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Regulation of Insulin Secretion and β-Cell Mass by Activating Signal Cointegrator 2†

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Activating signal cointegrator 2 (ASC-2) is a transcriptional coactivator of many nuclear receptors (NRs) and other transcription factors and contains two NR-interacting LXXLL motifs (NR boxes). In the pancreas, ASC-2 is expressed only in the endocrine cells of the islets of Langerhans, but not in the exocrine cells. Thus, we examined the potential role of ASC-2 in insulin secretion from pancreatic β-cells. Overexpressed ASC-2 increased glucose-elicited insulin secretion, whereas insulin secretion was decreased in islets from ASC-2$^{+/-}$ mice. DN1 and DN2 are two dominant-negative fragments of ASC-2 that contain NR boxes 1 and 2, respectively, and block the interactions of cognate NRs with the endogenous ASC-2. Primary rat islets ectopically expressing DN1 or DN2 exhibited decreased insulin secretion. Furthermore, relative to the wild type, ASC-2$^{+/-}$ mice showed reduced islet mass and number, which correlated with increased apoptosis and decreased proliferation of ASC-2$^{+/-}$ islets. These results suggest that ASC-2 regulates insulin secretion and β-cell survival and that the regulatory role of ASC-2 in insulin secretion appears to involve, at least in part, its interaction with NRs via its two NR boxes.

Type 2 diabetes is the most common form of diabetes and is a disorder of glucose homeostasis resulting from insulin resistance and relative insulin deficiency (52). Insulin secretion is affected by metabolic signals and transcription factors that are necessary for proper differentiation and growth of various types of pancreatic islet cells (10). The main intracellular signals for insulin secretion derive from glucose metabolism. Glucose metabolism generates oscillations in the ATP/ADP ratio, which lead to the opening and closing of ATP-sensitive K^+ channels and produce subsequent oscillations in membrane potential, cytoplasmic calcium concentration, and insulin release (7).

Studies of human mutations and knockout mice revealed several transcription factors that play crucial roles in pancreatic development, such as PDX-1 (also known as IPF-1) (21), Nkx2.2 (50), Pax4 (47), neurogenin3 (15), and NeuroD (40). The significance of transcription factors in pancreatic development was further reinforced by the analysis of mutations in patients with maturity-onset diabetes of the young (MODY). MODY is a monogenic type of diabetes characterized by early onset, autosomal dominant inheritance and impaired insulin secretion. Although the MODY2 gene encodes a glucokinase specific to the liver and β-cells (3), the remaining five MODY genes encode the transcription factors hepatic nuclear factor 1α (HNF-1α) (MODY3) (58), HNF-1β (MODY5) (18), HNF-4α (MODY1) (57), PDX-1/IPF-1 (MODY4) (48), and NeuroD1 (MODY6) (36). These transcription factors require coactivator or corepressor proteins that modulate the transcriptional machinery to activate or repress transcription. However, the precise roles of coactivators and corepressors in β-cell metabolism and function are only beginning to be elucidated. Recently, PPARγ coactivator 1 (PGC-1), a transcriptional coactivator of nuclear receptors (NRs), was shown to suppress β-cell energy metabolism and insulin release (59).

ASC-2 (activating signal cointegrator 2), also called AIB3, TRBP, RAP250, NRC, PRIP, and NCoA-6, is a recently isolated transcriptional coactivator molecule. It stimulates transactivation by NRs, AP-1, NF-κB, SRF, and numerous other transcription factors (5, 26, 29, 34, 62). Interestingly, ASC-2 contains two nuclear receptor interaction domains (NR boxes), both of which are dependent on the integrity of their core LXXLL sequences (30). The C-terminal NR box specifically interacts with liver X receptors (LXRs), whereas the N-terminal NR box binds numerous other NRs. ASC-2 null embryos exhibited growth retardation, hypoplastic heart development, defective placentation, and embryonic lethality between 8.5 and 12.5 days postcoitus (2, 28, 35, 61); this indicates that ASC-2 is an important coactivator molecule in vivo, especially

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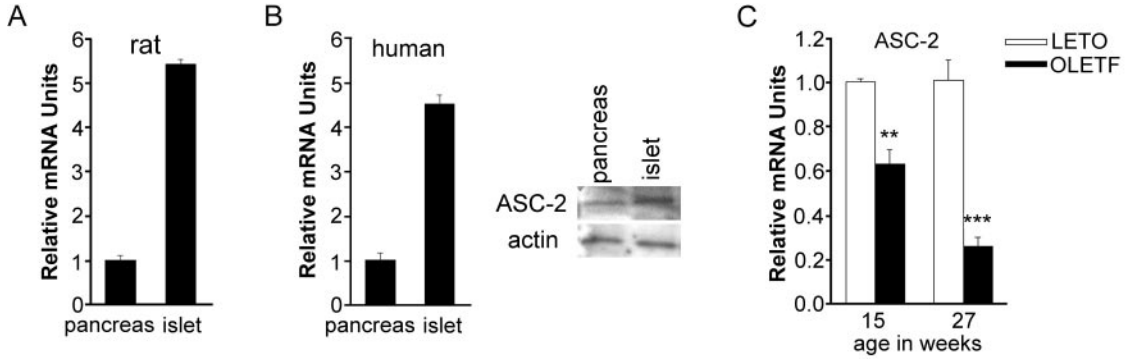

FIG. 1. Expression of ASC-2 in pancreatic islets of rats and humans. (A) Relative abundance of ASC-2 transcripts in islets and whole pancreases from SD rats. Total RNA was extracted from pooled tissues ($n = 3$ to 5); 1 μg per tissue was used for reverse transcription and subsequent QPCR. (B) Relative abundance of ASC-2 was determined in islets and whole pancreas from human by QPCR and Western blotting. (C) ASC-2 expression is lower in islets from diabetic OLETF rats. Total RNA was extracted from pooled islets and subjected to QPCR, as was done in panel A. **, $P < 0.005$; ***, $P < 0.001$.

in embryonic development. However, because of this early lethality, the use of these knockout mice in further studies of the physiological functions of ASC-2 has been limited. Thus, we took an alternative transgenic (Tg) approach, in which dominant-negative fragments of ASC-2 were overexpressed in mice (24, 25). Tg mice overexpressing ASC-2 fragment DN1 (the ASC-2 residues 849 to 929 containing the N-terminal LXXLL motif) but not DN1/m (in which the LXXLL sequences were mutated to LXXAA to disable receptor binding) were significantly impaired in many signaling pathways mediated by retinoic acid and other receptors. In this case, DN1 competitively blocked the interaction of these receptors with the full-length endogenous ASC-2 (24). Similarly, Tg mice overexpressing ASC-2 fragment DN2 (the ASC-2 residues 1,431 to 1,511 containing the C-terminal NR box) were impaired in their transactivation by LXRs (25). These two dominant negatives, DN1 and DN2, appeared to be specific to ASC-2 because they were not competed by other LXXLL-type coactivators such as SRC-1 and TRAP220. These transgenic studies not only revealed a crucial role of ASC-2 in eye development and liver lipid metabolism but also identified ASC-2 as a physiologically important transcriptional coactivator of LXRs and other NRs that interact with the two NR boxes in ASC-2.

Interestingly, it was recently described that endocrine cells in the islets of Langerhans express ASC-2, whereas exocrine cells do not (60). This led us to investigate the possible roles of ASC-2 in the pancreatic islets. Here we demonstrate that ASC-2 plays important roles in insulin secretion and β-cell mass maintenance and that the regulating role of ASC-2 in insulin secretion is mediated by its interaction with NRs. This study identifies an important new coactivator for β-cell biology and suggests that reduced expression or activity of ASC-2 may lead to an impairment of insulin secretion, a hallmark of type 2 diabetes.

MATERIALS AND METHODS

Animals. Generation methods for DN2 Tg (25) and ASC-$2^{+/-}$ mice (28) have been described previously. Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Research Institute (Japan); Sprague-Dawley (SD) rats were purchased from Orient, Ltd. (Korea). Animals were maintained in a temperature-controlled facility with a 12-h-light/12-h-dark cycle and ad libitum access to water and regular rodent chow. Genotyping of DN2 Tg and ASC-$2^{+/-}$ mice was performed by PCR analysis of genomic DNA from tail snips. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences.

Islet isolation and transfection. Islet isolation was accomplished by collagenase digestion and differential centrifugation through Ficoll gradients using a modification of procedures previously described for rat islets (49). After isolation, islets were cultured for 4 h in 11 mM glucose RPMI 1640 medium (containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum, pH adjusted to 7.4 with NaOH) before being handpicked and cultured at 37°C with 5% CO_2 and saturated humidity. Islets of similar size were picked by viewing with a microscope, divided into experimental groups, and transfected with ASC-2, DN1, DN2, or pcDNA3 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Measurement of insulin and C-peptide secretion from islets. After islet isolation, islet cells were incubated in 11 mM glucose RPMI 1640 medium overnight, transferred to 5.5 mM glucose RPMI 1640 medium, and transfected with ASC-2 or treated with ligand. These islet cells were washed twice with Krebs-Ringer/bicarbonate-HEPES buffer (KRBH; 115 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10 mM HEPES) containing 2% bovine serum albumin. Fifty islets were put into each well of 12-well plate and preincubated in KRBH containing 2% bovine serum albumin for 1 h. After an additional hour of incubation with 2.5 mM glucose, supernatants were collected and used to determine basal insulin and C-peptide levels. The same islet cells were stimulated with 6 or 25 mM glucose KRBH buffer for 1 h and were then assayed for stimulatory insulin and C-peptide levels. T0901317 (1 μM) was used to determine insulin and C-peptide secretion by LXR activation. Insulin and C-peptide concentrations in the supernatant were measured by radioimmunoassay (rat insulin and C-peptide kits from LINCO Research Inc., Charles, MO; mouse insulin kit from Shibayagi Co., Gunma, Japan). The cell pellet was assayed for DNA content to normalize insulin secretion by islet mass.

Analysis of gene expression by QPCR. Total RNA was isolated from rat or mouse islet cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Purified RNA was treated with DNase I (10 U/μg of total RNA) and reverse transcribed (Moloney murine leukemia virus reverse transcriptase; Promega, Madison, WI) according to the protocols supplied by the manufacturers. Quantitative gene expression analysis was performed on an ABI PRISM 7000 machine (Applied Biosystems, Foster City, CA) using the SYBR green PCR master mix. PCR primers were designed using Primer Express 2.0 software with the manufacturer's default settings and were validated for identical efficiencies. In 96-well optical plates, 12.5 μl SYBR green master mix was added to 12.5 μl cDNA (corresponding to 50 ng of total RNA input) and 200 nM forward and reverse primers in water. Plates were heated for 10 min at 95°C. Next, 40 PCR cycles consisting of 15 s at 95°C and 60 s at 60°C were applied. 18S rRNA was used as the internal control. Ratios of target gene and 18S rRNA expression levels were calculated by subtracting the C_T of the target gene from the C_T of 18S rRNA and raising 2 to the power of this difference (31). C_T values

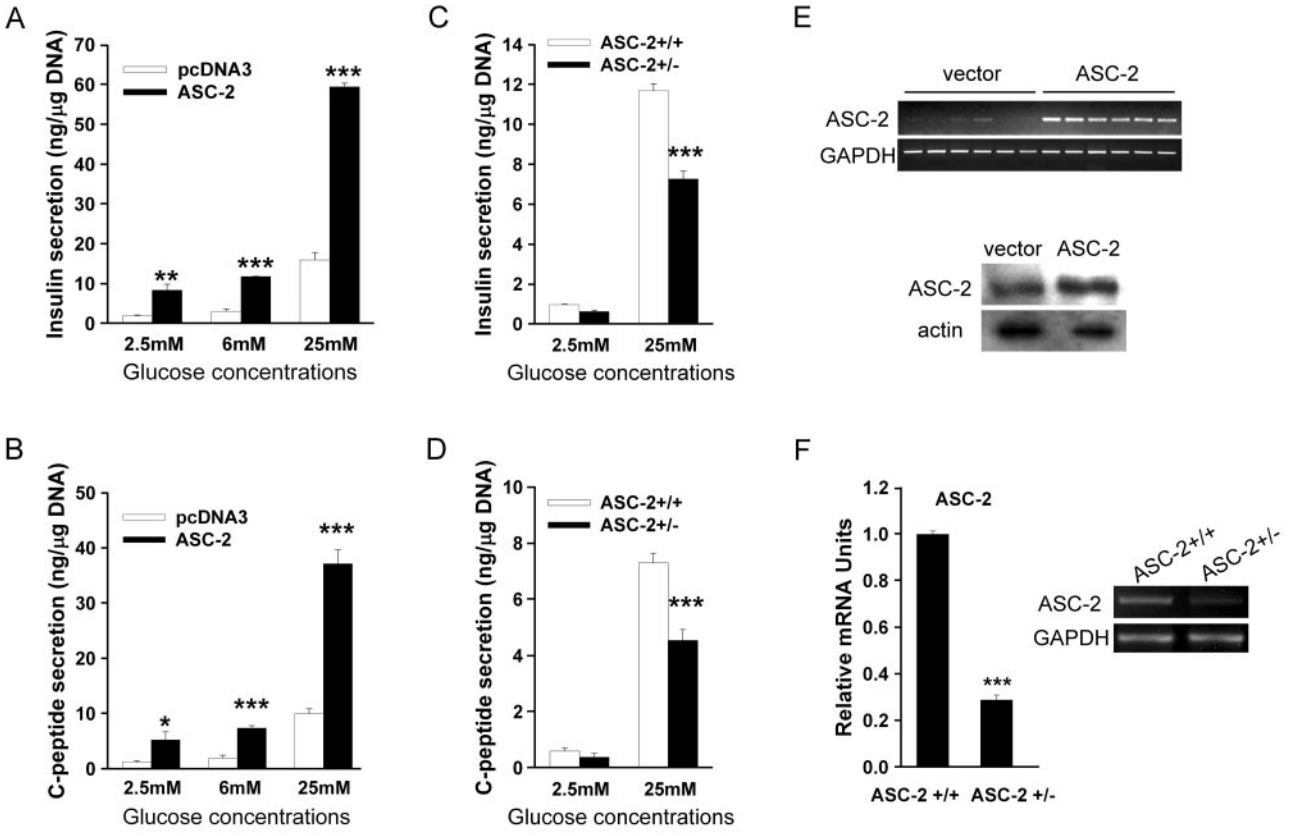

FIG. 2. Effect of ASC-2 expression level on insulin and C-peptide secretion. (A and B) Ectopic expression of ASC-2 increased glucose-elicited insulin and C-peptide secretion from rat islets. Isolated rat islets were transfected with pcDNA3 or ASC-2 and stimulated with 6 mM and 25 mM glucose. Insulin secretion was measured by radioimmunoassay. (C and D) Insulin secretion and C-peptide secretion were lower in ASC-$2^{+/-}$ islets. (E) Expression of transfected ASC-2 was confirmed by conventional RT-PCR and Western blotting. (F) Quantitation of the ASC-2 transcript was performed in ASC-$2^{+/+}$ and ASC-$2^{+/-}$ islets by QPCR and conventional RT-PCR. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

are defined as the number of PCR cycles at which the fluorescent signal reaches a fixed threshold. Target gene mRNA levels are thus expressed relative to 18S rRNA levels. The following oligonucleotide primers (forward and reverse, respectively) were used: ASC-2, 5′-ATGGCTTCTGAGAGCGTGGA-3′ and 5′-AGGTCGAGAGGATCTTTTTCGAT-3′; glucokinase, 5′-CAGGCTGACACCCAACTGC-3′ and 5′-CGCTGCCCTCCTCTGATTC-3′; HNF-4α, 5′-AGCAGAGATGAGCCGTGTGTC-3′ and 5′-GGCAAGACCAGCTCATCGAG-3′; LXRα, 5′-GGGAGGAGTGTGTCTTATCAG-3′ and 5′-GAGCGCCTGTTACACTGTTGC-3′; LXRβ, 5′-CCACCATCGAGATCATGTTG-3′ and 5′-TCTCGTGGTTGTAGCGTCTGG-3′; TXNIP, 5′-CGAGTCAAAGCCGTCAGGAT-3′ and 5′-TTCATAGCGCAAGTAGTCCAAAGT-3′; cyclin D2, 5′-GGGCTTCAGCAGGATGATGA-3′ and 5′-TGTCAGGGCATCACACGTG-3′; and 18S rRNA, 5′-AGTGCGGGTCATAAGCTTGC-3′ and 5′-GGTGTGTACAAAGGGCAGCC-3′.

Islet extraction and ATP measurements. Islets were pelleted, extracted in 30 to 50 μl of 0.1 M NaOH–0.5 mM EDTA, incubated at 60°C for 20 min, and stored at −80°C. The ATP concentrations were determined using a luciferin-based luminometric assay (44).

Chromatin immunoprecipitations. The chromatin immunoprecipitation (ChIP) assay was performed as described previously (41) with minor modifications. Briefly, islet cells were treated with 1% formaldehyde for 10 min at room temperature followed by 20 min at 4°C. The cells were washed with cold phosphate-buffered saline and resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 1% sodium dodecyl sulfate, and 10 mM EDTA. Soluble chromatin from islet cells was prepared by sonication and immunoprecipitated with anti-ASC-2 antibodies. The final DNA extractions were amplified using primers that encompass the P2 region of HNF-4α promoter and generate a 192-bp PCR product. The primers used were 5′-AGTGGACCTGAGGTCTAGCCAGG-3′ and 5′-CCGGGAGCCAGTCCACCAGGA-3′.

Cell culture and luciferase reporter assay. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections were performed in 24-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Ten nanograms of pCMV-βgal plasmid was included in each transfection experiment to control for the efficiency of transfection. The luciferase activity values were normalized to β-galactosidase activity as an internal control. pHNF4-tk-Luc and pcDNA3-hHNF4α were kindly provided by A. Fukamizu (17).

Intraperitoneal glucose and insulin tolerance test. For the intraperitoneal glucose tolerance test, mice were injected intraperitoneally with 2.0 g/kg body weight of glucose after a 16-h overnight fast. Blood samples were taken at various time points and were used to measure blood glucose concentrations with a glucose analyzer (YSI Inc., Yellow Springs, OH). For the intraperitoneal insulin tolerance test, mice were injected intraperitoneally with human regular insulin at 0.5 U/kg body weight after a 6-h fast.

Preparation of pancreas sections and immunohistochemistry. Pancreas was rapidly dissected from mice after anesthesia. Isolated pancreas was fixed in either Bouin's solution or 4% formaldehyde, embedded in paraffin, sectioned, and stained as described below. Sections were treated with 0.3% H_2O_2 and 0.5% trypsin before incubation with specific antibodies directed against insulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), glucagon (Santa Cruz Biotechnology Inc., Santa Cruz, CA), bromodeoxyuridine (BrdU) (MBL Co., Nagoya, Japan), Bcl-xL (Transduction Laboratories, Lexington, KY), or Bad (Transduction Laboratories, Lexington, KY). Preparations were then incubated with a goat biotinylated anti-rabbit immunoglobulin G (IgG) (for insulin, glucagons, and Bcl-xL), a horse biotinylated anti-mouse IgG (for Bad), goat tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (for BrdU), or goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (for insulin staining of 2-week-old mice). For immunoperoxidase staining, the avidin-biotin complex (ABC) method was performed using Vectastain Elite ABC reagent (Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine tetrahydrochloride (DAB) as a substrate (DakoCytomation, Carpinteria, CA). For in vivo cell

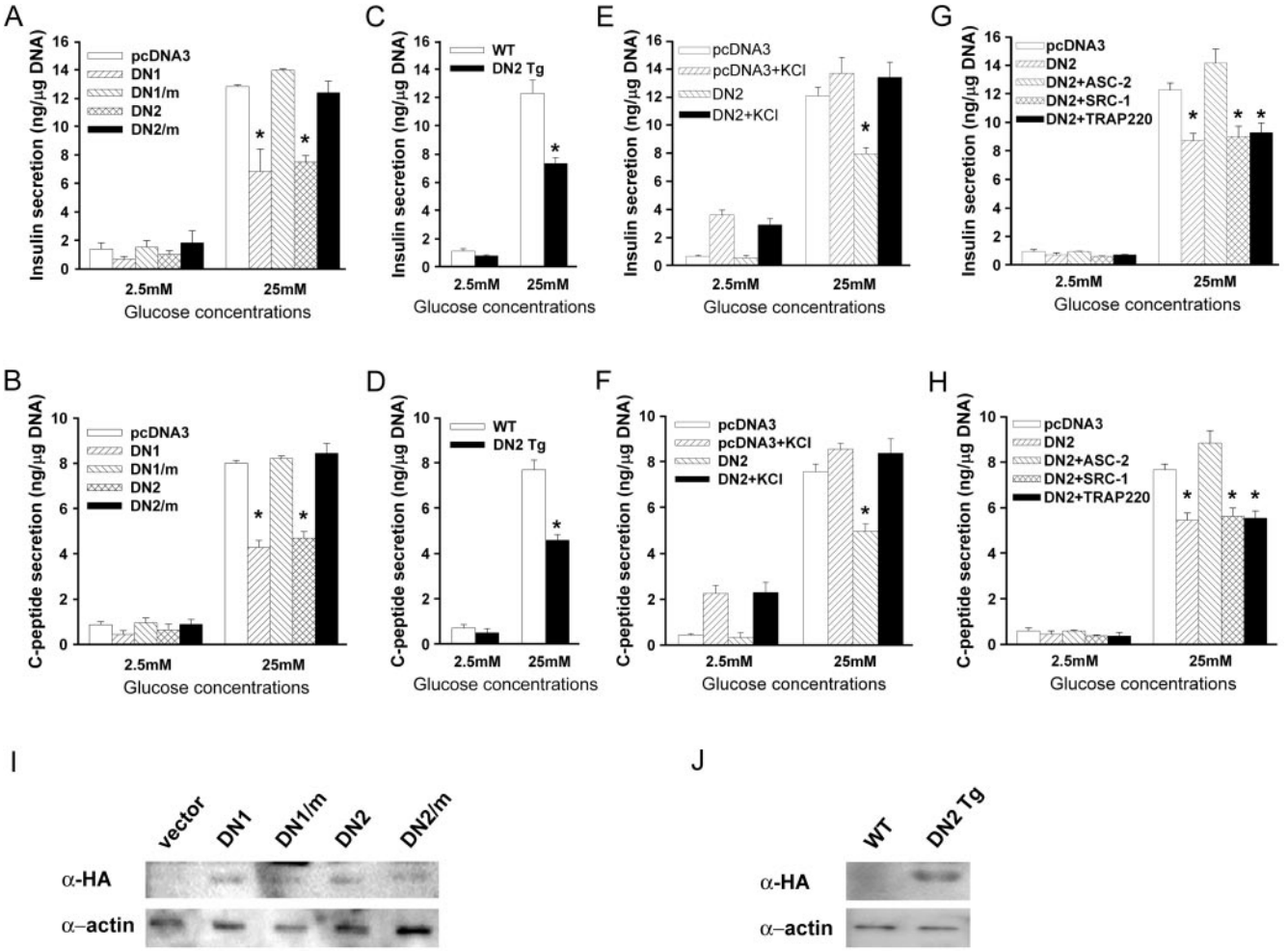

FIG. 3. Effects of ASC-2 dominant-negative mutants on glucose-elicited insulin and C-peptide secretion. (A and B) Two dominant-negative mutants (DN1 and DN2) and their inert mutants (DN1/m and DN2/m) were transfected into rat islet cells; insulin secretion and C-peptide secretion were then measured by radioimmunoassay. (C and D) Islet cells were isolated from DN2 Tg mice; insulin secretion and C-peptide secretion in response to glucose were measured. WT, wild type. (E and F) KCl (35 mM) was added to stimulate basic exocytosis of insulin-containing secretory granules. (G and H) The suppressive effect of DN2 was reversed by cotransfection with ASC-2, but not by cotransfection with SRC-1 or TRAP220. (I and J) Expression of transfected DNs and transgenic DN2 was determined by Western blotting. Each of the DNs was hemagglutinin (HA) tagged. α-HA and α-actin, anti-HA and antiactin antibodies, respectively. *, $P < 0.001$.

proliferation assays, 2-week-old mice were intraperitoneally injected with BrdU (100 mg/kg body weight) 6 h prior to sacrifice. For the apoptosis assay, the DeadEnd colorimetric kit (Promega, Madison, WI) for terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) was used.

Measurement of β-cell mass. β-Cell mass was measured according to the method described previously (51). Briefly, pancreatic weight was measured, and four random cross-sections of the pancreas were obtained. After insulin staining, the areas of the β-cells and the total pancreas were determined by planimetry using Image Tool for Windows, version 1.28 (The University of Texas Health Science Center, San Antonio). β-Cell mass (mg) was determined as pancreatic weight × (the area showing insulin positivity/total pancreatic area).

Statistical analysis. Results are expressed as mean ± standard error. Differences between groups were analyzed by analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

Relative expression of ASC-2 in pancreatic islets compared to whole pancreas. ASC-2 is expressed ubiquitously in many tissues, including testis, ovary, uterus, brain, thymus, white fat, heart, kidney, lung, muscle, and liver (60). However, its expression differs substantially across different tissues and varies significantly between cell types. Immunohistology of the mouse pancreas showed that all endocrine cells in the islets of Langerhans exhibit strong ASC-2 immunoreactivity, whereas exocrine cells that make up the majority of the pancreas show very weak reactivity (60). In accordance with this, we demonstrated, by quantitative reverse transcription (RT)-PCR (QPCR), that isolated islets expressed ASC-2 at a four- to sixfold-higher level than whole pancreas in rat and human (Fig. 1A and B), even though the difference (fold) was much lower than that of insulin (see Fig. S1A in the supplemental material). Western analysis also showed that ASC-2 protein is expressed preferentially in islets as compared to whole pancreas (Fig. 1B). We also assessed ASC-2 expression in the OLETF rat, an established polygenic animal model of human type 2 diabetes associated with impaired β-cell function (22). The LETO rat was

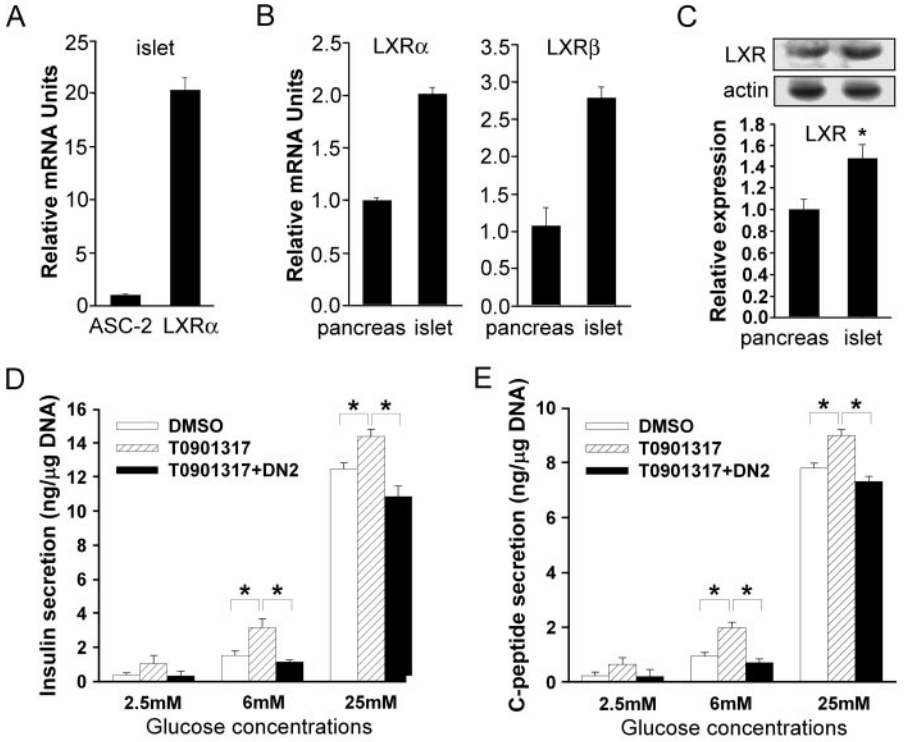

FIG. 4. Regulation of LXR-activated insulin secretion by ASC-2. (A) The expression level of LXRα was compared with that of ASC-2 in islets by QPCR. (B) The mRNA levels of LXRα and LXRβ were determined in the whole pancreas and isolated islets by QPCR. (C) The protein levels of LXRs were determined in the whole pancreas and isolated islets by Western blotting. The intensities of bands were quantified by the VersaDoc imaging system (Bio-Rad). (D and E) An LXR-specific ligand, T0901317, increased glucose-elicited insulin and C-peptide secretion, and the increases were repressed by DN2. *, $P < 0.05$.

used as a control. In isolated islets (see Fig. S1B in the supplemental material), mRNA levels of ASC-2 were found to be significantly lower in OLETF rats at 15 weeks of age. This difference became more dramatic at 27 weeks (Fig. 1C). Similarly, insulin mRNA level was decreased in OLETF rats (see Fig. S1C in the supplemental material). These results suggest that ASC-2 may play a role in the maintenance of β-cell function and in the prevention of diabetes.

Effects of ASC-2 on glucose-stimulated insulin secretion from islets. Because ASC-2 is expressed significantly in pancreatic endocrine cells (with the majority of these being insulin-producing β-cells), we evaluated the effects of ASC-2 on glucose-stimulated insulin secretion using primary islet cells isolated from SD rats. As shown in Fig. 2A and B, ectopic expression of ASC-2 significantly increased insulin and C-peptide release from rat islets, both at basal and stimulatory glucose concentrations. The ectopic expression of ASC-2 was confirmed at both the protein and mRNA levels (Fig. 2E and see Fig. S2A in the supplemental material). To further confirm the role of ASC-2 in insulin secretion, the same tests were performed in islet cells isolated from ASC-$2^{+/-}$ mice. As expected, decreased expression of ASC-2 in islets was associated with a significant decrease in glucose-stimulated insulin and C-peptide secretion (Fig. 2C, D, and F). On the other hand, the changes of ASC-2 expression level did not affect intracellular insulin mRNA/protein contents or glucagon release from the islets (see Fig. S3 in the supplemental material). Taken together, these data indicate that ASC-2 has a stimulatory effect on glucose-elicited insulin secretion from islet cells.

Impairment of glucose-stimulated insulin secretion by DN1 and DN2. The receptor-interacting domains of nuclear receptor coactivators, including ASC-2, RIP140, and SRC family members, contain a conserved LXXLL motif (where L is leucine and X is any amino acid) commonly known as the NR box (8). The NR box is necessary and sufficient to mediate coactivator binding to ligand-bound NRs. ASC-2 contains two LXXLL motifs. The N-terminal motif (NR box 1) binds to a broad range of NRs, whereas the C-terminal LXXLL motif (NR box 2) specifically interacts with LXRα and LXRβ. DN1 and DN2 are the fragments of ASC-2 that encompass the N-terminal and C-terminal NR boxes, respectively; each functions as a dominant-negative mutant for transactivation by NRs that interact with each of these LXXLL motifs in ASC-2 (24, 25).

To investigate the dependency of insulin secretion on the LXXLL motifs of ASC-2, these two dominant-negative mutants were expressed in rat islet cells by transfection. As shown in Fig. 3A and B, both DN1 and DN2 significantly inhibited glucose-stimulated insulin and C-peptide secretion. Furthermore, DN1/m and DN2/m, in which the LXXLL sequences were mutated to LXXAA to disable receptor binding, showed no differences from the control. The equivalent expression of each dominant-negative mutant in transfected rat islets was confirmed by Western blot and QPCR (Fig. 3I and see Fig.

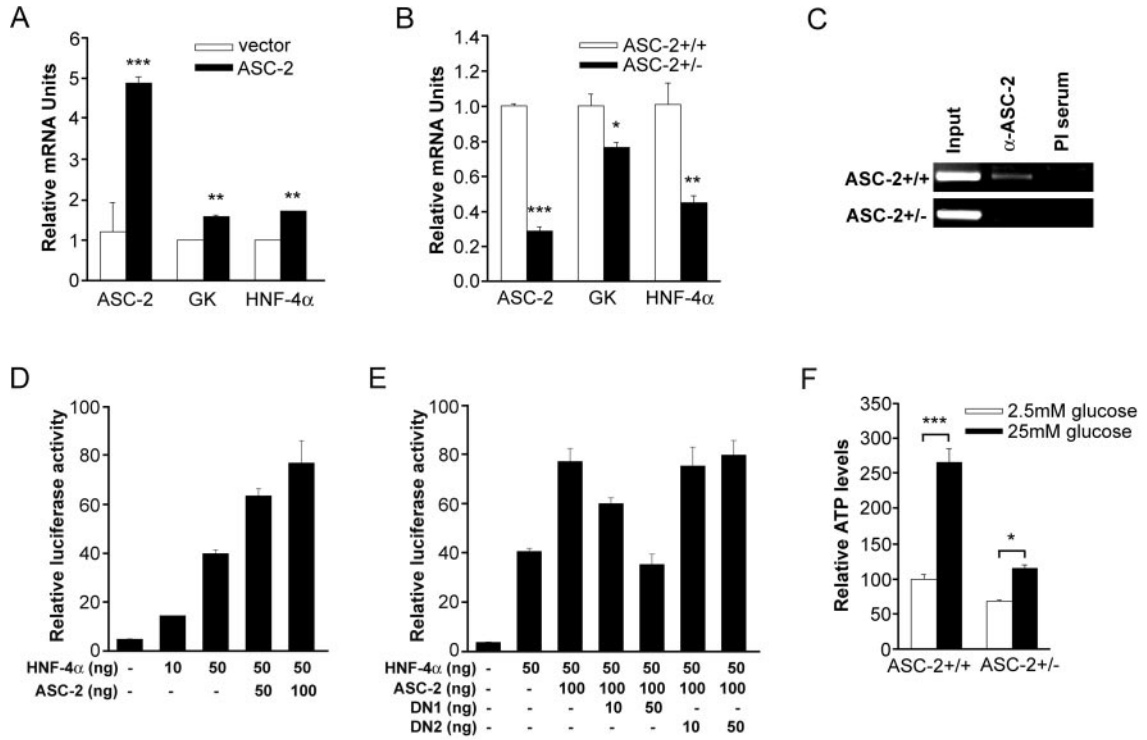

FIG. 5. Effect of ASC-2 on β-cell gene expression and ATP production. (A) Expression levels of glucokinase (GK) and HNF-4α were compared in ASC-2-transfected and pcDNA3-transfected rat islet cells by QPCR. (B) Expression levels of glucokinase and HNF-4α were compared in ASC-$2^{+/+}$ and ASC-$2^{+/-}$ mouse islets by QPCR. (C) ASC-2 was recruited to P2 promoter of HNF-4α in ASC-$2^{+/+}$ islets, but not in ASC-$2^{+/-}$ islets. Soluble chromatin from formaldehyde-fixed islets was prepared and immunoprecipitated with anti-ASC-2 antibodies (α-ASC-2) or preimmune (PI) serum. (D and E) Effect of ASC-2 on HNF-4α transcriptional activity. HeLa cells were cotransfected with HNF4-tk-Luc reporter plasmid and HNF-4 expression plasmid together with expression vectors for ASC-2, DN1, or DN2. (F) Glucose-induced ATP production was reduced in ASC-$2^{+/-}$ islets. Islets from ASC-$2^{+/+}$ and ASC-$2^{+/-}$ mice were stimulated with glucose and then extracted for measurement of ATP. ATP concentrations were measured by a luminometric assay. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

S2B in the supplemental material). These results suggest that the effect of ASC-2 on insulin secretion depends on the integrity of the LXXLL motifs in ASC-2, and LXRs and other NRs that interact with NR boxes 2 and 1 of ASC-2, respectively, might be involved with the role of ASC-2 in glucose-stimulated insulin secretion.

To further study the mechanism underlying the role of ASC-2 in insulin secretion, we examined the role of DN2 which specifically interacts with LXRs (30, 33, 34, 55). First, the effect of DN2 was confirmed using DN2 Tg mice. Islet cells isolated from DN2 Tg mice, in which DN2 expression was confirmed by Western blotting and QPCR (Fig. 3J and see Fig. S4 in the supplemental material), showed decreased insulin secretion in response to glucose (Fig. 3C and D). However, no significant difference in intracellular insulin mRNA/protein contents or glucagon release was found in DN2 islets (see Fig. S5 in the supplemental material). In addition, no significant difference was seen in the magnitude of the secretory response when a depolarizing concentration of KCl (35 mM) was used to induce basic exocytosis of insulin-containing secretory granules (Fig. 3E and F), indicating that the exocytosis machinery remained intact in DN2-expressing islets. The decrease in glucose-stimulated insulin secretion by DN2 was reversed by cotransfection of ASC-2, but not by other LXXLL-type coactivators such as SRC-1 and TRAP220 (Fig. 3G and H). These results are consistent with our previous study employing a cotransfection reporter assay and ChIP (25), in which DN2 was shown to be a specific dominant-negative mutant for ASC-2, but not for other LXXLL-type coactivators.

The effects of DN2 on insulin secretion led us to examine the role of LXRs in islets. To investigate the involvement of LXRs in insulin secretion from β-cells, we determined the presence of LXRs in islets. As shown in Fig. 4A, LXRα was expressed at a higher level than ASC-2 in rat islet cells. Both LXRα and LXRβ were preferentially expressed in islets relative to exocrine pancreas, like ASC-2 (Fig. 4B and C). Importantly, an LXR-specific ligand, T0901317, significantly increased insulin secretion from islet cells, and the increases in insulin secretion by T0901317 were abolished by DN2, the LXR-specific dominant-negative fragment of ASC-2 (Fig. 4D and E). Taken together, these results indicate that ASC-2 increases glucose-stimulated insulin secretion, at least in part, through its coactivator function with LXRs.

Effects of ASC-2 on β-cell glucose metabolism. To further investigate the molecular basis for the increased insulin secretion by ASC-2, we performed QPCR to examine the levels of mRNAs encoding several β-cell genes known to be involved in insulin secretion. Ectopic expression of ASC-2 increased the mRNA levels of both glucokinase and HNF-4α (Fig. 5A). In contrast, ASC-$2^{+/-}$ islets showed a significant reduction in the expression

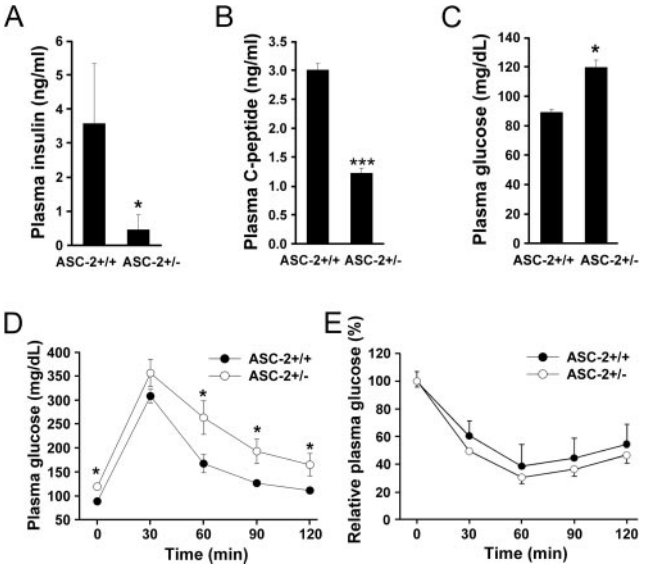

FIG. 6. Impaired glucose tolerance in ASC-$2^{+/-}$ mice. (A and B) Plasma insulin and C-peptide levels in ASC-$2^{+/+}$ and ASC-$2^{+/-}$ mice after 6 h of fasting in 20-week-old male mice ($n = 5$). (C) Plasma glucose levels in ASC-$2^{+/+}$ and ASC-$2^{+/-}$ mice after 16 h of fasting in 20-week-old male mice ($n = 4$). (D) Intraperitoneal glucose tolerance tests were performed after 16 h of fasting in 20-week-old male mice ($n = 4$). (E) Effects of ASC-$2^{+/-}$ on insulin sensitivity. Intraperitoneal insulin tolerance tests were performed after 6 h of fasting in 20-week-old male mice ($n = 4$). *, $P < 0.05$.

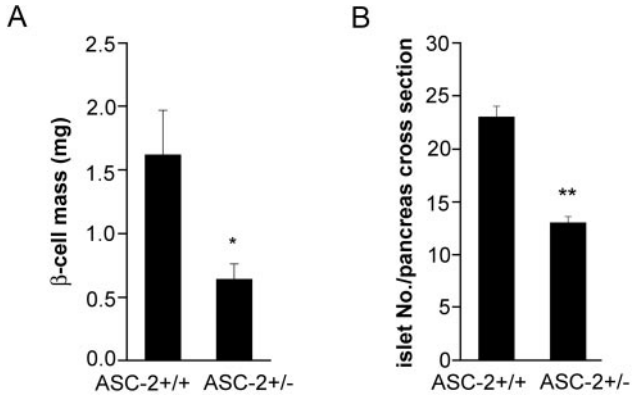

FIG. 7. Reduced β-cell mass and islet number in ASC-$2^{+/-}$ mice. (A) The mass of islet cells from 20-week-old mice was estimated using insulin immunoreactivity, as described in Materials and Methods (ASC-$2^{+/+}$, $n = 7$; ASC-$2^{+/-}$, $n = 5$). (B) The number of islets was counted in complete pancreatic sections at low magnification. *, $P < 0.05$; **, $P < 0.005$.

of these genes (Fig. 5B). No significant differences in islet mRNA expression for Glut2, PGC-1, and UCP-2 were observed in either of these experiments (data not shown). Using the ChIP assay, we next investigated whether control of glucokinase and HNF-4α by ASC-2 in islet cells is direct. Pancreatic HNF-4α uses two promoter regions, P1 and P2, and pancreatic glucokinase has a β-cell-specific promoter, which is located ~35 kb upstream from the glucokinase hepatic promoter (11, 39). ChIP analysis of mouse islet chromatin by using anti-ASC-2 antibodies revealed that ASC-2 was recruited to the P2 promoter region of HNF-4α in ASC-$2^{+/+}$ islet cells (Fig. 5C). This recruitment of ASC-2 to the P2 region was not detected in ASC-$2^{+/-}$ islets. In contrast, ASC-2 was not recruited to P1 of HNF-4α nor to the β-glucokinase promoter region (data not shown). These results suggest that the decrease of HNF-4α expression in ASC-$2^{+/-}$ islets is due to the reduced recruitment of ASC-2 to the HNF-4α promoter, while the effects of ASC-2 on the expression of glucokinase may be indirect.

We also determined the effect of ASC-2 on HNF-4α transcriptional activity. As shown in Fig. 5D, ASC-2 coactivated HNF-4α transcriptional activity in a dose-dependent manner. Interestingly, DN1 repressed coactivation of HNF-4α by ASC-2, whereas DN2 had no effect (Fig. 5E). These results suggest that HNF-4α could be involved in the regulation of glucose metabolism by ASC-2 through interaction with NR box 1.

Glucokinase is the first enzyme in the glycolytic pathway in β-cells and acts as a glucose sensor (37). HNF-4α plays a critical role in the transcriptional regulation of genes involved in glucose metabolism in both hepatocytes and pancreatic β-cells (45). Defects in the process of glucose oxidation in β-cells, such as reduced activity of glycolytic enzymes and increased glucose cycling, are considered key contributory factors in insulin secretion dysfunction because insulin secretion is tightly coupled to the rate of glucose metabolism (6, 20, 23, 42, 54, 56). Therefore, the altered expression of metabolic genes in ASC-$2^{+/-}$ islets may influence glucose-induced oscillations in ATP levels, which in turn are coupled to insulin secretion. As expected, the increase of ATP levels by exposure to high glucose concentrations was lower in ASC-$2^{+/-}$ islets than in ASC-$2^{+/+}$ islets (Fig. 5F). These results suggest that the reduction of glucose-elicited insulin secretion in ASC-$2^{+/-}$ islets may be a consequence of the diminished ability to drive ATP production in response to glucose stimulation.

Glucose intolerance in ASC-$2^{+/-}$ mice. The decrease in glucose-elicited insulin secretion in ASC-$2^{+/-}$ islets prompted us to investigate the systemic effects of ASC-2 on glucose and insulin homeostasis. As shown in Fig. 6A and B, fasting plasma insulin and C-peptide levels were significantly lower in ASC-$2^{+/-}$ mice than in wild-type mice. On the other hand, fasting plasma glucose levels were higher in ASC-$2^{+/-}$ mice (Fig. 6C). These data indicate that reduced ASC-2 levels result in impaired glucose homeostasis in the whole animal. To further examine the effects of ASC-2 deficiency on glucose homeostasis, intraperitoneal glucose and insulin tolerance tests were performed. Upon glucose challenge, ASC-$2^{+/-}$ mice showed statistically higher glucose levels than wild-type mice (Fig. 6D). On the other hand, there was no significant difference in insulin tolerance between ASC-$2^{+/-}$ and ASC-$2^{+/+}$ mice (Fig. 6E), suggesting that the impaired glucose tolerance in ASC-$2^{+/-}$ mice is primarily attributable to insufficiency of insulin secretion.

Reduced islet mass in ASC-$2^{+/-}$ mice. A defect in insulin secretion could arise not only from an alteration in β-cell metabolism, but also from a reduction in the size or number of islets. Interestingly, we consistently observed fewer islets (on average, about 110 islets) in ASC-$2^{+/-}$ mice than in ASC-$2^{+/+}$ mice (on average, about 200 islets). The sizes of isolated ASC-$2^{+/-}$ islets were more variable than those of wild-type islets, even though we hand-picked islets of similar size to ensure a fair comparison in the insulin secretion assay described above. To investigate whether the systemic effects on glucose toler-

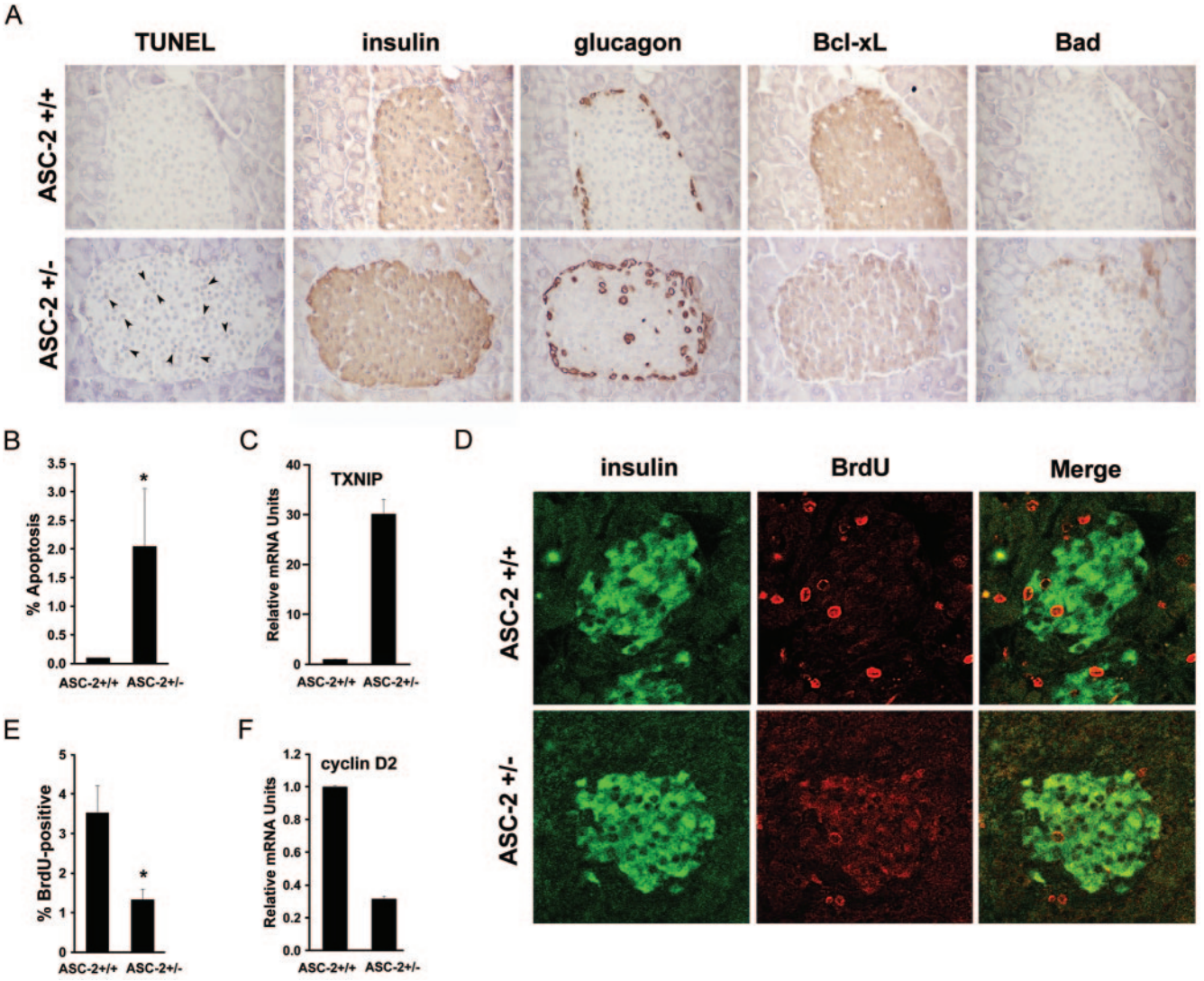

FIG. 8. Increased apoptosis and impaired proliferation in ASC-$2^{+/-}$ islets. (A and B) Immunohistochemistry was performed with antibodies to apoptosis-related proteins, insulin, and glucagon using pancreatic sections of 20-week-old ASC-$2^{+/+}$ ($n = 5$) and ASC-$2^{+/-}$ ($n = 5$) mice. Apoptotic cells were measured by the TUNEL assay and are indicated by arrowheads. (C) The mRNA levels of TXNIP were determined by QPCR using islets isolated from 20-week-old ASC-$2^{+/+}$ ($n = 5$) and ASC-$2^{+/-}$ mice ($n = 5$). (D and E) An in vivo cell proliferation assay was performed by BrdU injection and immunostaining with an anti-BrdU antibody. Representative micrographs are presented for BrdU immunostainings of pancreatic sections from 2-week-old ASC-$2^{+/+}$ ($n = 5$) and ASC-$2^{+/-}$ ($n = 5$) mice, and the percentage of BrdU-positive cells is indicated. (F) The mRNA levels of cyclin D2 were determined by QPCR using pancreas of 2-week-old ASC-$2^{+/+}$ ($n = 3$) and ASC-$2^{+/-}$ mice ($n = 3$). *, $P < 0.05$.

ance were associated with any changes in the morphology of the pancreas, we carried out immunohistochemical studies. Analysis of pancreatic sections using an anti-insulin antibody revealed that ASC-$2^{+/-}$ mice showed reduced β-cell mass, which was due to an approximately 50% decrease in islet number (Fig. 7). However, there was not a significant decrease in the average area of individual islets. In addition, glucagon staining showed that the peripheral distribution of α-cells was disturbed in ASC-$2^{+/-}$ mice (Fig. 8A).

To further determine whether reduced β-cell mass was due to increased apoptosis or impaired proliferation, a TUNEL assay and BrdU immunostaining, respectively, were performed. The number of TUNEL-positive cells was significantly higher in the pancreas of ASC-$2^{+/-}$ mice (Fig. 8A and B). In addition, immunohistochemistry revealed that the expression of antiapoptotic Bcl-xL was decreased; whereas the expression of proapoptotic Bad was increased in ASC-$2^{+/-}$ islets. Furthermore, thioredoxin-interacting protein TXNIP mRNA was increased in ASC-$2^{+/-}$ islets (Fig. 8C). TXNIP was recently reported to be a proapoptotic β-cell gene that is elevated in insulin resistance/diabetes and upregulated by glucose (38). On the other hand, proliferation rates in ASC-$2^{+/-}$ islets were lower, as measured by in vivo BrdU incorporation (Fig. 8D and E). In accordance with this, cyclin D2, one of the key components of the cell cycle machinery, was reduced in ASC-$2^{+/-}$ pancreas (Fig. 8F). Cyclin D2 was shown to be uniquely required for β-cell replication and the proper expansion of β-cell mass during postnatal development (14). Taken together, these results indicate that reduced islet mass in ASC-$2^{+/-}$ mice

is due to both decreased proliferation and increased apoptosis of pancreatic endocrine cells.

DISCUSSION

The present study was initiated to explore the function of islet-specific expression of ASC-2 in the pancreas. Two major findings were produced by this study. First, ASC-2 regulates glucose-stimulated insulin secretion, at least partially, through alterations in the expression of some β-cell metabolic genes. Second, ASC-2 deficiency predisposes islets to apoptosis and reduces proliferation in vivo, leading to a significant decrease in β-cell mass and islet number.

Since ASC-2 is a general transcriptional coactivator, it is likely to affect β-cell metabolism by interacting with a set of specific transcription factors bound to pertinent promoters. Among these transcription factors, the LXRs are promising because DN2, an LXR-selective dominant-negative form of ASC-2, inhibits insulin secretion stimulated by T0901317, an LXR-specific synthetic ligand. In addition, the present study agrees with a recent study showing that activation of LXRs increases the level of glucokinase protein in MIN6 cells and stimulates insulin secretion in pancreatic β-cells (12). LXR activation could also increase ATP production in response to glucose stimulation (see Fig. S6 in the supplemental material). Therefore, it is assumed that activation of LXR with ASC-2 leads to increases in glucose utilization, ATP production, and insulin secretion from β-cells.

However, activation of LXRs is not the sole mechanism by which ASC-2 stimulates insulin secretion. We found that ASC-2 leads to a modest increase in HNF-4α expression and coactivates its transcriptional activity. Furthermore, coactivation of HNF-4α by ASC-2 was specifically repressed by DN1, but not by DN2. HNF-4α is a transcription factor that plays a critical role in the transcriptional regulation of glucose metabolism in pancreatic β-cells, and its mutations result in MODY (57). A recent study demonstrated that deletion of HNF-4α in β cells resulted in diminished first-phase insulin secretory response and impaired glucose tolerance (16). Collectively, it is suggested that ASC-2 increases insulin secretion from the pancreatic islet by coactivating HNF-4α and LXR via NR box 1 and NR box 2, respectively. It is also notable that Pdx1$^{+/-}$ mice show increased islet apoptosis and striking changes in islet architecture (19). Mice with genetically engineered reductions in Pdx1 levels have impaired glucose tolerance; moreover, mice in which Pdx1 has been genetically inactivated in more than 80% of the β-cells develop diabetes (1, 4, 9, 32, 53).

Our data show that both islet mass and islet number were lower in the pancreases of ASC-2$^{+/-}$ mice than in ASC-2$^{+/+}$ mice. We also found that apoptosis was increased and proliferation was decreased in the ASC-2$^{+/-}$ islets. The involvement of ASC-2 in the apoptotic process has been suggested previously by two other groups (35, 43). ASC-2$^{-/-}$ mouse embryo fibroblasts grow at a reduced rate and exhibit apoptosis. This apoptosis is inhibited by zVAD-fmk, an irreversible pancaspase inhibitor, suggesting a role for caspases in the apoptosis of ASC-2$^{-/-}$ MEFs (35). The other group reported a slight increase of apoptosis in the terminal end buds of ASC-2-deficient mammary glands relative to wild-type mammary glands (43). ASC-2 is also known to be important in the activation of cellular antiapoptotic mediators such as Nur77, NF-κB, c-Fos, c-Jun, and CREB (26, 29, 46). We demonstrated here that antiapoptotic Bcl-xL, proapoptotic Bad, and TXNIP were involved in the apoptosis of ASC-2$^{+/-}$ islets. Taken together, these results suggest that ASC-2 may have a general role in apoptosis. In regards to proliferation, it is noteworthy that ASC-2 transactivates E2F-1-driven gene transcription and increases the stability of E2F-1, which plays an important role in cell cycle progression (27). Furthermore, E2F-1$^{-/-}$ mice showed impaired proliferation of islets (13).

Since the maintenance of adequate insulin secretion requires the balance of β-cell proliferation and death, our findings identify ASC-2 as an important factor in the pathophysiology of diabetes. Consistent with this, we found that ASC-2 expression was lower in OLETF rats, an animal model of human type 2 diabetes with defective β-cell function. We also found that islet architecture was disrupted in ASC-2$^{+/-}$ mice. The specialized architecture and relative distribution of islet cell types may also play an important role in islet function and survival. Whether this is a cause or an effect of enhanced apoptosis and diminished proliferation remains unclear. Together, these results suggest that ASC-2 plays important roles in both the maintenance of an adequate pool of healthy islets as well as in β-cell metabolism. All of these factors, including an increase in apoptosis, abnormal regulation of islet population and β-cell mass, and a decrease in glucose utilization, might contribute to the increased plasma glucose level seen in ASC-2$^{+/-}$ mice. Of particular importance in our study is that ASC-2 increases insulin secretion from the pancreatic islets by coactivating transcription factors including HNF-4α and LXRs, which is mediated by interactions with N-terminal and C-terminal LXXLL motifs. Finding the means to increase the expression or activity of ASC-2 or to modulate interaction between ASC-2 and these transcription factors may provide a new therapeutic strategy to prevent or treat type 2 diabetes mellitus.

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