Original Article

The HMG Box Transcription Factor Sox4 Contributes to the Development of the Endocrine Pancreas

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To investigate the role of the Sry/hydroxymethylglutaryl box (Sox) transcription factors in the development of the pancreas, we determined the expression pattern of Sox factors in the developing mouse pancreas. By RT-PCR, we detected the presence of multiple Sox family members in both the developing pancreas and mature islets and then focused on two factors, Sox2 and Sox4. The expression field of Sox2, which plays a role in the maintenance of some stem cell populations, included the developing duodenum, but Sox2 was specifically excluded from the pancreatic buds. In contrast, Sox4 was detected broadly in the early pancreatic buds and eventually became restricted to the nuclei of all islet cells in the adult mouse. Mice homozygous for a null mutation of the *sox4* gene showed normal pancreatic bud formation and endocrine cell differentiation up to embryonic day 12.5. Beyond that date, cultured pancreatic explants lacking *sox4* failed to form normal islets. Instead, a markedly reduced number of endocrine cells were found scattered through the explant. We show here that several Sox transcription factors are expressed in the developing pancreas and in the islet, and that one of these factors, Sox4, is required for the normal development of pancreatic islets. *Diabetes* 54:3402–3409, 2005

Scattered through the exocrine pancreas, the islets of Langerhans are highly organized clusters of endocrine cells comprised of four distinct cell types: the glucagon-secreting α-cells, the insulin-secreting β-cells, the somatostatin-secreting δ-cells, and the pancreatic polypeptide–secreting cells. Because of the essential role of the islet hormones in energy metabolism, defects in the development, maintenance, or function of the endocrine pancreas have serious consequences, including diabetes.

The pancreas forms from the endoderm in the region of the foregut/midgut junction and is first visible in mice at embryonic day 9.5 (e9.5) (1). The first endocrine cells of the pancreas appear at around e9.5 in the dorsal pancreatic bud. These cells express glucagon; a few insulin-expressing cells appear ~1 day later. At ~e13–14, the pancreas undergoes a distinct change termed the secondary transition, characterized by the appearance of ductal cells, exocrine cells, and delta cells and a rapid expansion of the insulin-expressing cells. The endocrine cells found in the pancreas before the secondary transition have clear differences from mature islet cells and may result from developmental pathways that are distinct from those that produce the more mature endocrine cells that arise following the secondary transition (2–6). By e18, the first pancreatic polypeptide–expressing cells appear, and the endocrine cells organize into distinct islets with the β-cells forming the central core. Around the same time, β-cell neogenesis declines, but the simultaneous onset of β-cell replication continues to expand the β-cell population and enlarge the forming islets (7).

This process of endocrine cell determination and differentiation depends on the proper sequential actions of multiple transcription factors (8). The basic helix-loop-helix transcription factor Neurogenin3 initiates endocrine differentiation and is both necessary and sufficient to drive the differentiation of the undifferentiated pancreatic epithelial cells to an endocrine fate (9–13). Neurogenin3 expression is transient but activates the expression of the factors that complete endocrine differentiation, including the basic helix-loop-helix transcription factor NeuroD1 and the homeodomain transcription factors Pax4 and Nkx2.2 (14–18), all three of which are required for proper β-cell differentiation and islet development (19–21). Nkx2.2 in turn activates expression of the homeodomain transcription factor Nkx6.1, which is also required for normal β-cell differentiation (5,19). In addition, Neurogenin3 activates, either directly or indirectly, the expression of several other homeodomain transcription factors that play a role in islet development (12,14,22). Interestingly, with the exception of Nkx2.2, none of these factors downstream of Neurogenin3 are required for the differentiation of the endocrine cells that appear before the secondary transition.

In order to extend our understanding of the transcriptional cascade controlling the differentiation of the endocrine pancreas, we examined the role of members of the Sry/hydroxymethylglutaryl (HMG) box (Sox) family of transcription factors. Members of the Sox family contrib-

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Received for publication 13 June 2005 and accepted in revised form 30 August 2005.

M.E.W. is employed by and holds stock in Metabolex.

HMG, hydroxymethylglutaryl; UCSF, University of California at San Francisco.

ute to the development of a variety of tissues (23,24), and several Sox factors have been detected in the developing pancreas (25–30). We found that *sox4* was the most highly expressed Sox gene in the endocrine pancreas in the mouse and therefore examined its role in the development of the endocrine pancreas.

RESEARCH DESIGN AND METHODS

Mice were housed on a 12-h light-dark cycle with controlled climate, according to the regulations of the University of California, San Francisco. Midday on the day on which the vaginal plug was discovered was considered e0.5 in the timing of embryo collection. CD1 mice were obtained from Charles River Laboratories and used for all studies on normal mice. The *sox4* mutant mice have been described previously (31). The *sox2* mutant mice were generated by disrupting the *sox2* locus with the β*geo* gene by homologous recombination in embryonic stem cells (32).

Glucose tolerance tests. Six-month-old mice were fasted for 12 h then injected intraperitoneally with glucose at 1 mg/kg body wt. Blood was sampled from the tail vein before injection of glucose and at the time points indicated after injection up to 180 min. Blood glucose was measured using a Glucometer 2m (Bayer).

RT-PCR. Total RNA was isolated with the RNAqueous kit (Ambion) from the indicated cells and tissues. First-strand cDNA was prepared from 1–3 μg of total RNA with the Invitrogen SuperScript first-strand cDNA synthesis kit and oligo dT primers.

First-strand cDNA served as a template for PCR amplification as previously described (10) using the following degenerate primers derived from conserved amino acid sequences within the HMG DNA-binding domain of all Sox family members: 5′primer: cagctctagaCCIATGAAYGCITTYATGGTIGG and 3′primer: ggacctcgagGICKRTAYTTRTARTCIGG. PCR products were cloned into the pBlueScript (KS+) vector (Stratagene) and sequenced.

For TaqMan real-time PCR quantification, 30 ng of cDNA were used as a template. All primer/probe sets for TaqMan RT-PCR were validated with a standard curve by using serial cDNA dilutions. Assays for *sox2* were performed with ABI mSox2 assay-on-demand and ABI TaqMan Universal PCR Master Mix. All other assays used 200 μmol/l dNTP, 5.5 mmol/l $MgCl_2$, 500 nmol/l forward/reverse primers, and 100 nmol/l probe in 50 μl for 40 cycles with the ABI TaqMan Gold Kit. Quantification was performed on an Applied Biosystems Prism 7700 sequence detection system. Results are reported relative to the level of mouse Histone3.3A mRNA, which was used as an internal control for cDNA quantity and quality in all assays. Sequences of all primers and probes are available on request.

Pancreatic bud culture. The dorsal and ventral pancreatic primordia and attached stomach and foregut were dissected from e11.5 embryos and placed on Matrigel matrix without phenol red (Becton Dickinson) contained within Millicell culture plate inserts (Millipore). The explants were incubated in 50% DME H-16, supplemented with nonessential amino acids, 1.4 g/l glucose, 0.11 g/l sodium pyruvate, 0.365 g/l linoleic acid, 1.2 g/l NaHCO3, 10% FBS, penicillin/streptomycin, fungizone, and insulin/transferrin/selenium supplement (Sigma) in 12-well plates. Explants were cultured for 8 days at 37°C in 5% CO_2. Media was changed every 48 h.

After 8 days in culture, the fetal pancreas explants were photographed, fixed in 2.5% paraformaldehyde for 10 min at room temperature, dehydrated through graded ethanols, embedded in paraffin (paraplast), and sectioned at 5 μm.

Immunohistochemical and immunofluorescence assays. Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (33). For β-galactosidase staining, embryos harvested on the dates indicated were fixed for 20–45 min on ice in 2% paraformaldehyde and 0.1% glutaraldehyde. Tissues were incubated overnight at 37° with X-gal (1 μg/ml) as previously described (34). Gross embryos and dissected pancreases were visualized using a Leica dissecting microscope and imaged with a Spot RT digital camera and Openlab software. The tissues were fixed again in 4% paraformaldehyde, paraffin embedded, and sectioned at 5 μm.

Primary antisera employed in the assays were diluted in 5% goat serum/PBS at the following dilutions: guinea pig anti-insulin (Linco), 1:4,000; mouse anti-glucagon (Sigma), 1:10,000; rabbit anti-Nkx 6.1 (5), 1:6,000; rabbit anti-Sox4 (Chemicon), 1:2000; mouse monoclonal anti-Nkx2.2 (35) (Developmental Studies Hybridoma Bank, Iowa City, IA), 1:100; guinea pig anti-pancreatic polypeptide (Linco), 1:1,000; rat anti-somatostatin (Chemicon), 1:1,000; guinea pig anti–pdx-1 (10), 1:2000; rabbit anti–pdx-1 (10), 1:2000; guinea pig anti-neurogenin3 (10), 1:4,000; rabbit anti-Ki67 (Novocastra Laboratories), 1:500; and rabbit anti-cleaved caspase-3 (Cell Signaling), 1:100.

Sox4 antigen was produced by inserting the coding sequence for amino acids 131–410 from mouse Sox4 downstream of the glutathione-S-transferase coding sequence in the pGEX-2T vector (Pharmacia). The resulting fusion protein was purified from *E. coli* and injected into rabbits. Dilutions of 1:2,000 were used for staining, and preimmune sera from the same animal gave no staining at the same concentration.

Secondary antibodies (Jackson ImmunoResearch Laboratories) for immunofluorescence were used at the following concentrations: Cy3-conjugated goat anti-rabbit and anti-mouse, 1:800; fluorescein isothiocyanate–conjugated goat anti-rabbit, anti–guinea pig, anti-hamster, and anti-mouse, 1:200; and Cy2-conjugated donkey anti-rat, 1:200. For peroxidase staining, slides were incubated with goat anti–guinea pig biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200.

All slides were visualized with a Zeiss Axioskop II microscope and imaged with a Hamamatsu ORCA100 digital camera and Openlab software.

In situ hybridization. For RNA in situ hybridization analysis of paraffin sections (5 μm), adult mouse pancreas was processed, sectioned, and hybridized with digoxigenin-labeled riboprobes as previously described (10). Digoxigenin-labeled sense and antisense riboprobes were detected with alkaline phosphatase–coupled anti-digoxigenin antibodies using BM purple (Boehringer Mannheim) as color substrate. The *sox4* sense and antisense riboprobes were transcribed in vitro from a fragment consisting of bp 1,427–1,776 of mouse *sox4* (accession number X70298). The sense probe gave no significant signal on adult mouse pancreas.

Microarray gene expression analysis. Slides carrying ~22,000 mouse cDNAs were generated by the Mouse Microarray Consortium at University of California at San Francisco (UCSF). A total of 20,000 of the clones corresponded to FANTOM1 set of cDNAs from RIKEN (http://fantom.gsc.riken.jp/). The remaining clones were amplified from personal stocks of various UCSF laboratories. The complete list of the clones can be obtained from the UCSF Mouse Microarray Consortium web site at http://mmc.ucsf.edu.

Pancreatic buds were extracted from *sox4* mutant mice at e12.5. Buds from homozygous mutants and wild-type embryos were pooled separately, and total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with

TABLE 1
Expression of selected genes in *sox4*+/+ vs. *sox4*−/− pancreases at e12.5

Gene name	Unigene	Fold difference*
Ring finger protein 122	Mm0.29532	2.26 ± 0.27
Insulin 1	Mm0.46269	1.84 ± 0.05
Cell division cycle 6 homolog (Cdc6)	Mm0.20912	1.74 ± 0.10
Peroxiredoxin 2	Mm0.70130	1.61 ± 0.10
Cyclin-dependent kinase 4 (Cdk4)	Mm0.6839	1.44 ± 0.08
Insulin 2	Mm0.4946	1.42 ± 0.09
Glutathione peroxidase 1	Mm0.1090	1.42 ± 0.09
Nkx6.1	Mm0.193072	1.38 ± 0.08
NeuroD1	Mm0.4636	0.79 ± 0.07
Transforming growth factor-β receptor 1	Mm0.197552	0.69 ± 0.03
Cyclin D1	Mm0.273049	0.68 ± 0.03
Gastrin	Mm0.4767	0.65 ± 0.02
Sox11	Mm0.41702	0.63 ± 0.08
Neurogenin3	Mm0.57236	0.61 ± 0.02
Growth hormone	Mm0.343934	0.58 ± 0.03
DEAD box polypeptide 3, Y-linked	Mm0.302938	0.51 ± 0.04
DEAD box polypeptide 4	Mm0.12818	0.50 ± 0.02
Metallothionein 1 (Mt1)	Mm0.192991	0.50 ± 0.05
BCL2 interacting protein 1 (Bnip3)†	Mm0.2159	0.49 ± 0.03
Metallothionein 2 (Mt2)	Mm0.147226	0.43 ± 0.02
Apoptotic chromatin condensation inducer 1	Mm0.297078	0.26 ± 0.02
Alpha fetoprotein	Mm0.358570	0.25 ± 0.02

Data are the means of six ratios ± SEM. *Fold difference was calculated by taking the ratio of the signal from *sox4*+/+ RNA relative to *sox4*−/− RNA. †This gene was spotted twice on the array, and the fold difference shown is the mean of both spots.

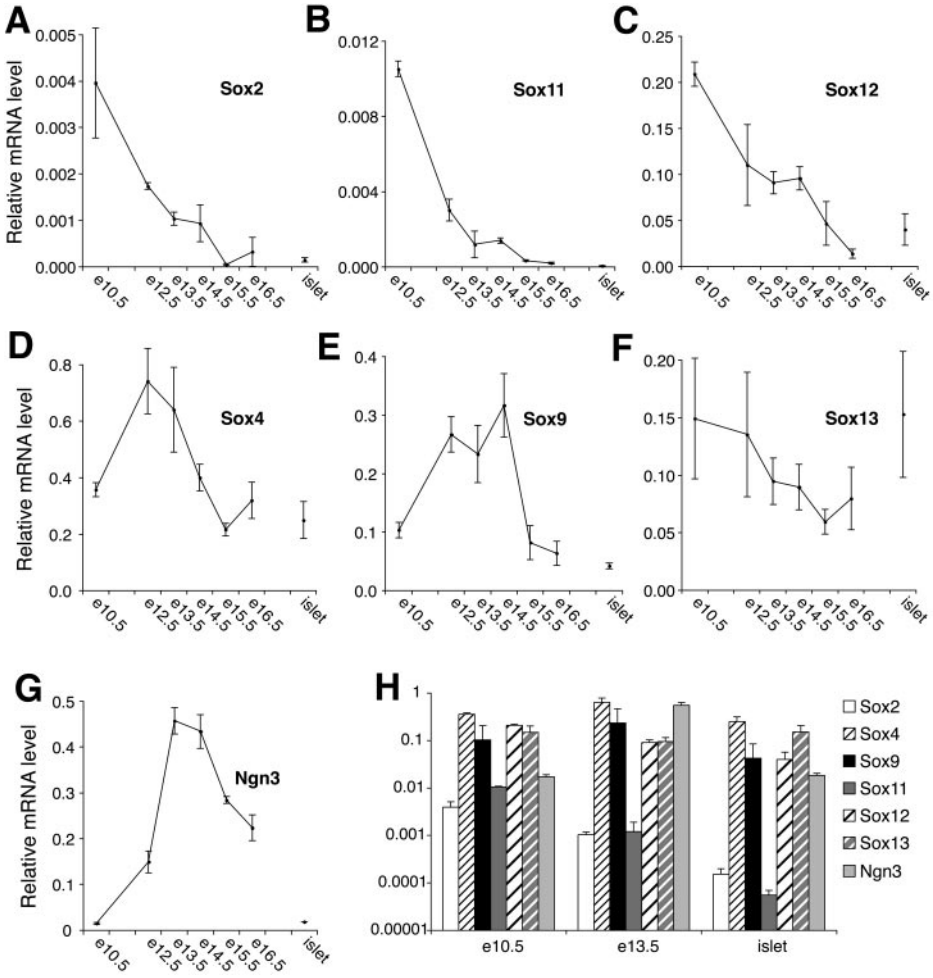

FIG. 1. Temporal expression patterns of Sox factors in the developing pancreas. Expression levels of the indicated genes in mouse pancreas were measured by real-time TaqMan RT-PCR at the embryonic dates shown and in adult islets. Results represent mean ± SEM from three independent experiments and are expressed relative to levels of mouse Histone3.3A mRNA, which was used as an internal control for cDNA quantity and quality in all assays. Ngn3, Neurogenin3.

RNase-free DNase according to the manufacturer's protocol. With the MessageAmp aRNA Kit (Ambion), 2 μg of total RNA were amplified. As previously described (14), 5 μg of amplified RNA from *sox4*−/− and *sox4*+/+ embryos were labeled with Cy3 and Cy5, respectively. Labeled samples were hybridized and washed as previously described (36). Microarrays were scanned with an Axon 400B scanner (Axon Instruments), and the data were analyzed using microarray analysis software Genepix v.4 (Axon Instruments) and the NOMAD microarray database system (http://ucsf-nomad.sourceforge.net/). Two hybridizations were performed from amplified RNA from each of three independent amplifications and used to calculate the mean fold difference and SEM shown in Table 1.

RESULTS

Expression of Sox family members in the pancreas. To survey the Sox genes expressed in the pancreas, we designed degenerate oligonucleotides that recognized all known mammalian Sox family members and used them to amplify cDNA from mouse fetal pancreas and adult islets by PCR (26). Of 50 independent PCR products from adult islets, 69% encoded Sox4 and the remainder encoded Sox10, 11, 12, and 15. From cDNA harvested from fetal pancreas at embryonic day 15.5 (e15.5), 25% of sequenced products encoded Sox4; Sox9, 11, and 15 were highly represented (>10%); and Sox5, 7, 8, 10, 12, 13, and 21 were also present. We also detected cDNA encoding Sox2 from fetal pancreas at e10.5. All but Sox2 and 12 have been reported previously in the pancreas (25–30).

To assess the temporal expression pattern of selected Sox mRNAs, we used TaqMan real-time RT-PCR and included Neurogenin3 for comparison (Fig. 1). The mRNAs encoding Sox2, 11, and 12 were present at e10.5 (Fig. 1*A–C*) but rapidly declined before the secondary transition at e13, although modest levels of *sox12* mRNA persisted in adult islets. The expression levels of mRNA encoding Sox2 and 11 were significantly lower than the other mRNAs tested (Fig. 1*H*). Levels of mRNA encoding Sox4 peaked during the secondary transition and persisted at high levels in adult islets (Fig. 1*D*). The expression pattern of mRNA encoding Sox9 most closely mirrored that of Neurogenin3, with a marked peak during the secondary transition (Fig. 1*E*). The expression level of the mRNA encoding Sox13 varied the least over the period tested and along with Sox4 remained at high levels in adult islets (Fig. 1*F*).

These RT-PCR data cannot establish which cells within the pancreas express each mRNA. To assess the spatial expression pattern of *sox2* within the pancreas, we examined mouse embryos heterozygous for a *sox2* allele disrupted by the chimeric β*geo* gene encoding a β-galactosidase/neomycin fusion protein. Cells expressing the *sox2* gene were identified with the β-galactosidase substrate X-gal, and the pancreas was identified by immunohistochemical staining for the Pdx1 homeodomain transcription factor (Fig. 2). At e9.5, the foregut, especially the developing stomach, stained strongly for β-galactosidase activity, and the proximal midgut stained more weakly (Fig. 2*A*), but the dorsal pancreatic bud did not express β-galactosidase (Fig. 2*B*). At e10.5, staining in the foregut was reduced, and by e16.5 there was no apparent staining of gut epithelium. There was no staining in the pancreas at either e10.5 or e16.5 (data not shown). Therefore,

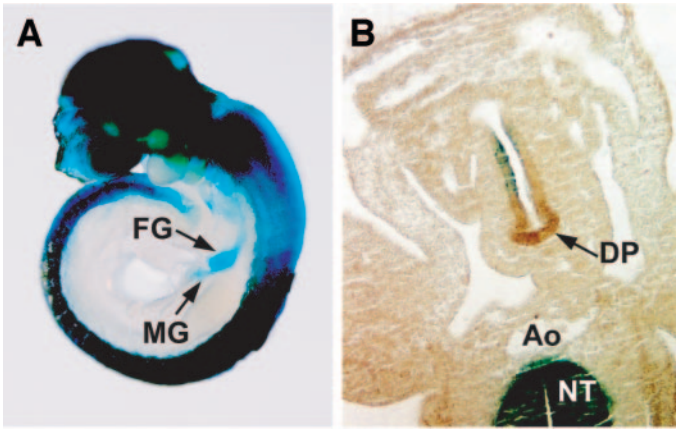

FIG. 2. Sox2-driven β-galactosidase expression in the developing endoderm of Sox2-lacZ knock-in mice. Heterozygous embryos were harvested at e9.5 and stained for β-galactosidase activity (blue). Sox2 was expressed (*A*) in the foregut (FG) as well as the proximal midgut (MG) regions of the gut tube at e9.5. However, when transverse sections of e9.5 embryos were stained for Pdx1 (brown), Sox2 was specifically excluded from Pdx1-positive cells in the dorsal pancreatic bud (DP) (*B*). Ao, aorta; NT, neural tube.

in the early pancreatic bud, when *sox2* mRNA was detected in samples from the developing pancreas (Fig. 1*A*), no evidence of *sox2* gene activity was detected within the pancreatic epithelium. The *sox2* mRNA detected by RT-PCR may have resulted from contamination of the pancreatic samples with mRNA from the adjacent gut. We cannot completely rule out the possibility, however, that very rare cells express significant levels of *sox2* mRNA in the pancreas after e9.5 or that the early expression of Sox2 in the prepancreatic gut may have effects later in development.

Nuclear expression of Sox4 protein was detected broadly in the pancreas epithelium throughout embryonic development (data not shown). In the adult pancreas, however, both in situ hybridization and immunofluorescence detected Sox4 expression only in islets (Fig. 3). Immunofluorescent costaining for Sox4 along with each of the islet hormones demonstrated the presence of Sox4 in the nuclei of all islet cells (Fig. 3*B–E*).

Islet development in Sox4 null embryos. To test the role of Sox4 in pancreatic development, we examined the development of the pancreas in mice with a targeted disruption of the *sox4* gene (31). Adult heterozygous *sox4* mutant mice appeared normal, with no difference in weight from their wild-type littermates. By intraperitoneal glucose tolerance test at age 6 months, the heterozygotes tended to have higher glucose levels than their wild-type littermates despite weighing less (Fig. 4) ($P = 0.06$ at 30 min by two-tailed Student's t test).

We examined the developing pancreas in these mice at stages up to e12.5 and found no morphological differences in the pancreas between homozygous null animals and wild-type littermates. Assessment of insulin, glucagon, and Pdx1 expression by immunohistochemistry also revealed no differences between *sox4*−/−, *sox4*+/−, and wild-type littermates at this age (data not shown). To test for changes in gene expression, we labeled cDNA from e12.5 pancreas from sox4−/− and their wild-type littermates and compared the expression patterns by hybridization on microarrays containing ~22,000 mouse cDNAs (Table 1). Although by immunohistochemistry we detected no difference in staining for insulin protein at e12.5, the microarray studies detected a consistent reduction in both *insulin1* and *insulin2* mRNA levels in the mutant pancreases. Among other mRNA reduced in the mutants, messages encoding the homeodomain transcription factor Nkx6.1 and antioxidant enzymes (peroxiredoxin 2 and glutathione peroxidase 1) were also significantly reduced in the mutants. Interestingly, the mRNAs encoding Sox11 and both islet bHLH proteins (Neurogenin3 and NeuroD1) were increased in the mutants. The complete gene expression dataset has been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2539.

Unfortunately, homozygous *sox4* mutant embryos do not survive beyond e14 because of cardiac defects (37). At e13.5, however, the pancreas has just reached the secondary transition, and very few insulin-expressing cells can be detected before that time. Therefore, we cultured explants of pancreas from e11.5 embryos for 8 days under conditions that allowed islet development to continue ex vivo (38) (Fig. 5). Explants from Sox4 null animals grew just as well in culture as those from wild-type and heterozygous littermates. After culture, explants were fixed, embedded, and stained for pancreatic markers by immunohistochemistry.

All explants developed equal amounts of acinar pancreas, but Sox4−/− pancreatic explants developed fewer endocrine cells than those from wild-type or heterozygous littermates, although all four endocrine cell types were detected (Fig. 5 and data not shown). The few endocrine cells present showed some tendency to cluster in the Sox4−/− explants but did not generate well-formed islets with a core of β-cells surrounded by α-cells, whereas classic islets formed in explants from wild-type and Sox4+/− embryos. Insulin and glucagon-expressing cells in the explants were quantified, and Sox4−/− animals were found to have significantly reduced numbers of insulin-expressing cells (Fig. 5*I*). Assessment of the frequency of replication and apoptosis of the insulin-positive cells by staining for KI67 and activated caspase 3, respectively, demonstrated no change in the cultured Sox4−/−

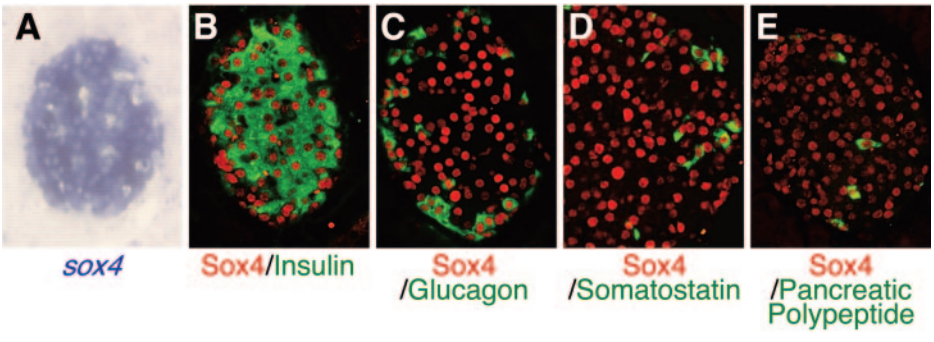

FIG. 3. Sox4 expression in the adult islet. In situ hybridization with a *sox4* anti-sense probe (*A*) specifically labeled all islet cells in an 8-week-old adult mouse pancreas. Immunofluorescent staining for Sox4 (red) in the adult mouse pancreas demonstrated expression in the nuclei of cells expressing insulin (green) (*B*), glucagon (green) (*C*), somatostatin (green) (*D*), and pancreatic polypeptide (green) (*E*).

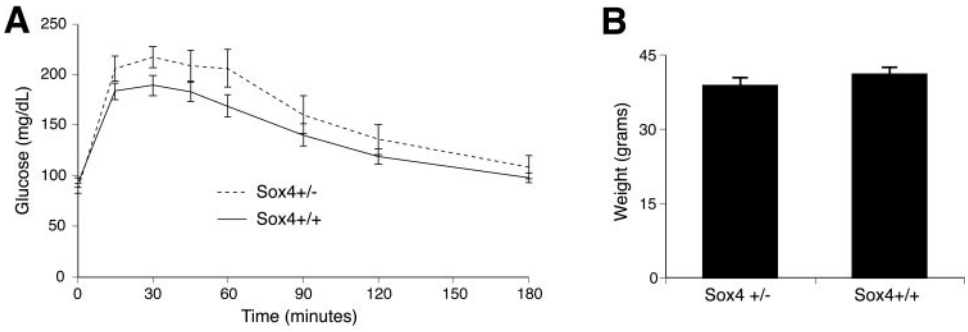

FIG. 4. Intraperitoneal glucose tolerance tests. After intraperitoneal injection of 1 g/kg of glucose (*A*), the rise in blood glucose levels was the same in 6-month-old male *sox4*+/− (dashed lines, $n = 21$) and *sox4*+/+ (solid lines, $n = 24$) mice. *B*: Mean weight for the same animals at 6 months old is shown. Error bars represent SEM.

pancreatic explants compared with control explants (data not shown).

Nkx2.2 and Nkx6.1 expression in Sox4 mutant mice. Animals lacking the transcription factors Nkx6.1 and Nkx2.2 have defects in β-cell formation (5,19). To determine whether the function of Sox4 in endocrine pancreas development lies upstream of these factors, we looked for Nkx2.2 and Nkx6.1 expression in the cultured Sox4−/− pancreatic explants and found that both factors were still expressed in the appropriate endocrine cells in Sox4−/− bud cultures (Fig. 6). It should be noted that the number of cells expressing the β-cell–specific factor Nkx6.1 was significantly reduced in concordance with both the reduction in β-cells and the microarray studies showing a reduction in *nkx6.1* mRNA at e12.5 (Table 1). Nkx2.2 is specifically required for the differentiation of insulin-producing cells in the fetal mouse pancreas (19). Real-time RT-PCR analysis of e17.5 pancreas RNA from animals lacking Nkx2.2 (19) showed that *sox4*, as well as *sox9* and *sox13*, was still expressed in the absence of Nkx2.2 (Fig. 7). These data suggest that these Sox factors do not lie directly downstream of Nkx2.2 and that the pancreatic

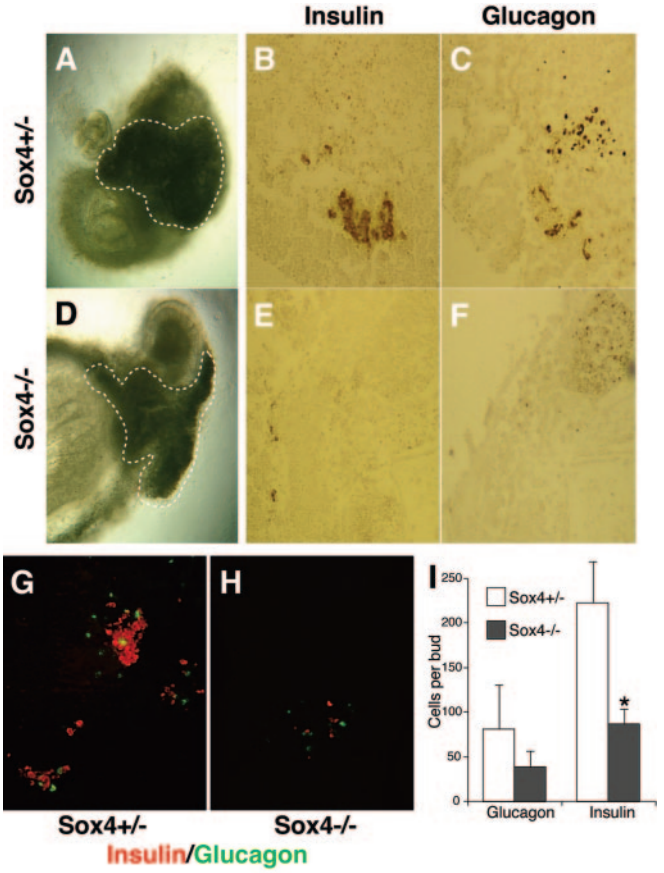

FIG. 5. Endocrine differentiation in cultured pancreatic explants. The pancreas was removed from *sox4*+/− (*A–C*, *G*) and *sox4*−/− (*D–F*, *H*) mouse embryos at e11.5 and cultured for 8 days. In *A* and *D*, the developing pancreas after 8 days in culture is outlined by the dashed line. The *sox4*−/− had markedly reduced staining for insulin (peroxidase staining in brown) (*E*) and variably reduced staining for glucagon (brown) (*F*) compared with *sox4*+/− explants (*C* and *B*, respectively). Serial sections were used for staining in panels *B*, *C*, *E*, and *F*. Immunofluorescent costaining for insulin (red) and glucagon (green) shows the same effect (*G*, *H*). These differences were quantified by counting the numbers of insulin- and glucagon-positive cells in every other section of peroxidase stained sections of the explants from *sox4*+/− ($n = 6$) and *sox4*−/− ($n = 6$) explants. (*F*). Error bars indicate SEM. *Statistically significant decrease in the number of insulin-positive cells in the *sox4*−/− explants ($P = 0.018$ by two tailed Student's *t* test).

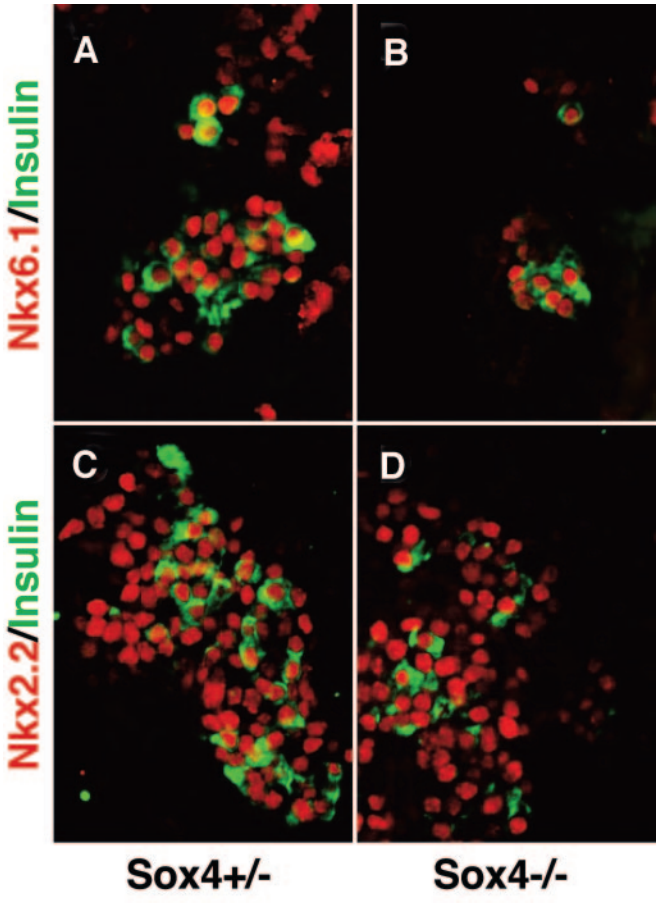

FIG. 6. Expression of Nkx6.1 and Nkx2.2 in cultured pancreatic explants. The pancreas was removed from mouse embryos at e11.5 and cultured for 8 days. Cells expressing insulin (green) also expressed both Nkx2.2 (red) (*A* and *B*) and Nkx6.1 (red) (*C* and *D*) in both *sox4*+/− (*A* and *C*) and *sox4*−/− explants.

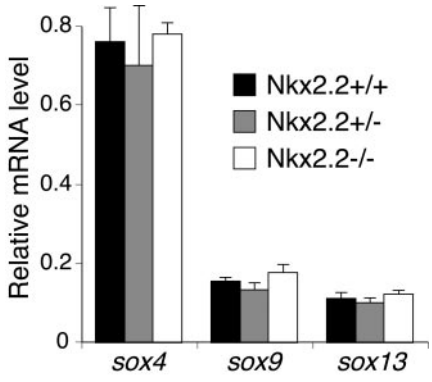

FIG. 7. Expression patterns of Sox factors in *nkx2.2*−/− pancreas. Expression levels of the indicated genes in the pancreases of *nkx2.2*+/+, *nkx2.2*+/−, and *nkx2.2*−/− mouse embryos were measured by real-time TaqMan RT-PCR at e17.5. Results represent mean ± SEM from three independent experiments and are expressed relative to levels of mouse Histone3.3A mRNA, which was used as an internal control for cDNA quantity and quality in all assays.

phenotype of the Nkx2.2 null animals does not result from the loss of these factors.

DISCUSSION

Members of the Sox family of transcription factors, including the 20 mammalian members, have been implicated in the development of a variety of tissues at all stages of development (23,24). In the developing mouse pancreas, we detected the expression of 11 Sox factors, Sox4, 5, 7, 8, 9, 10, 11, 12, 13, 15, and 21. All of these but Sox21 have been identified previously in the vertebrate pancreas, and in addition, Sox factors 17, 18, and 30 were detected previously by RT-PCR with gene-specific primers from the embryonic mouse pancreas (26). Although this suggests that the majority of Sox factors are expressed in the pancreas, some mRNAs that have been detected only by RT-PCR may not be expressed at significant levels or may originate from cells that do not derive from the pancreatic epithelium, such as mesenchymal cells, endothelial cells, migrating neurons, or circulating blood cells.

Sox2 plays a critical role in the maintenance of certain stem cell populations in vertebrates. It is expressed in the oocyte, persists through the blastocyst including the inner cell mass from which embryonic stem cells are derived, and is required for the further development of these multipotent precursors that will eventually form all of the tissues of the embryo (34). In studies in embryonic stem cells, Sox2 expression correlates with maintenance of the undifferentiated, multipotent precursor state (39,40), and in collaboration with the octamer transcription factor Oct3/4, supports a gene expression program that is critical for both maintaining the undifferentiated state and multipotency (40–43). Sox2 is also expressed in populations of neural stem cells in the central nervous system of the adult, where it appears to play a role similar to that in embryonic stem cells (44–48).

Because the endocrine cells of the pancreas share common developmental pathways with neurons, common gene expression patterns, and similar functions, it has been proposed that a population of cells similar to the neural stem cells may exist in the adult pancreas. In fact, cells expressing the intermediate filament nestin, a marker of neural stem cells (49), have been detected in the adult pancreas (50), although many of these cells are not of endoderm origin and do not appear to be stem cells (51–53). Our data in the mouse pancreas, however, indicates that Sox2-expressing cells are extremely rare, if they exist at all. Unlike the central nervous system, the mouse pancreas does not appear to contain Sox2-expressing stem cells.

In contrast, Sox4 is expressed abundantly in the pancreas, initially broadly and eventually restricted to the mature islet cells, and its predominant function appears to occur late in endocrine cell differentiation. Sox4 expression has been detected previously in a variety of developing and adult tissues in mammals, including central nervous system, thymus, heart, lung, gonads (54), and gut (55), as well as the pancreas (26). A role has been demonstrated for Sox4 in the development of B lymphocytes, the cardiac outflow tract, (31) and the central nervous system (56). In the pancreas, the pancreatic explant data demonstrate that Sox4 is required for the proper expansion of the endocrine cell population, especially β-cells, after the secondary transition.

Among the changes observed in gene expression in the Sox4 mutant embryos, we detected an increase in *sox11* mRNA. This increase in Sox11 may be partial compensation for the loss of Sox4. Among mammalian Sox factors, Sox11 is the most similar to Sox4. They share homologies throughout their sequence and 95% identity in the amino acid sequence of their HMG domains, and together with Sox12, which we also detected in the developing pancreas and the mature islet, they have been classified as class C Sox proteins (24). Early in development, Sox11 is expressed in many tissues, including the pancreatic epithelium (26,29), but, as we observed in the pancreas, its expression rapidly declines as the embryo matures (29). In the development of the central nervous system, transient Sox11 expression frequently precedes Sox4 in the same populations of neural precursors (56). In the absence of Sox11, pancreas development is modestly impaired, although islet development has not been studied in these animals (29).

Because we found that Nkx6.1 expression was reduced in the pancreases of Sox4 mutant embryos at e12.5, it is possible that the reduction in β-cell number in the Sox4 mutant explants may result from a decrease in Nkx6.1, since Nkx6.1 mutant embryos have a similar, although more dramatic, decrease in β-cell number after the secondary transition (5). Two lines of reasoning, however, suggest that Sox4 does not lie upstream of Nkx6.1 in β-cell differentiation. First, the expression of Nkx6.1 appears normal in those β-cells that do successfully differentiate in the Sox4 mutant explants. Second, the reduction in Nkx6.1 may simply result from the reduction in β-cells. Expression levels of Nkx6.1 protein are initially fairly uniform in the pancreatic bud, but by e12.5 there are already distinct gradations in nuclear expression of Nkx6.1 protein in different pancreatic cell populations (5). Given the lag between transcription and protein accumulation, differences in mRNA levels may be even greater, with the highest levels of *nkx6.1* mRNA likely in β-cells or β-cell progenitors. Insulin mRNA half-life is ~100 h, so it may take a long time after the initial differentiation of a β-cell before enough insulin accrues to allow its detection with anti-insulin antibodies. Therefore, although there is no reduction in insulin-expressing cells in Sox4 mutant embryos at e12.5, the number of cells that have initiated both *nkx6.1* and *insulin* gene transcription and would normally be detectable a day later may already be reduced. Consis-

tent with this interpretation, we also saw a reduction in transcripts for both *insulin1* and *insulin2* at e12.5 in the Sox4 mutant embryos (Table 1). These data are consistent with the assumption that the morphologic and immunohistologic changes that characterize the secondary transition in the pancreas at e13 are preceded by changes in the gene expression program a day earlier.

Interestingly, we now know of several transcription factors that contribute to endocrine cell differentiation after, but not before, the secondary transition, including the homeodomain transcription factors Pdx1, Pax4, and Nkx6.1 (5,21,57–59). In embryos lacking any of these three factors, the early endocrine cells differentiate apparently normally, but starting at the secondary transition there is a marked deficit in the formation of new endocrine cells of one or more types. This distinct difference between the gene expression pathways that lead to the differentiation of endocrine cells before and after the secondary transition has led to the conclusion that the two populations may reflect distinct lineages (5,6). These differences may have important implications for attempts to produce β-cells, since all pathways for the formation of insulin-producing cells may not be equivalent, and it may be necessary to follow the Pax4/Nkx6.1/Pdx1/Sox4 pathway to produce functionally mature β-cells for treating diabetes.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK2134 and DK61245, cores from National Institutes of Health Grant P30 DK063720, Larry L. Hillblom Foundation Grant No. 2002/1(E), Juvenile Diabetes Research Foundation fellowship awards 3-98-341 (to M.E.W.) and 3-2004-276 (to F.C.L.), and an American Diabetes Association Mentor-Based Postdoctoral Fellowship Grant (to Y.K.).

We thank members of the German and Hebrok laboratories, John Rubenstein, and Didier Stainier for helpful advice and criticism. We thank the UCSF Mouse Microarray Consortium for supplying mouse cDNA arrays.

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