to β-actin to ensure that the same amount of protein was presented in each lane (*lower panel*). The *bar graphs* present the relative c-Myc protein amounts, normalized against β-actin intensity. B, HEK293 cells were cotransfected with the c-Myc reporter (Del1) together with a Pdx1 expression construct or a control plasmid. Luciferase activities were determined 36 h after transfection, shown as relative activity normalized with pRL-TK (Promega) values. Data were presented as the mean ± SD determined from three transfections. The total amounts of plasmids in each well were uniform by adding pcDNA3.0 vector. C, Map of the c-Myc promoter showing the restriction sites used for generating nested deletions (Del constructs). The APC- and β-catenin-responsive elements (TBE1/2) were near *Kpn*I and *Pvu*II enzyme sites. The *horizontal lines* represent the sequences in each reporter construct, which were placed upstream of a minimal promoter and luciferase cassette. P1 and P2 are start sites of transcription; P2 is the major start site. D, 293 cells were cotransfected with the indicated reporters plus Pdx1 or control plasmids. Luciferase activity was determined as before. *, $P < 0.05$. NS, No significant difference.

Pdx1 inhibited β-catenin-mediated transcriptional activity

To confirm observations made with the c-Myc promoter reporter constructs, we examined the ability of Pdx1 to inhibit β-catenin signaling. Pdx1 was cotransfected with Tcf reporter plasmid pGL-OT, which contains three tandem copies of TBE upstream of a minimal promoter, into 293 cells. As shown in Fig. 2A, the induction of Pdx1 suppressed the luciferase activity by nearly 70%. We also used LiCl, an inhibitor of glycogen synthase kinase (GSK)-3β activity, to increase endogenous β-catenin levels and detect its transactivation potential after transfection with Pdx1 (28–30). β-Catenin activity was elevated by 4-fold in empty vector-transfected cells, whereas this effect was significantly reduced in cells transfected with Pdx1. LiCl treatment also activated the c-Myc promoter-Luc constructs by 3- to 4-fold in the absence of Pdx1. In contrast, 70% of c-Myc activity was lost with coexpression of Pdx1 (data not shown). The specificity of Pdx1-mediated effects on Tcf reporter was confirmed by using pGL-OF, which harbors mutated Tcf binding sites and was not influenced by Pdx1. Because it has been identified that the chibby expression could repress endogenous β-catenin transcriptional activity to a rather low level in HEK293 cells (31), we further explored the regulatory effect of Pdx1 on exogenous β-catenin-mediated transcriptional activity. Because Pdx1 could repress human cytomegalovirus immediate early promoter activity (32), β-catenin cDNA was inserted under human elongation factor-1 promoter (pEF), which was not influenced by Pdx1 expression (Fig. 2B). In 293 cells, exogenous β-catenin expression led to up-regulation of the pGL-OT activity by nearly 9.5-, 25-, 45-,

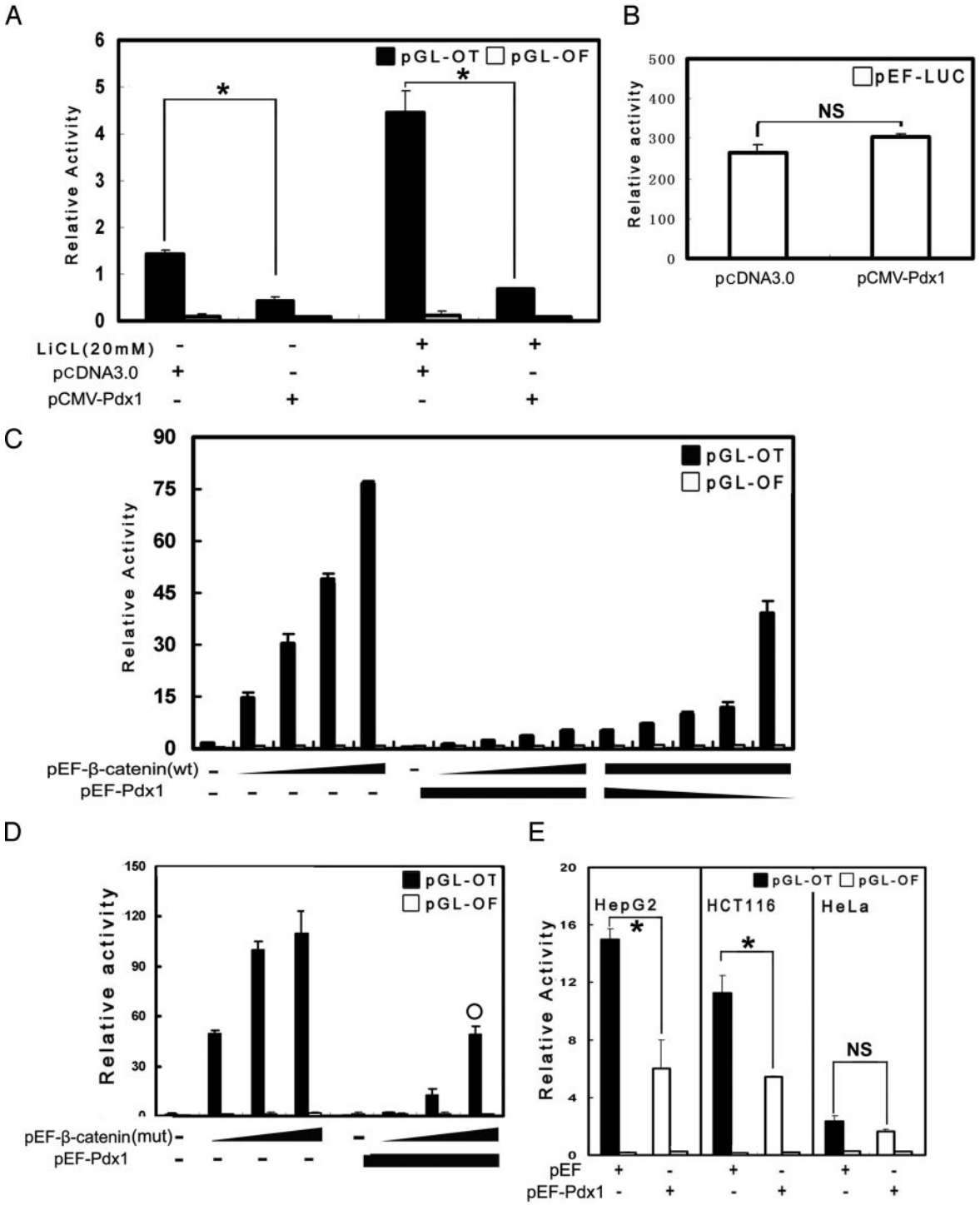

FIG. 2. Pdx1 suppressed β-catenin/Tcf signaling activity. β-Catenin/Tcf transcriptional activity was indicated by pGL-OT luciferase activity. pGL-OF, which contains mutant Tcf binding sites, was used as a negative control for pathway specificity. A, Twenty hours after transfection, 293 cells were treated with 20 mM LiCl for 24 h before cells harvested. B, 293 cells were cotransfected with luciferase reporter plasmids harboring the human elongation factor-1 promoter, Pdx1, and control as indicated. Luciferase activity was assayed 24 h after transfection. C, 293 cells were transfected with pGL-OT or pGL-OF reporter plasmids (150 ng), pRL-TK plasmid (1.5 ng), and the increasing amounts of pEF-β-catenin vector (20, 50, 100, 200 ng), pEF-Pdx1 plasmid (10, 50, 150 300,100 ng) as indicated. D, 293 cells were transfected with pGL-OT or pGL-OF reporter plasmids (150 ng), pRL-TK plasmid (1.5 ng), and the increasing amounts of constitutively active form of β-catenin (S37A) vector (0, 50, 100, 200 ng), pEF-Pdx1 plasmid (0, 400 ng) as indicated. *Open circle* indicated the restoration of β-catenin activity with high amount of mutant β-catenin. E, Three different tissue-derived cell lines, HepG2, HCT116, and HeLa, were cotransfected with pGL-OT or OF together with Pdx1 or control vector as indicated. Luciferase activity was measured 24 h after transfection. *, $P < 0.05$. NS, No significant difference.

and 57-fold at 20, 50, 100, and 200 ng, respectively (Fig. 2C). When pEF-β-catenin was cotransfected with pEF-Pdx1, the luciferase activity was drastically reduced at any amounts of β-catenin used (20–200 ng). In addition, exogenous expression of Pdx1 also imposed a substantial inhibition of the pGL-OT promoter activity in a dose-dependent manner.

To further test whether Pdx1 could modulate the transcriptional activities of the degradation-resistant mutants of β-catenin, we cotransfected S37A mutant β-catenin construct, which lost the ability of GSK-3β-dependent phosphorylation and degradation, together with Pdx1 and the Tcf reporter plasmids pGL-OT into HEK293 cells. Cotransfection of Pdx1 significantly reduced S37A mutant form-induced Tcf reporter activation, indicating that the inhibitory effect of Pdx1 on β-catenin activity is independent of its degradation (Fig. 2D). To confirm these results, we then determined the effects of Pdx1 on β-catenin signaling in cells with aberrant β-catenin activation. As expected, expression of Pdx1 reduced pGL-OT reporter activity by 60 and 50% in HepG2 and HCT116 cells, respectively, but not in Hela cells in which nuclear β-catenin activity was negligible (Fig. 2E). In addition, as shown in Fig. 2D, high amounts of mutant β-catenin partially restored Pdx1-mediated inhibition, which might result from the overcapacity of β-catenin signal pathway by so many mutant β-catenin in cells, simultaneously. Taken together, these results suggested that Pdx1 could exert specifically inhibitory effect on β-catenin-mediated transcriptional activity.

Pdx1 down-regulated the β-catenin-induced signaling in β-cells

Further evidence supporting a role for Pdx1 came from experiments using RNA interference (RNAi) techniques to abolish Pdx1 expression. Pdx1 short hairpin RNA expression vectors were generated in the pSuper-background (Fig. 3A). As shown in Fig. 3B, The expression of the Pdx1 protein was evidently reduced in cells transfected with pSuper-Pdxi construct (90% reduction) in a dose-dependent manner, compared with those transfected with pSuper-GFPi or pSuper-null constructs. Moreover, a 3-fold increase in pGL-OT reporter activity was observed in the presence of pSuper-Pdxi construct, and no apparent effects were detected when transfected with pSuper-null construct (Fig. 3C). We also performed the RNAi experiments in β-TC 6 cells, an islet-derived insulinoma cell line, and obtained 2-fold increase of pGL-OT reporter activity.

To characterize the capacity of Pdx1 in regulation of c-Myc expression under physiological conditions, we developed Pdx1-expressing and Pdx1 shRNA-expressing adenoviruses both carrying GFP marker and investigated their effects on the pancreas- or islet-derived cell lines. Infection of Pdx1-negative ASPC-1 pancreas cancer cells with Ad-Pdx1 adenovirus at MOI of 20 yielded nearly 90% of culture cells positive for GFP (Fig. 3D), and the expression level of Pdx1 was verified by Western blot analysis, which also resulted in a nearly 2-fold decrease of c-Myc protein (Fig. 3E). On the contrary, the infection of Pdx1-positive islet β-TC6 cells with Ad-Pdxi efficiently knocked down endogenous Pdx1 expression and led to an increase of c-Myc protein levels up to 3-fold. Similar results were obtained in normal islet cells 48 h after treatment with Ad-Pdxi (Fig. 3E, *right panel*).

Pdx1 expression had no effect on β-catenin expression and localization

To explore the molecular mechanism underlying Pdx1-mediated suppression of β-catenin signaling activity, cDNAs encoding enhanced GFP (EGFP) and Pdx1-EGFP were stably transfected into HEK293 cells, respectively (Fig. 4, A and B). The fluorescence was distributed throughout the entire cell with EGFP expression (Fig. 4Ab), whereas Pdx1-EGFP was observed primarily in the nucleus (Fig. 4d). Consistently, 60% of the pGL-OT luciferase activity was lost in HEK293-Pdx1-EGFP cells (Fig. 4C). However, the mRNA or protein level of β-catenin was not affected on Pdx1 expression (Fig. 4, D and E), even in the presence of LiCl or epoxomicin.

Several studies have reported that extranuclear sequestration of β-catenin results in the reduction of β-catenin/Tcf-mediated transcriptional activity (33–35). Thus, we examined the issue of whether nuclear trafficking of β-catenin is blocked by Pdx1 expression. Nuclear and cytoplasm protein extractions were obtained from HEK293-EGFP and HEK293-Pdx1-EGFP cells. Western blotting showed that free cytoplasm and nuclear β-catenin levels in HEK293-EGFP and HEK293-Pdx1-EGFP cells were equivalent after treatment with LiCl, epoxomicin, or ALLN. In addition, treatment of the cells with epoxomicin resulted in an apparent increase in β-catenin levels with the typical pattern of multiple ubiquitylated bands, which were detected in both cytoplasm and nuclear fraction (Fig. 4F). These results indicated that Pdx1 had no effect on β-catenin expression and its subcellular distribution.

The amino acid (1–209) of Pdx1 was crucial for its effect on Tcf reporter activity

To investigate the functional domains of the Pdx1 protein necessary for regulating β-catenin/Tcf transcriptional activity, the various fragments of the gene were fused in frame to the pEGFP-N2 vector, as depicted in Fig. 5A. Expression of EGFP and different Pdx1-EGFP deletion constructs was analyzed by immunoblotting (Fig. 5B). We also examined the cellular localizations of full-length and mutant forms by a fluorescence microscopy. Consistently with previous report (36), the fusion proteins containing amino acids 150–209 of Pdx1 localized mostly in the nucleus (Fig. 5C). We next examined which domain in Pdx1 was necessary for its regulatory role of β-catenin-dependent transcriptional activity. The EGFP fusion protein expression plasmids, together with pGL-OT reporter plasmid were cotransfected into 293 cells and luciferase activities were determined 24 h after administration of LiCl. In agreement with previous study, about 50% of activity was reduced with Pdx1 (full length) expression. The activity of pGL-OT was also inhibited by mutants Pdx1-BCD [amino acids (aa) 80–283)], Pdx1-BC (aa80–209), or Pdx1-ABC (aa1–209). In contrast, little if any further effect on the reporter activity was conferred by expression of Pdx1-AB (aa1–149), Pdx1-A (aa1–79), Pdx1-B (aa80–149), or Pdx1-C (aa150–209) (Fig. 5D). Similar results were also observed with the use of c-Myc promoter reporter construct (Del1) (Fig. 5E). Interestingly, the most significant effect was obtained with the Pdx1-ABC (1–209) mutant, which caused an approximately 75% reduction of the activation. Taken together, these results indicated that amino-terminal domain (aa1–149) and homeodomain (aa150–209) of Pdx1 were

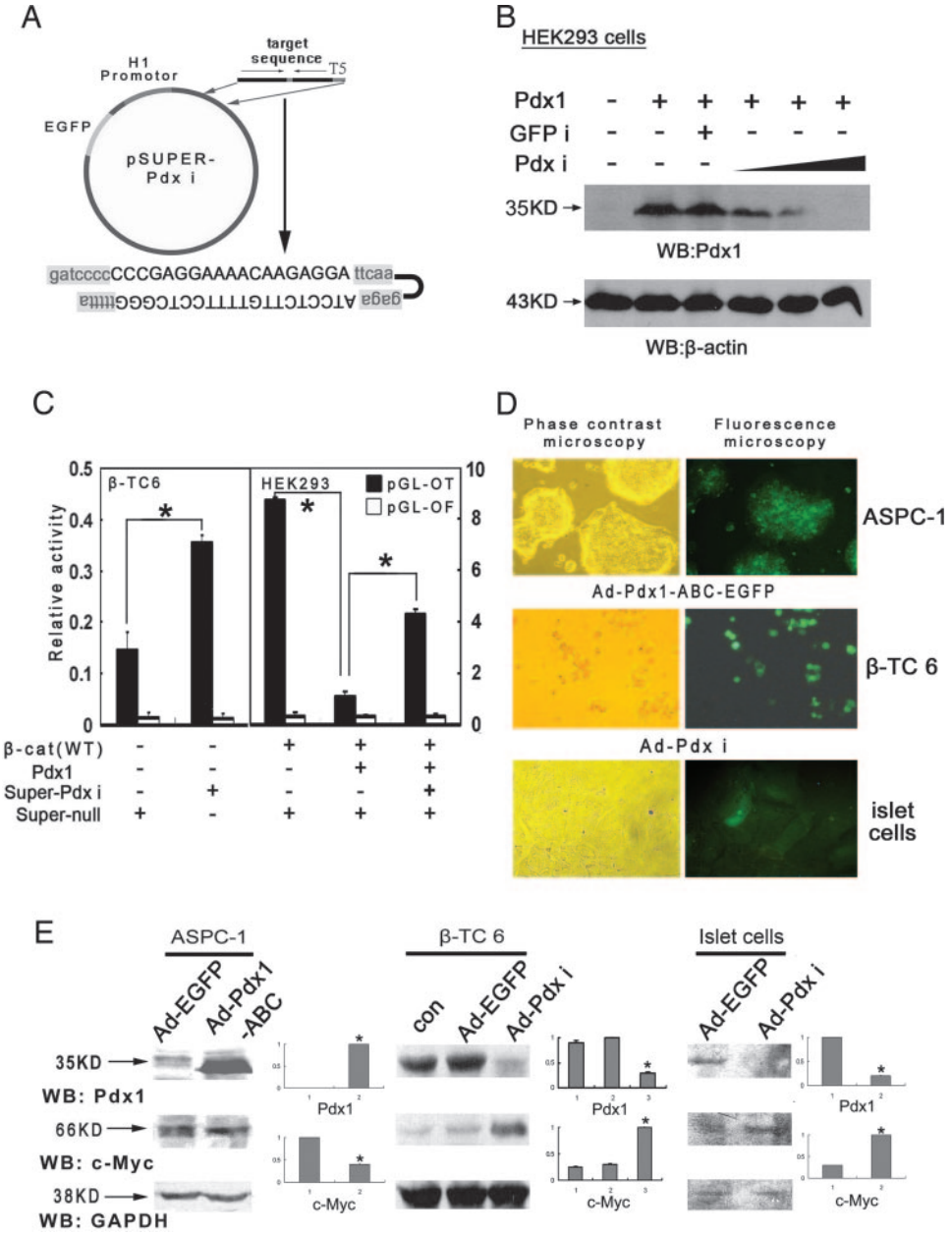

FIG. 3. Pdx1 down-regulated the β-catenin-induced signaling in β-cells. A, Schematic representation of pSuper-Pdx-i. The *capital letters* represent the specific sequence targeting the murine Pdx1 transcription. B, The expression of Pdx1 in transfected 293 cells was analyzed by Western blot (WB) with anti-Pdx1 antibody in the presence of pSuper-GFPi or pSuper-Pdxi constructs. C, HEK293 or β-TC6 cells were transfected with pSuper-Pdxi or Pdx1 as indicated in the presence or absence of pEF-β-catenin as indicated. pGL-OT reporter activity was measured 24 h after transfection. D, ASPC-1 or β-TC6 and primary islet cells were infected with Ad-Pdx1 or Ad-Pdx-i at MOI of 20 or 30 and 20, respectively. Twenty-four hours after infection, cells were analyzed with a phase-contrast microscopy, and EGFP expression was checked with fluorescence microscopy. E, The expression of Pdx1, c-Myc, and GAPDH in ASPC-1, β-TC6 or islet cells was analyzed by Western blot (WB) 48 h after infection. The *bar graphs* present the relative Pdx1 or c-Myc protein amounts, normalized against GAPDH intensity. *, $P < 0.05$. NS, No significant difference; Con, control.

crucial for its repression on β-catenin/Tcf-mediated transcriptional activity.

Pdx1-down-regulated β-catenin signaling could be rescued by the expression of Tcf4

Because β-catenin could form a complex with p300 in the presence of Tcf4 (37), which is essential for c-Myc transcription activation (38), we reasoned that Pdx1 may exert its repression on the β-catenin/Tcf4/P300 complex directly. To test this proposal, 293 cells were transfected with PDX-1, and an immunoprecipitation analysis was performed with p300 and β-catenin-specific antibodies. The precipitated proteins were probed for the presence of P300, β-catenin, and Pdx1. The addition of Pdx1 profoundly reduced the amount of β-catenin and P300 coprecipitating with either p300 or β-catenin (Fig. 6A). In contrast, Pdx1 did not affect P300 or β-catenin protein expression. As shown in Fig. 6B, similar results were observed in β-TC6 cells after Ad-Pdxi infected.

To further investigate the issue of which component is crucial for Pdx1-mediated suppression of β-catenin/Tcf4 transcription activity, we introduced Pdx1 together with pEF-β-catenin, pEF-P300 or pEF-Tcf4 constructs into HEK293 cells in the presence of LiCl and monitored the pGL-OT activity after 48 h. As shown in Fig. 6C, the expression of Pdx1 could significantly repress the β-catenin and P300-induced signal activity. The fold repression of pGL-OT activity (pEF *vs.* pEF-Pdx1) was increased by approximately 4- or 5-fold in the presence of β-catenin or P300, compared with the control vector. However, the fold repression was obviously reduced to nearly 1-fold with full-length Tcf4 ex-

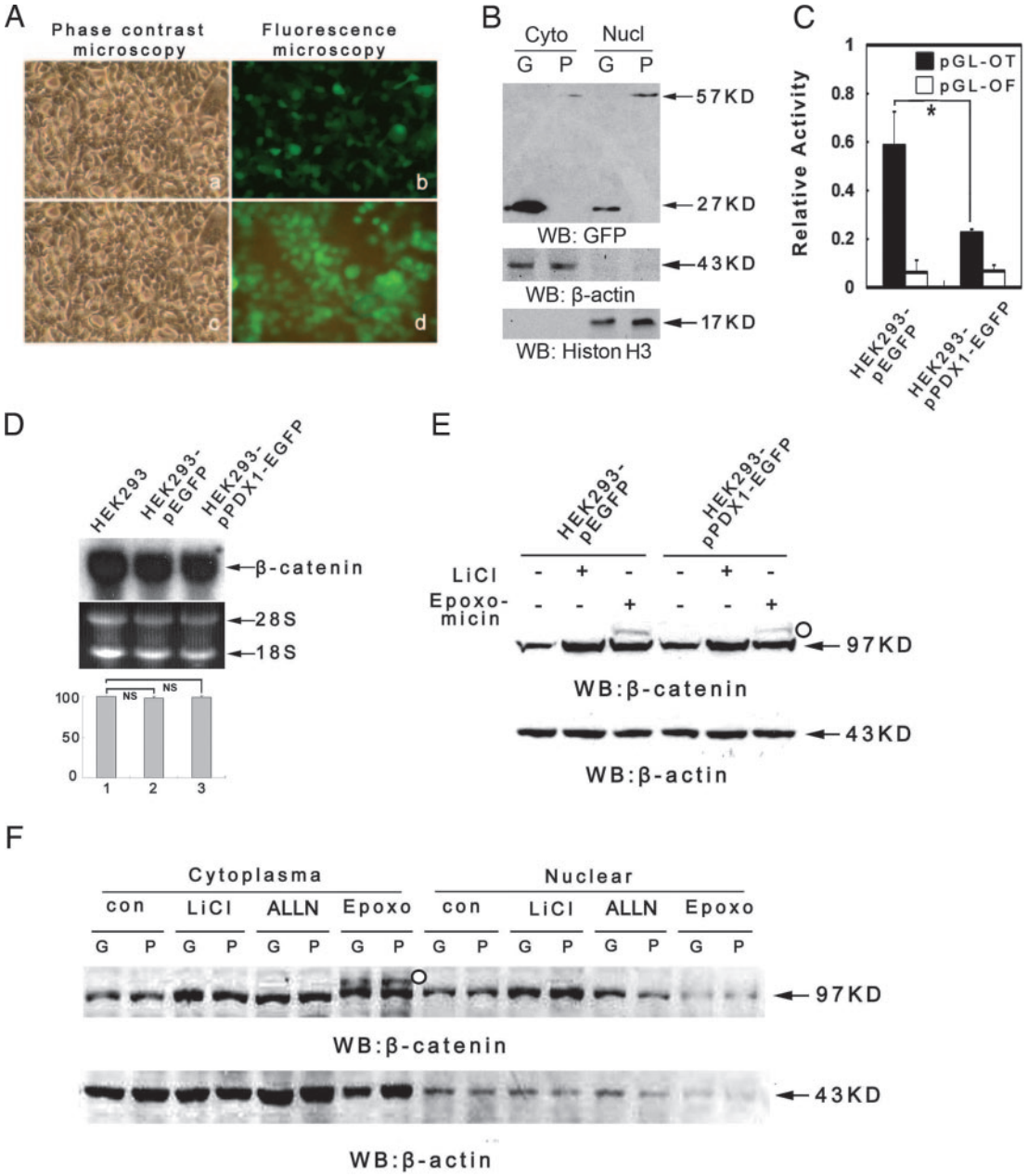

FIG. 4. Pdx1 expression had no effect on endogenous β-catenin levels or intracellular distribution. A, 293 cells were transfected with indicated plasmids. After selection of the G418-resistant colonies, HEK293-EGFP and HEK293-Pdx1-EGFP were observed with a fluorescence microscope. a and b, HEK293-EGFP cells; c and d, HEK293-Pdx1-EGFP cells. B, Expression of Pdx1-EGFP and EGFP in nuclear (Nucl) and cytoplasm (Cyto) was analyzed by Western blot with anti-EGFP antibody, anti-β-actin (specific to cytoplasm), and anti-histone H3 (specific to nuclear) antibodies. C, pGL-OT or pGL-OF was transfected into HEK293-EGFP or HEK293-Pdx1-EGFP normalized with pRL-TK. Luciferase activities were determined 36 h after transfection. D, β-Catenin mRNA levels were analyzed by Northern blot. Total RNA (30 μg) was isolated from HEK293, HEK293-EGFP, and HEK293-Pdx1-EGFP and then hybridized to a probe specific for β-catenin. The *bar graphs* present the relative mRNA amounts in three cells, normalized against 28S/18S intensity with those of untransfected cells being arbitrarily set at 100. E, 293 stable cells were treated with 20 mM LiCl, and 100 nM epoxomicin for 24 and 6 h before cells were harvested for Western blot analysis (WB) with the antibodies indicated. F, Two stable cells were grown in absence or presence of LiCl, ALLN (10 μM), or epoxomicin for 20, 6, and 6 h. Cytoplasma and nuclear were examined by Western blot analysis with the anti-β-catenin antibody. Con, Control. G, HEK293-EGFP; P, HEK293-Pdx1-EGFP. *Open circle* indicated the ubiquitylated bands of β-catenin. *, $P < 0.05$.

pression (Fig. 6, C and E). Similar results were observed with the use of c-Myc promoter reporter construct (Del1). As shown in Fig. 6D, the introduction of Tcf4 abolished the effect of Pdx1 on Del1 reporter activity efficiently. Notably, only induction of Tcf4 could activate β-catenin signal pathway besides β-catenin and P300 in β-TC6 cells as expected (Fig. 6F). Consistently, ChIP assay revealed that β-catenin was indeed recruited less to the promoter regions of c-*myc* gene in islet β-TC6 cell pool than in Ad-Pdxi-infected cell pool (Fig. 6G).

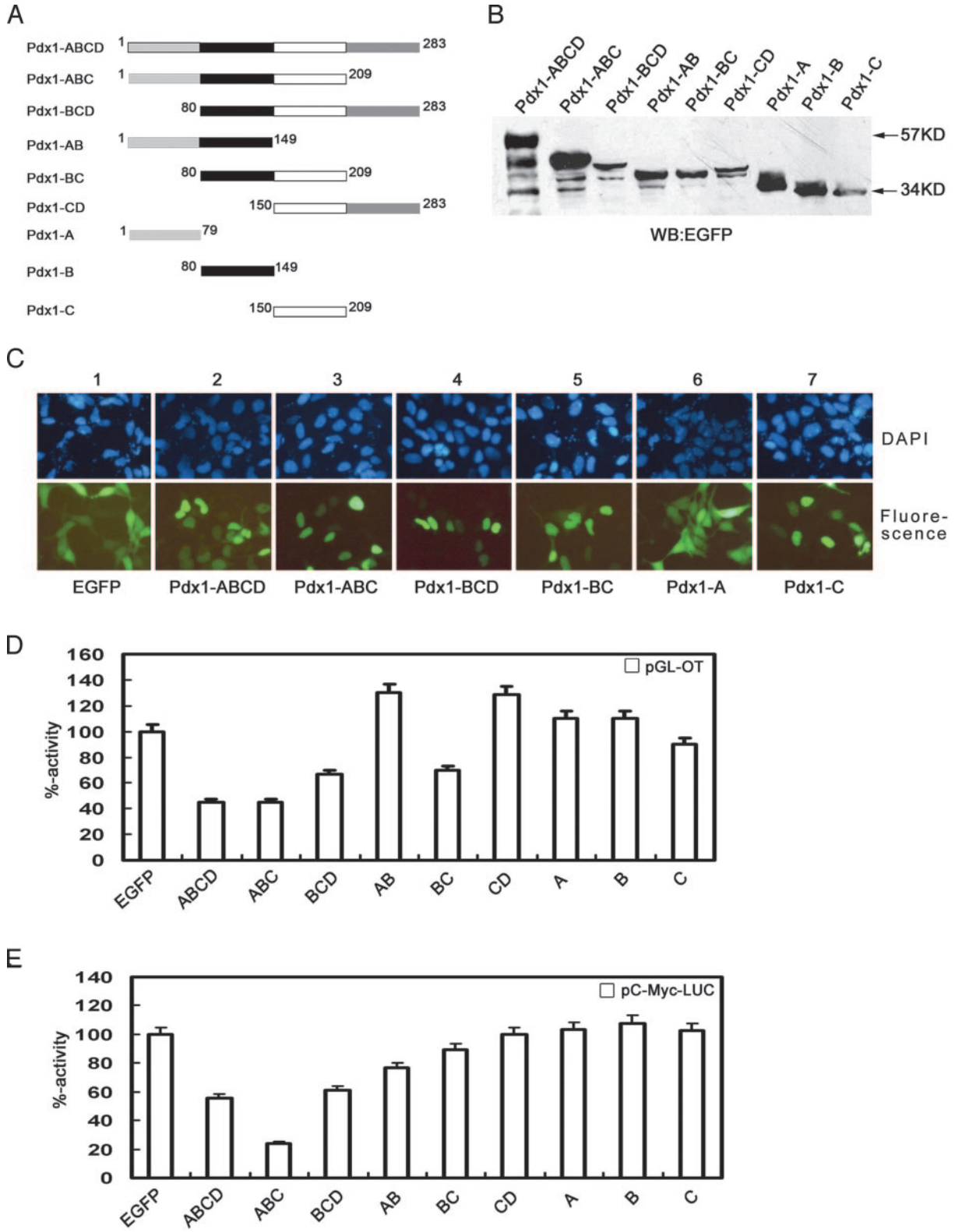

FIG. 5. The amino acids (1–209) of Pdx1 were necessary for its inhibitory effect on β-catenin activity. A, Scheme of Pdx1 deletion constructs. Amino acid positions of the deletion end points were given. All constructs were fused with EGFP protein at the C terminus. B, Expression of the Pdx1 deletion mutants was analyzed by Western blotting (WB) with anti-EGFP antibody. C, The intracellular location of Pdx1 mutant constructs were determined under fluorescence microscopy (*bottom panels*) and further compared with DAPI staining (*upper panels*) in the same field. D, 293 cells were transfected with pGL-OT and Pdx1 mutant vectors. Reporter gene activity with EGFP was arbitrarily assigned the value of 1. E, Same experiment as in D with pGL-OT replaced by c-Myc promoter reporter (Del1).

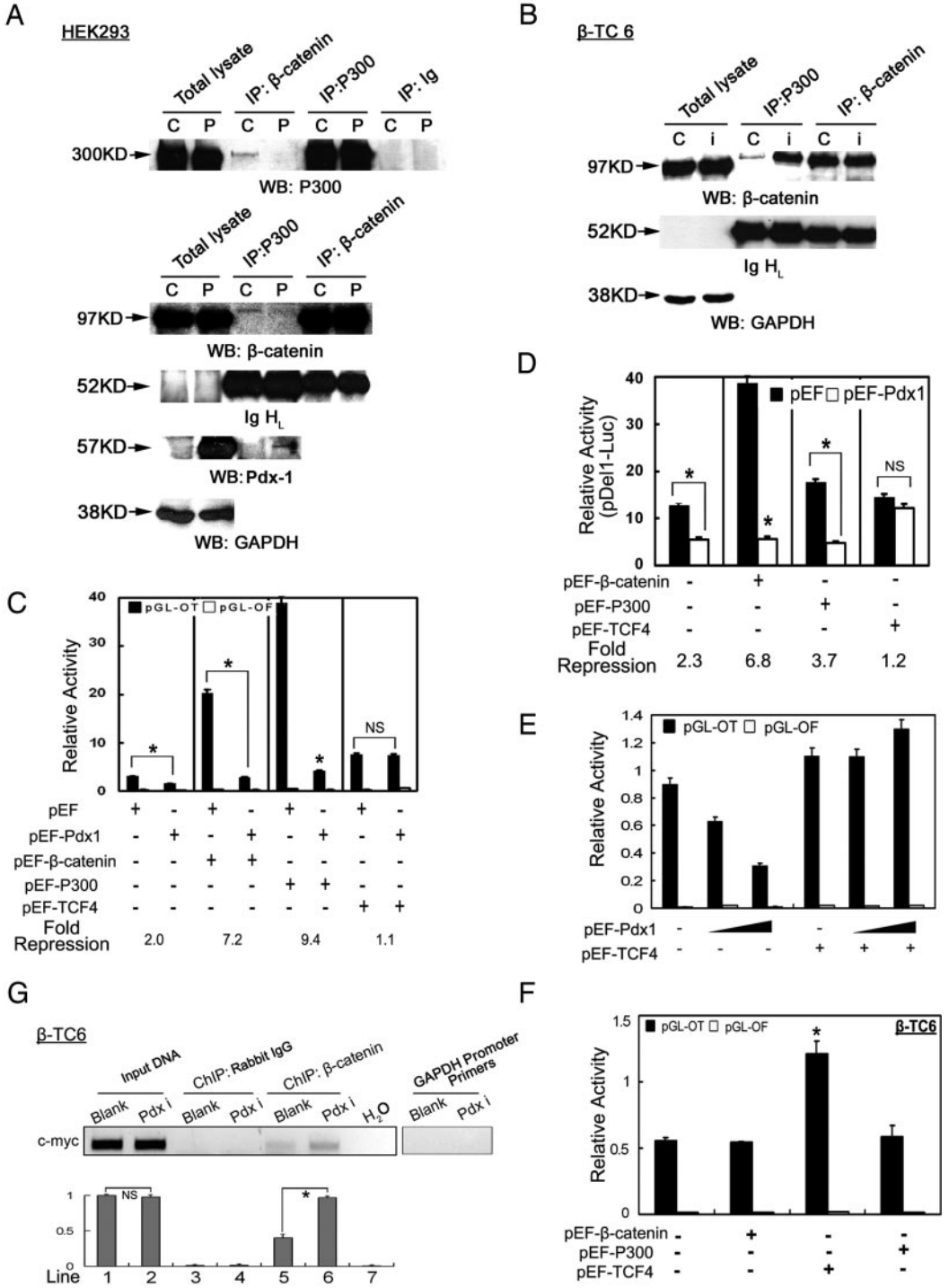

FIG. 6. The expression of Tcf restored the Pdx1-mediated repression of β-catenin activity. A and B, HEK293 or β-TC6 cells was introduced with Pdx1 or Pdxi and harvested after 36 h for immunoprecipitation (IP) and Western blot (WB) analyses. The cells were lysed in radioimmunoprecipitation assay buffer and incubated overnight with antiserum to Pdx1, P300, β-catenin, or normal IgG (Ig). Detection was performed using enhanced chemiluminescence after incubation with a horseradish peroxidase-conjugated secondary antibody. C, 293 cells were transfected with pGL-OT or pGL-OF and pEF-β-catenin, pEF-P300, or pEF-Tcf4 together with pEF-Pdx1. Luciferase activity was assayed 24 h after transfection. D, 293 cells were transfected with pDel1-Luc and pEF-β-catenin, pEF-P300, or pEF-Tcf4 together with pEF-Pdx1. E, 293 cells were transfected with pGL-OT, pGL-OF, and pEF-Tcf4 or pEF together with increased amounts of Pdx1-EGFP (50, 200 ng) in the presence of pEF-β-catenin (150 ng). F, β-TC 6 cells were transfected with β-catenin, Tcf4, or p300 together with pGL-OT or pGL-OF. Luciferase assay was performed as before. G, ChIP done with chromatin from β-TC 6 cell pool using β-catenin-specific antibody or preimmune rabbit IgG as a negative control for precipitation (*upper panel*). Analysis was done using specific primer for promoter region of c-Myc containing TBE. As controls, one fiftieth of the starting chromatin (input), water (negative), and GAPDH promoter primers were used. *Each column* represents the relative density of certain band. *, $P < 0.05$. NS, No significant difference.

Taken together, our data strongly indicated that Pdx1 inhibited c-Myc gene transcription by preventing communication between the P300 coactivator and key DNA-bound activators, like β-catenin and Tcf4, and that could be rescued by the introduction of Tcf4.

Pdx1 regulated pancreatic cell growth and apoptosis through repressing c-Myc expression

Because overexpression of c-Myc could perturb islet development with alteration in β-cell proliferation and apoptosis that result in early-onset diabetes or neoplastic lesion (15), we thus explored whether Pdx1-mediated repression of c-Myc transcription was involved in these processes. After infection with recombination adenovirus Ad-Pdx1 and treatment with TNF-α/CHX, DAPI staining showed clear evidence of condensed and fragmented nuclei in ASPC-1/Ad-EGFP and ASPC-1/null cells, compared with that in ASPC-1/Ad-Pdx1 cells (Fig. 7A). Meanwhile, significant more apoptotic nuclei were found in Ad-Pdxi-infected *vs.* Ad-Blank β-TC6 and primary islet cells (Fig. 7, D and F). To clearly highlight the impact of c-*myc* in Pdxi-mediated increase in apoptosis, RNA interference was performed to repress c-Myc in cells expressing Pdxi. Three stealth siRNA duplexes were applied to inhibit the transcription of c-Myc duplex R3 could significantly interfere the c-Myc protein level in β-TC6 cells, in comparison with R1 and R2 (Fig. 7C).

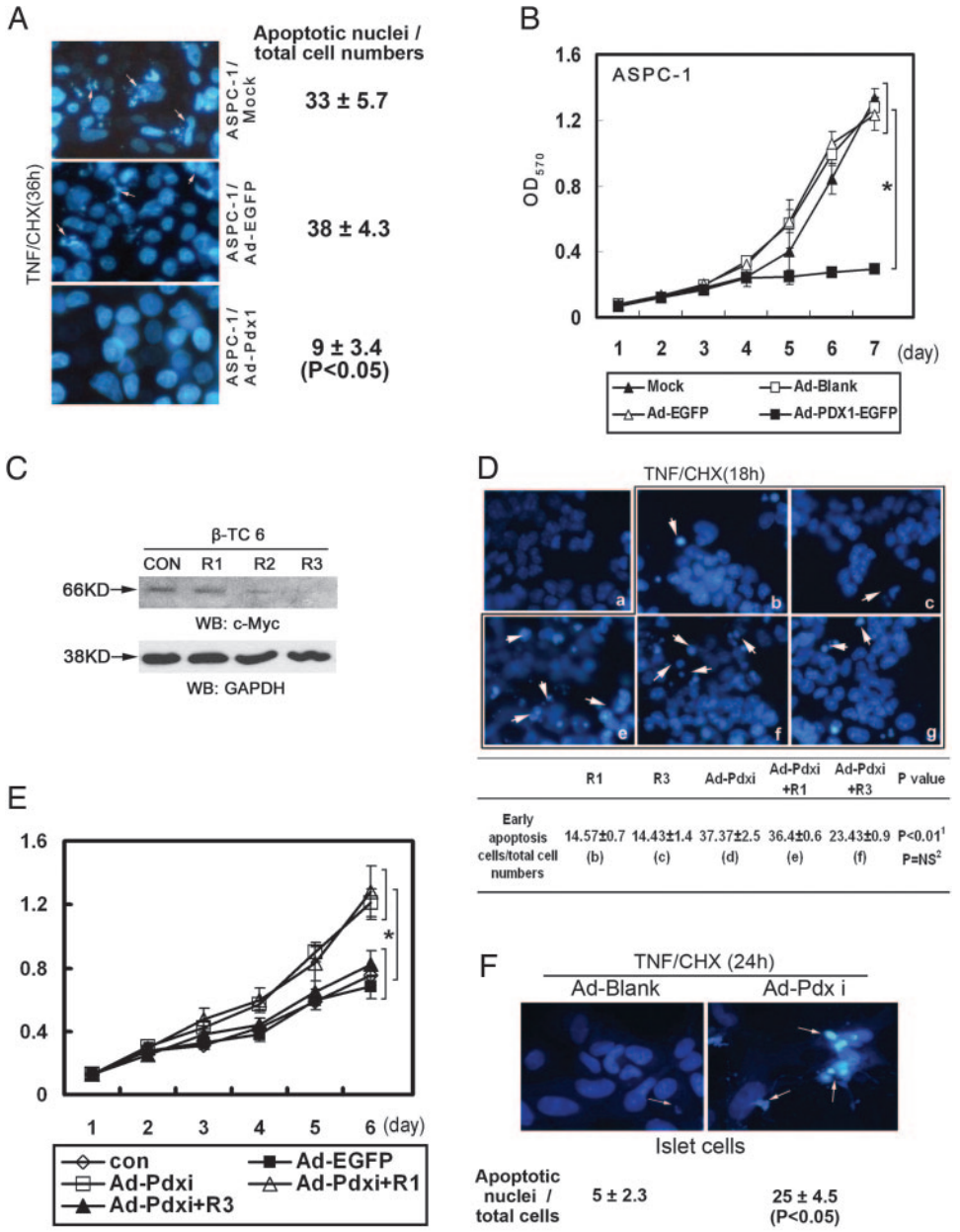

FIG. 7. Pdx1 regulated cell growth and apoptosis through repressing c-Myc expression. A, Nuclear condensation of ASPC-1/mock, Ad-EGFP, and Ad-Pdx1-ABC cells was detected by fluorescence microscopy analysis 36 h after TNF-α (25 ng/ml) and CHX (1 μg/ml) treatment. B, The proliferation of ASPC-1 cells infected with indicated adenovirus was measured by CCK-8 cell proliferation assay. C, Stealth siRNAs, R1, R2, and R3, were introduced into β-TC6 cells, Western blotting (WB) was performed to examine their inhibition on c-Myc protein. D, Apoptotic evaluation of β-TC6 cells determined through annexin V-based apoptotic detection kit. Corresponding images to the numeric counts are presented. a, Normal cells without TNF/CHX treatment; b, c, d, e, f, or g was treated with TNF/CHX for 18 h after the induction of R1, R3, Ad-Pdxi, Ad-Pdxi, and R1 or Ad-Pdxi and R3, respectively. All values are mean ± SD for three independent experiments. *Superscript 1*, *P* values between Ad-Pdxi and Ad-Pdxi and R3; *superscript 2*, *P* values between Ad-Pdxi and Ad-Pdxi and R1. NS, No significant difference; CHX, cycloheximide. E, The proliferation of β-TC6 cells was measured by CCK-8 as before. Con, Control. F, Nuclear condensation of primary islet cells was analyzed by fluorescence microscopy 24 h after TNF/CHX treatment. The numbers of apoptotic nuclei were counted in three random fields (>100 cells) each well, and all values are mean ± SD for three independent experiments. *White arrows* showed the condensed and fragmented nuclei. *, $P < 0.05$.

Annexin V-based apoptotic detection assay was performed to monitor the progression of apoptosis in Ad-Pdxi infected β-TC6 cells. As shown in Fig. 7D (*bottom panel*), the induction of R3 significantly repressed cytokine-evoked apoptosis in Pdxi-infected cells ($P < 0.01$).

Subsequently we evaluated the proliferation of Pdx1-negative or -positive cell lines infected with Ad-Pdx1 or Ad-Pdxi, respectively. As shown in Fig. 7B, exogenous expression of Pdx1 in ASPC cells led to a dramatic reduction in cell growth rate as compared with cells infected with the control viruses (null, Ad-blank, and Ad-EGFP). On the contrary, knockdown of endogenous Pdx1 expression in β-TC6 cells significantly promoted cell proliferation (Fig. 7E), which could be restrained by the induction of c-Myc RNAi, R3. Taken together, these findings further indicated that Pdx1-mediated repression of c-Myc transcription is capable of regulating pancreatic β-cell proliferation and apoptosis.

Discussion

In diabetic patients and animal models, β-cells have been found to lose the unique differentiation that optimizes glucose-induced insulin synthesis and secretion (12, 39, 40). This loss may result from the reduced expression and/or activity of the β-cell transcription factor Pdx1 and the increased expression of the transcription factor c-Myc, a potent stimulator of cell growth (15). Normal adult islets have low c-Myc expression (16), but the transcription of c-Myc is induced in diabetic rats after partial pancreatectomy and rats made hyperglycemic with glucose clamps *in vivo* and *in vitro* (12, 39). Overexpression of c-Myc in β-cells of transgenic mice exerted a markedly deleterious effect on islet development, with abnormality of β-cell proliferation, disruption of islet formation, and down-regulation of insulin gene expression that result in early onset diabetes and neonatal lethality (15). However, the molecular mechanism responsible for the increased c-Myc expression in diabetes has not yet been elucidated.

It is well known that c-Myc is directly regulated by β-catenin/Tcf complex via TBE and plays a vital role in β-catenin-mediated cell transformation and proliferation (18). In this study, we presented evidence that Pdx1 could suppress c-Myc gene transcription by inhibiting β-catenin/Tcf-mediated transcriptional activation in different cell lines with or without aberrant β-catenin activation, which was independent of β-catenin degradation sensitivity or cellular distribution. To localize the Pdx1 effective domain, various Pdx1 mutants were cotransfected with reporter constructs to analyze their abilities of repression. Interestingly, the Pdx1 mutant (aa1–209) lacking the carboxy-terminal domain retained a strong capacity to suppress the Tcf reporter activity, whereas a Pdx1 mutant encompassing the homeodomain and carboxy-terminal domain (aa150–283), but lacking the amino-terminal 149 amino acids, did not. It has been shown that a binding site for p300 exists within the amino-terminal domain of Pdx1 between amino acids 1 and 143 (37). Thus, it seems that the interaction of Pdx1 and p300 may be crucial in Pdx1-dependent transcription suppression. Moreover, the transcription of c-Myc was regulated by a large complex, which consists of β-catenin, Tcf4/lymphoid-enhancing factors, and p300. Tcf4 that has the DNA binding domain serves as a connector bridging p300 and β-catenin *in vivo*. So the regulated interactions of Pdx1 or Tcf4 with the coactivator p300 are likely important for the transcriptional activation of their target genes (37, 41). In this study, we found that β-catenin/P300 complex levels were reduced by Pdx1 to a similar low in the P300 and β-catenin immunoprecipitation in both HEK293 and β-TC6 cell lines. Meanwhile, β-catenin was indeed recruited less to the promoter regions of c-Myc gene in islet β-TC6 cell pool than in Pdx1 interference cell pool in ChIP assays, and the induction of Tcf4 could rescue the c-Myc reporter activity to a normal level after Pdx1 treatment dramatically. These findings indicated that a substantial component of the observed Pdx1-mediated suppression of β-catenin/Tcf transcriptional activation is mediated by either preventing assembly or destabilizing the p300 complex formed with Tcf4 and β-catenin. In addition, Pdx1 (aa1–209) mutant demonstrated a stronger inhibitory effect on c-Myc promoter activity than did the wild-type Pdx1, suggesting that the Pdx1 carboxy-terminal domain may contribute to conformational states that regulate interactions with p300.

The function of Pdx1 is essential for normal glucose metabolism and insulin production in humans and rodents. Mutations in Pdx1 are associated both with maturity-onset diabetes of the young and the development of type 1 diabetes (4, 5, 42–44). Recently Thomas and colleagues (37) reported that point mutation Pdx1 (S66A/Y68A) impaired its ability to interact with the coactivator p300, thus leading to the reduced Pdx1-mediated transcriptional regulation. We proposed that the reduction of Pdx1 and p300 complex level results in the up-regulation of β-catenin/Tcf-mediated c-Myc transcription that may eventually contribute to β-cell dysfunction. In support of this hypothesis, expression of Pdx1 was found to decrease progressively with increasing hyperglycemia in the 90% partial pancreatectomy model, in parallel with an obvious induction of c-Myc expression accompanied by β-cell hypertrophy and loss of β-cell differentiation (12). Additionally, prolonged culture in low glucose also induced a large increase in c-Myc expression, which was attributed to low-glucose-induced β-cell apoptosis (17). Because culture in low glucose could also induce Pdx1 cytoplasm translocation, it is possible that the attenuated interactions of Pdx1 and p300 in the nuclei make more p300 available for β-catenin/Tcf target gene activation. However, the mechanistic insight to this possibility warrants further study.

c-Myc is known as a potent inducer of apoptosis. Activation of c-Myc in adult, mature β-cells induces uniform β-cell proliferation but is accompanied by overwhelming apoptosis that rapidly results in diabetes (15, 17, 39). Suppression of c-Myc-induced apoptosis by coexpression of Bcl-x(L) exposes multiple neoplastic attributes of Myc and allows it to drive tumor progression (16), which were consistent with previous studies from Brun *et al.* (13) about the role of c-Myc in promoting β-cell proliferation and survival in rat and human islets. Moreover, it has been recently reported that haploinsufficiency of Pdx1 may be responsible for increased apoptosis of islet cells in $Pdx1^{+/-}$ mice (45). We postulated that dysregulation of Pdx1-mediated suppression of the c-Myc gene transcription might contribute to the observed

changes in differentiation and hypertrophy during pancreatic β-cell oncogenesis. Consistent with this suggestion, we have found a decrease of apoptosis and proliferation, in parallel with a reduction of c-Myc protein level, in Ad-Pdx1-infected-ASPC-1 pancreas cancer cells on TNF treatment. Conversely, knockdown of endogenous Pdx1 expression in insulinomas β-TC6 cells led to an expansion in the cell number and cytokine-evoked apoptosis depended on the induction of c-Myc transcription. These observations were consistent with studies that Pdx1 repression in adult mice was associated with impaired expression of insulin and glucagons, increased cell proliferation predominantly in the exocrine pancreas and up-regulation of genes implicated in pancreas regeneration, leading to diabetes within 14 d (46).

In conclusion, the data presented here established that Pdx1 could suppress the transcription of c-Myc through inhibiting the β-catenin/Tcf signal pathway and pose an important mechanism by which the Pdx1 regulates pancreas development and normal pancreatic islet function. Moreover, our results suggested a novel model for further understanding the development of diabetes.

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