GDF11 modulates NGN3+ islet progenitor cell number and promotes β-cell differentiation in pancreas development

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Accepted 21 September 2004

Development 131, 6163-6174
Published by The Company of Biologists 2004
doi:10.1242/dev.01535

Summary

Identification of endogenous signals that regulate expansion and maturation of organ-specific progenitor cells is a major goal in studies of organ development. Here we provide evidence that growth differentiation factor 11 (GDF11), a member of the TGF-β ligand family, governs the number and maturation of islet progenitor cells in mouse pancreas development. *Gdf11* is expressed in embryonic pancreatic epithelium during formation of islet progenitor cells that express neurogenin 3. Mice deficient for *Gdf11* harbor increased numbers of NGN3+ cells, revealing that GDF11 negatively regulates production of islet progenitor cells. Despite a marked expansion of these NGN3+ islet progenitors, mice lacking *Gdf11* have reduced β-cell numbers and evidence of arrested β-cell development, indicating that GDF11 is also required for β-cell maturation. Similar precursor and islet cell phenotypes are observed in mice deficient for SMAD2, an intracellular signaling factor activated by TGF-β signals. Our data suggest that *Gdf11* and *Smad2* regulate islet cell differentiation in parallel to the Notch pathway, which previously has been shown to control development of NGN3+ cells. Thus, our studies reveal mechanisms by which GDF11 regulates the production and maturation of islet progenitor cells in pancreas development.

Key words: *Gdf11*, TGF-β, Pancreas, Islet of Langerhans, Insulin, Stem cell

Introduction

Pancreatic endocrine cells comprising the islets of Langerhans produce insulin (β-cells), glucagon (α-cells), somatostatin (∂-cells), or pancreatic polypeptide (PP cells). These endocrine cells derive from progenitor cells that express the basic helix-loop-helix transcription factor encoded by neurogenin 3 (*Ngn3*; *Neurog3* – Mouse Genome Informatics; also known as *Atoh5* and *Relax*) (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Jensen et al., 2000a; Jensen et al., 2000b; Gu et al., 2002; Ravassard et al., 1997). The number of NGN3+ islet progenitor cells peaks at embryonic day (E) 15.5, then declines until birth, at which point pancreatic NGN3+ cells are undetectable and islets with mature architecture form. Elucidating the cellular and molecular mechanisms that regulate production and maturation of islet progenitor cells will be important for understanding pancreas development.

Both positive and negative regulators control the transient amplification of NGN3+ islet progenitor cells in the developing pancreas. Loss-of-function studies in mice, and in vitro analyses suggest that HNF6 (ONECUT1 – Mouse Genome Informatics) is a direct activator of *Ngn3* transcription (Jacquemin et al., 2000; Lee et al., 2001). By contrast, the product of hairy and enhancer of split 1 (HES1) probably functions as a direct repressor of *Ngn3* transcription (Jensen et al., 2000a,b; Lee et al., 2001), and prior studies provide evidence that pancreatic HES1 expression is controlled by Notch signaling in the pancreas (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Jensen et al., 2000b). However, it is not known if other intercellular signaling pathways regulate development of NGN3+ islet progenitor cells.

During islet cell maturation, the progeny of hormone-negative, proliferating NGN3+ cells exit the cell cycle, begin hormone production and aggregate to form polyclonal islets with stereotyped architecture (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Deltour et al., 1991). In neonatal mice, islets represent 2-3% of total pancreatic mass, and insulin-producing β-cells constitute about 80-90% of the islet. Experiments in mice have identified a sequence of transcription factors, including *Ngn3*, *Pax6, Neurod1, Nkx2.2, Nkx6.1* and *Isl1*, essential for maturation of β-cells (reviewed by Wilson et al., 2003). Thus far, experiments fail to demonstrate that a single transcription factor is sufficient to direct pancreas development. Rather, classical and modern studies reveal that islet cell maturation, apportioning of endocrine-to-exocrine cell ratio, and islet morphogenesis are orchestrated by multiple intercellular signals (Wessels and Cohen, 1967) (reviewed by Kim and Hebrok, 2001; Edlund, 2002; Ober et al., 2003; Wilson et al., 2003).

Prior studies of embryonic chicks and mice have implicated the transforming growth factor β (TGF-β) signaling pathways as regulators of pancreatic differentiation and islet development (Hebrok et al., 1998; Yamaoka et al., 1998; Shiozaki et al., 1999; Kim et al., 2000; Dichmann et al., 2003; Sanvito et al., 1995; Lee et al., 1995). Additionally, in vitro studies (Sanvito et al., 1994; Miralles et al., 1998; Miralles et al., 1999) have shown that TGF-β ligand activity may regulate the ratio of endocrine to exocrine cells during pancreas development. However, an endogenous pancreatic TGF-β ligand that regulates development of islet progenitor cells has not yet been identified. Here we provide evidence that growth differentiation factor 11 (GDF11; also known as BMP11) is a pancreatic TGF-β ligand that negatively regulates the production of $NGN3^+$ pancreatic islet precursor cells, and is required for regulating islet size, β-cell maturation and β-cell mass.

Materials and methods

Generation, breeding, and genotyping of mice

Targeted disruption of *Gdf11* (McPherron et al., 1999), *Smad2* (Nomura and Li, 1998), and *Hes1* (Ishibashi et al., 1995) has been previously described. *Gdf11* in adult mice and embryos was genotyped by PCR with the following forward (F) and reverse (R) primers: F=CAATGTCTGGGTGGGAGCCG; wt R=CCATGCCAG-GGATCTTGCCG; mutant R=CCTCTGAGCCCAGAAAGCGA-AGG. SMAD2 genotyping was performed by PCR to identify heterozygous animals with the following primers: F=ACAGACAA-TCGGCTGCTCTGATG; R=GATGGATACTTTCTCGGCAGGAG. *Hes1* genotyping was performed as previously described (Hirata et al., 2001). Genomic DNA was prepared from embryonic heads or yolk sacs and adult tails as previously described (Nomura and Li, 1998). Mice were maintained on a mixed 129/Sv-C57BL/6J hybrid genetic background.

Histological analysis

Digoxigenin-labeled RNA probes of *Gdf11* (McPherron et al., 1999), *Ngn3* (Dr D. Anderson, California Institute of Technology), and *Hes1* (Dr T. Vogt, Princeton University) were prepared and hybridized as previously described (Kim et al., 2000). To detect hybridization of digoxigenin-UTP (dig-UTP) labeled riboprobes to tissue sections, we used antibodies that detect dig-UTP and NBT/BCIP (blue) or INT/BCIP (rust brown, Roche, Indianapolis, IN) as previously described (Kim et al., 2000). Immunohistochemical analyses were performed as described (Kim et al., 2000; Kim et al., 2002). The primary antisera used were: rabbit anti-caboxypeptidase A (1:200, Biogenesis, Kingston, NH), guinea pig anti-glucagon (1:100, Linco, St Charles, Missouri), rabbit anti-ghrelin (1:400, Dr Lori Sussel, University of Colorado Health Sciences Center), rabbit anti-HES1 (1:500, Dr T. Sudo, Toray Industries Inc., Kamakura, Japan), rabbit anti-HNF6 (1:100, Dr G. Rousseau, Université Catholique de Louvain, Brussels), guinea pig anti-insulin (1:100, Linco), rabbit anti-insulin (1:100, ICN, Aurora, OH), hamster anti-mucin (1:200, NeoMarkers, Fremont, CA), mouse anti-NKX2.2 (both 1:25, Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-NKX6.1 (1:500, Dr M. German, University of California, San Francisco), rabbit anti-PDX1 (1:200, Dr C. Wright, Vanderbilt University, Nashville, TN), rabbit anti-PP and rabbit anti-somatostatin (both 1:200, Dako, Carpinteria, CA), and mouse anti-synaptophysin (1:50, BioGenex, San Ramon, CA). Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for immunofluorescent or peroxidase detection of primary antibodies were respective animal Ig conjuguated to Cy3 (1:800), FITC (1:200) or biotin (1:100). Ghrelin was detected using a biotinylated secondary antibody followed by streptavidin-Cy3 (1:1000, Jackson ImmunoResearch). To stain ductal cells, biotinylated lectin *Dolichos biflorus* agglutinin (DBA) was used at 1:200 (Vector Laboratories, San Bruno, CA) and visualized with streptavidin-FITC (1:200, Jackson ImmunoResearch). Immunoperoxidase detection was performed using Vectastain Elite ABC and DAB kits (Vector Laboratories). TUNEL assays were performed using the Fluorescein In Situ Cell Death Detection Kit (Roche). Immunofluorescent stains were imaged using a BioRad confocal microscope. Immunoperoxidase pictures were captured using a Zeiss Axiophot microscope.

Morphometric quantification of pancreas tissue and cell numbers

Cell counting, point-counting morphometry, and β-cell and α-cell mass determination were performed using standard morphometric techniques (Kim et al., 2002). To obtain representative results, all quantification of immunostaining was performed by counting numbers of positive-stained cells and dividing by the area of total pancreatic tissues using a standard 10×10 microscope grid. For quantification of cells expressing *Ngn3*, *Nkx6.1*, *Nkx2.2*, insulin, glucagon, *Hnf6*, *Hes1*, carboxypeptidase A, mucin, DBA and synaptophysin, pancreata were fixed, and sectioned to generate seven micron-thick tissue sections. Appropriately stained cells were counted in a minimum of 30 random microscopic fields obtained from at least three animals per genotype (if greater, then number indicated in the appropriate figure legend). All data represent the average of the indicated number of animals ± the standard error of the mean (s.e.m.). Two-tailed *t*-tests were conducted to determine statistical significance.

Results

Gdf11 expression in embryonic foregut

To analyze patterns of *Gdf11* expression in the embryonic pancreas and other foregut organs, we performed in-situ hybridizations with *Gdf11* antisense riboprobes. Prior studies demonstrated that *Gdf11* is expressed in the primitive streak region, tailbud mesoderm and neuroectoderm, but did not describe patterns of tissue expression in foregut organ anlagen (McPherron et al., 1999; Nakashima et al., 1999; Gamer et al., 1999; Liu et al., 2001; Wu et al., 2003). At E11.5 we detected *Gdf11* expression in pancreatic epithelial cells (Fig. 1A), and in posterior stomach epithelial cells (data not shown). Little to no *Gdf11* expression in pancreatic or stomach mesenchyme was detected. Thus, patterns of cellular expression suggest that *Gdf11* may regulate the development of visceral organs that form in the posterior foregut, including the stomach, spleen and pancreas.

At E13.5, we detected continued *Gdf11* expression throughout the pancreatic epithelial compartment (Fig. 1B). *Gdf11* was also detected in stomach epithelial cells adjacent to the spleen anlage, in duodenal epithelium (Fig. 1B) and, as previously reported, in renal epithelium (Fig. 1B inset) (Esquela and Lee, 2003). By E18.5, *Gdf11* expression was maintained in the acinar cell compartment, but appeared excluded from islets and ductal cells (Fig. 1C,D). Together, these studies support the hypothesis that GDF11 signaling may regulate embryonic pancreatic cell differentiation.

Gdf11 deficiency leads to malformations of the stomach, spleen and pancreas

We analyzed *Gdf11*-deficient mice (McPherron et al., 1999) to elucidate the role of *Gdf11* in development of the pancreas and adjacent organs. In $Gdf11^{-/-}$ embryos, inspection by

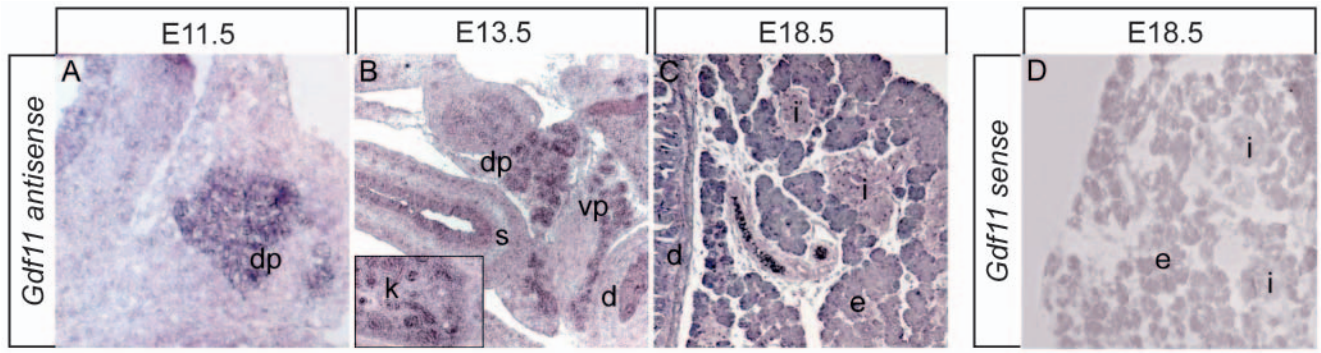

Fig. 1. Pancreatic *Gdf11* expression detected by in-situ hybridization. (A) Expression of *Gdf11* (purple) in dorsal pancreas epithelium at E11.5. Little-to-no expression of *Gdf11* mRNA is detected in pancreatic mesenchymal cells that surround the epithelium. (B) Expression of *Gdf11* (purple) in dorsal pancreas, ventral pancreas and stomach epithelium, duodenal epithelium and kidney (inset) at E13.5. (C) Expression of *Gdf11* (purple) in exocrine acinar cells and duodenal epithelium at E18.5. Little-to-no *Gdf11* expression was detected in islets. Blood cells are stained black in this panel. (D) Sense control at E18.5. Labels are the same as in C. d, duodenal epithelium; dp, dorsal pancreas epithelium; e, exocrine acinar cells; i, islets; k, kidney; s, stomach epithelium; vp, ventral pancreas epithelium.

stereomicroscopy revealed defects in stomach, spleen and pancreas morphology. Analysis of sectioned stomachs from *Gdf11*$^{-/-}$ neonates and their control littermates confirmed that there was a 2-fold reduction in the thickness of the gastric wall, accompanied by a reduction in the number of characteristic folds in the stomach, called gastric rugae (Fig. 2A,B). The spleen in *Gdf11*$^{-/-}$ mice was also invariably malformed (Fig. 2A,B, insets), similar to splenic defects seen in mice lacking the type IIB activin receptor (ACTRIIB; ACVR2B – Mouse Genome Informatics) (Oh and Li, 1997; Oh et al., 2002). The pancreas in *Gdf11*$^{-/-}$ mice appeared grossly normal at E11.5 (data not shown) but had an abnormally compact appearance by E12-13.5 and was malformed by postnatal day (P) 1 (Fig. 2C,D). Thus, consistent with its expression in foregut epithelium, GDF11 is required for morphogenesis of organs in the posterior foregut.

To elucidate the changes underlying pancreatic malformation in *Gdf11*$^{-/-}$ pancreata, we performed morphometric studies of the exocrine acini, ducts and endocrine cells, which comprise the three principal cell compartments in the pancreas. Analysis of sectioned *Gdf11*$^{-/-}$ pancreata stained with antiserum specific for carboxypeptidase A revealed a 13% decrease in pancreatic acinar cells (Fig. 2E-G). Thus, a requirement for GDF11 in partitioning of cells to an acinar fate during pancreas development may partially underlie the gross pancreatic malformation seen in *Gdf11*$^{-/-}$ mice. To assess the effects of *Gdf11* deficiency on endocrine cell number in the developing pancreas, we quantified cells expressing synaptophysin, a marker of endocrine cells in the pancreas (reviewed by Tomita, 2002; Heremans et al., 2002) and other neuroendocrine organs. Compared to wild-type littermates, the average number of synaptophysin^{+} cells was increased by 65% in *Gdf11*$^{-/-}$ mice (P<0.01) (Fig. 2H-J,N,O), revealing a role for Gdf11 in limiting endocrine cell number during pancreas development. The average number of synaptophysin^{+} cells was also slightly increased in heterozygous *Gdf11*$^{+/-}$ mice, but this increase was not statistically significant (Fig. 2J). To determine if *Gdf11* deficiency led to changes in ductal cell numbers, we quantified ductal cells marked by the lectin *Dolichos biflorus* agglutinin (DBA) (Kobayashi et al., 2002; Dor et al., 2004). We did not detect changes in the numbers of DBA^{+} cells (Fig. 2K-M,N,O) in *Gdf11*$^{+/-}$ or *Gdf11*$^{-/-}$ mice. In separate studies we also quantified ductal cells marked by antibodies that recognize mucin, a glycoprotein expressed by polarized epithelia lining the pancreatic ducts and ductules (Braga et al., 1992; Reid and Harris, 1998), and we did not detect changes in mucin^{+} cell numbers in *Gdf11*-deficient mice (data not shown). Thus, increased numbers of ductal cell subsets observed in *Gdf11*-deficient mice (described below) are not likely to result from general ductal cell expansion. We did not detect changes of cell proliferation or apoptosis in the pancreas of *Gdf11*$^{-/-}$ mice at E14.5, E17.5 or P1 (*Gdf11*$^{-/-}$ mice die on P1; McPherron et al., 1999), as determined by quantification of Ki67 expression, or TdT-mediated dUTP nick-end labeling (TUNEL) assays (Fig. 2P,Q, data not shown). Thus, it is unlikely that changes in programmed cell death or proliferation caused pancreatic malformations in mice lacking *Gdf11*. Consistent with these findings; overall pancreatic weight was unchanged in *Gdf11*$^{-/-}$ mice compared with controls (data not shown). Together, these results provide evidence that *Gdf11* modulates the relative proportions of acinar and endocrine cell numbers during mouse pancreas development.

Disrupted pancreatic islet development in *Gdf11*-deficient mice

Immunohistochemical analysis identified defects of pancreatic islet development in *Gdf11*-deficient mice. At birth, wild-type mouse pancreatic islets contain clustered insulin-producing β-cells surrounded by other hormone-producing cells, including cells producing glucagon, somatostatin or pancreatic polypeptide (Fig. 3A,E,I,L,O). In heterozygous *Gdf11*$^{+/-}$ mice at E17.5 and P1, the number of islets was slightly increased, but the islets were clearly smaller than in wild type (Fig. 3O,P). Total β-cell mass was not reduced (Fig. 3A,B,D,) but α-cell mass in *Gdf11*$^{+/-}$ mice was increased approximately 3-fold (Fig. 3E,F,H). After weaning, we did not detect alterations of glucose regulation in random-fed or glucose-challenged *Gdf11*$^{+/-}$ mice (data not shown). In *Gdf11*$^{-/-}$ mice, the mass of insulin^{+} cells was reduced approximately 2-fold (Fig. 3A,C,D), and the mass of glucagon^{+} cells was increased (Fig. 3E,G,H), resulting in islets of reduced size (Fig. 3Q). We did not observe

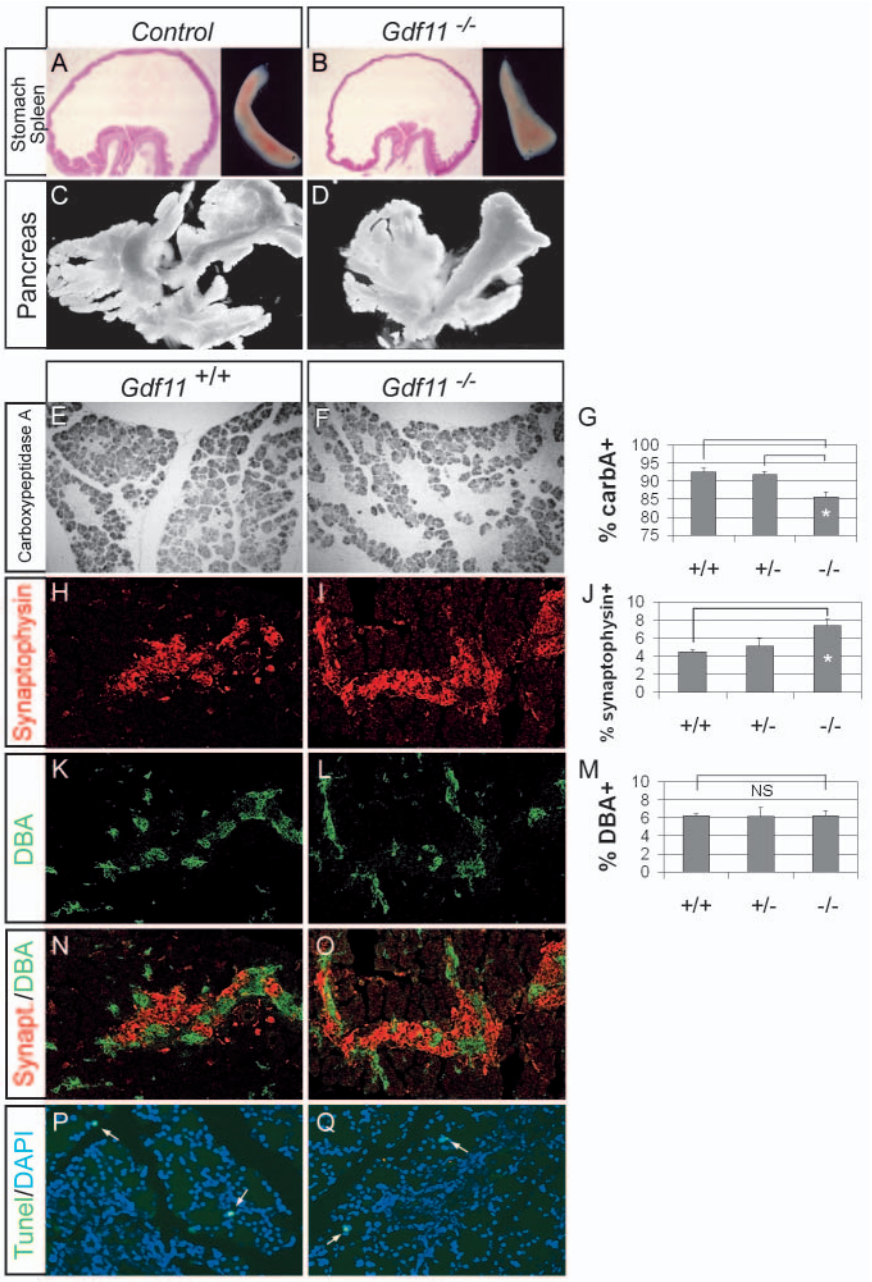

Fig. 2. Defective stomach, spleen and pancreas development in embryos deficient for *Gdf11*. '*Control*' indicates that the observed phenotype is indistinguishable in $Gdf11^{+/+}$ and $Gdf11^{+/-}$ embryos. (A,B) Sectioned stomachs stained with hematoxylin and eosin and whole mounted spleens (insets) from mice at P1. Note thinning of the stomach epithelial and mesenchymal layers and paucity of epithelial folds in the $Gdf11^{-/-}$ mouse. (A,B inset) Whole spleen preparations demonstrate spleen malformation in $Gdf11^{-/-}$ mice. (C,D) Whole mounted preparations of pancreas from $Gdf11^{+/-}$ and $Gdf11^{-/-}$ mice at P1. (E,F) Significantly reduced exocrine acinar cell development in *Gdf11*-deficient mice at E17.5. Acini are dark following immunoperoxidase staining with antiserum specific for carboxypeptidase A. (G) Quantification of morphometry showing significant reduction of exocrine acinar cell volume in $Gdf11^{-/-}$ mice relative to both wild-type and heterozygous $Gdf11^{+/-}$ mice. Data are shown as the average measurements from at least six mice of indicated genotypes±standard error of the mean. Asterisk indicates significance as $P<0.01$. (H,I) The endocrine cell compartment is significantly increased in $Gdf11^{-/-}$ mice relative to wild-type mice at E17, as assessed by immunofluorescent staining using the neuroendocrine marker, synaptophysin (red). (J) Quantification of synaptophysin staining in each of the indicated genotypes. The asterisk indicates significance as $P<0.05$ between wild-type and $Gdf11^{-/-}$ mice. There is no significant difference between wild-type and $Gdf11^{+/-}$ mice. (J) Duct cell number, as assessed by lectin DBA staining is normal in $Gdf11^{-/-}$ mice at E17 (green). (M) Quantification of DBA staining for all three genotypes. NS, not significant. (N,O) Merge of synaptophysin (red) and DBA (green) channels for wild-type and $Gdf11^{-/-}$ mice at E17.5, confirming that DBA^+ cells are distinct from $synaptophysin^+$ cells. (P,Q) Apoptotic pancreatic nuclei (green, arrows) labeled by TUNEL from E17.5 $Gdf11^{+/+}$ (P) and $Gdf11^{-/-}$ (Q) mice. All nuclei are simultaneously co-stained by DAPI (blue).

a significant change in the numbers of cells expressing somatostatin or pancreatic polypeptide in $Gdf11^{+/-}$ or $Gdf11^{-/-}$ mice (Fig. 3I-K,L-N). Together, these data provide evidence that GDF11 regulates islet cell fate and morphogenesis.

Accelerated formation and accumulation of $NGN3^+$ cells in *Gdf11*-deficient mice

The combination of increased pancreatic α-cell mass, and reductions in β-cell and acinar cell mass in *Gdf11*-deficient mice was reminiscent of phenotypes described in mice and chicks with increased pancreatic *Ngn3* expression (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Jensen et al., 2000b; Grapin-Botton et al., 2001). At E11.5, approximately 1 day after pancreatic $NGN3^+$ cells are first observed (Apelqvist et al., 1999; Gradwohl et al., 2000), we found increased numbers of pancreatic *Ngn3*-expressing cells in *Gdf11*-deficient embryos (Fig. 4A-C). By E15.5, there was a 3-fold increase of *Ngn3*-expressing cells in $Gdf11^{+/-}$ and $Gdf11^{-/-}$ mice compared with wild-type littermates (Fig. 4D-F,L) and this relative increase persisted until E17.5 (Fig. 4G-I). Thus, production and accumulation of *Ngn3*-expressing pancreatic cells was increased throughout the period of pancreatic islet development in $Gdf11^{+/-}$ and $Gdf11^{-/-}$ embryos.

Similar to those in the wild-type pancreas, $NGN3^+$ cells in *Gdf11*-deficient embryos were detected adjacent to cells expressing islet hormones such as insulin and glucagon (Fig. 4J,K). Like $NGN3^+$ cells in the wild-type pancreas, subsets of $NGN3^+$ pancreatic cells in *Gdf11*-deficient embryos expressed the proliferation antigen Ki67 (data not shown), and did not produce insulin or glucagon (Fig. 4J,K). It remains unclear if

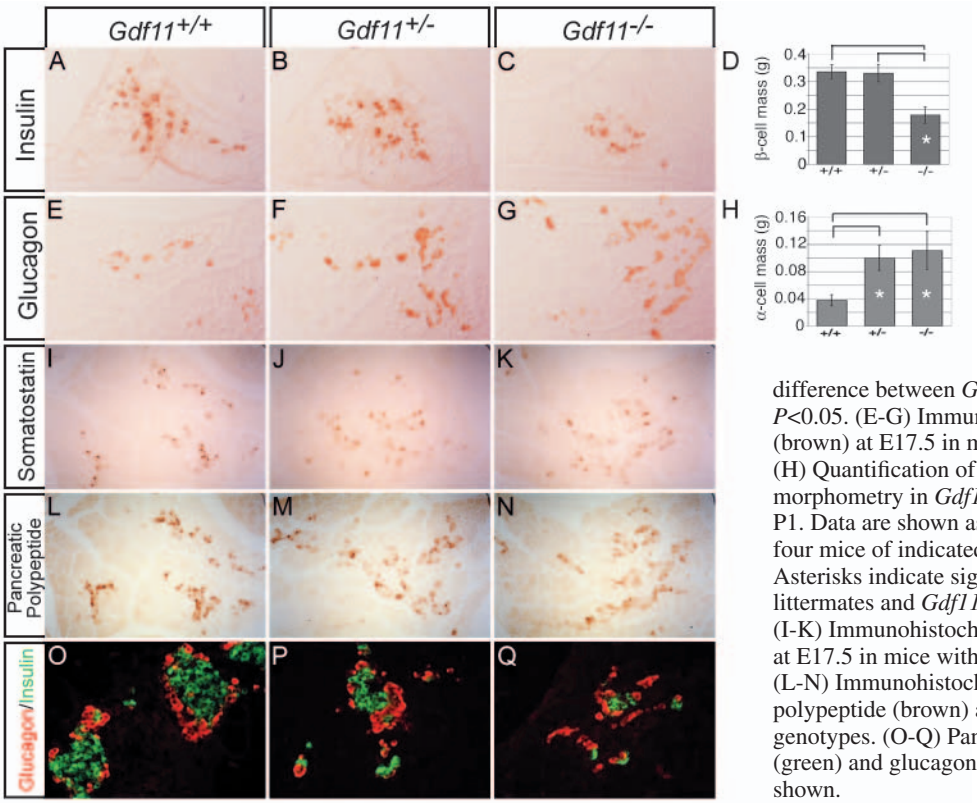

Fig. 3. Defective pancreatic islet β-cell and α-cell numbers in *Gdf11*-deficient mice. (A-C) Immunohistochemical detection of insulin (brown) at E17.5 in mice with the indicated genotypes. (D) Quantification of β-cell mass by point-counting morphometry in $Gdf11^{+/+}$, $Gdf11^{+/-}$ and $Gdf11^{-/-}$ pancreata at P1. Data are shown as the average measurements from at least four mice of indicated genotypes ± standard error of the mean. The asterisk indicates a significant difference between $Gdf11^{-/-}$ animals and $Gdf11^{+}$ littermates at $P<0.05$. (E-G) Immunohistochemical detection of glucagon (brown) at E17.5 in mice with the indicated genotypes. (H) Quantification of α-cell mass by point-counting morphometry in $Gdf11^{+/+}$, $Gdf11^{+/-}$ and $Gdf11^{-/-}$ pancreata at P1. Data are shown as the average measurements from at least four mice of indicated genotypes ± standard error of the mean. Asterisks indicate significant differences between wild-type littermates and *Gdf11* deficient animals at $P<0.05$. (I-K) Immunohistochemical detection of somatostatin (brown) at E17.5 in mice with the indicated genotypes. (L-N) Immunohistochemical detection of pancreatic polypeptide (brown) at E17.5 in mice with the indicated genotypes. (O-Q) Pancreatic islet cell expression of insulin (green) and glucagon (red) at P1. Representative samples are shown.

levels of *Ngn3* expression per cell are affected by *Gdf11* deficiency, and further studies are in progress to address this question. Nonetheless, our data show that NGN3+ cells in $Gdf11^{+/-}$ and $Gdf11^{-/-}$ mice are similar to wild-type NGN3+ cells according to several criteria.

To explore further the basis for accumulation of NGN3+ cells in mice lacking GDF11, we examined expression of HNF6, an established positive regulator of pancreatic *Ngn3* transcription (Jacquemin et al., 2000; Lee et al., 2001). The expression of HNF6 in ductal cells normally peaks around E15.5, coinciding with the peak in NGN3+ cell number (Jacquemin et al., 2000; Gannon et al., 2000; Gradwohl et al., 2000). By E17.5 the number of these HNF6+ cells declines, paralleling a decrease in NGN3+ cell number. Using anti-HNF6 antisera (Jacquemin et al., 2000), we observed two levels of HNF6 protein accumulation in the embryonic pancreas at E17.5. In acinar cells, low concentrations of HNF6 were detected in nuclei. By contrast, we observed dark nuclear staining of cells associated with, and comprising, ductal epithelia (Fig. 4M-O). Consistent with previous reports (Gannon et al., 2000), we found few HNF6+ cells associated with ducts in wild-type mice at E17.5 (Fig. 4M), corresponding with a pronounced decrease in numbers of NGN3+ cells in wild-type mice at this stage (Fig. 4G; production of both HNF6- and NGN3-antisera in rabbits precluded immunohistochemical co-localization studies). By contrast, we observed that the number of these HNF6+ cells was increased in $Gdf11^{+/-}$ and $Gdf11^{-/-}$ mice (Fig. 4N-P). These increases paralleled the accumulation of NGN3+ cells observed in these mice (Fig. 4H,I). Together, these data suggest that GDF11 may modulate the expression of HNF6 during NGN3+ cell development, although further quantitative studies of *Hnf6* mRNA levels are required to confirm this possibility.

Defective β-cell maturation in mice lacking GDF11

Despite increased numbers of NGN3+ islet precursor cells, the numbers of insulin-producing cells in $Gdf11^{-/-}$ mice was reduced (Fig. 3D). As we did not detect evidence of increased pancreatic cell death or reduced cell proliferation in $Gdf11^{-/-}$ mice, we postulated that maturation of β-cell precursors into insulin-producing cells was disrupted by the absence of GDF11 in these mice. To test this possibility, we examined expression of gene products in $Gdf11^{-/-}$ mice that regulate or define stages of β-cell maturation. In the mid- and late-gestational pancreas, NK-class homeodomain transcription factors NKX2.2 and NKX6.1 are expressed in subsets of islet precursor cells and in more mature hormone-expressing islet cells, including β-cells (Schwitzgebel et al., 2001; Sander et al., 2000; Sussel et al., 1998). In $Gdf11^{-/-}$ mice at E15.5, we observed significantly increased numbers of cells expressing NKX2.2 and NKX6.1 (Fig. 5A-F). Further analysis showed that all cells expressing NKX6.1, which is required for β-cell maturation (Sander et al., 2000), co-expressed synaptophysin, consistent with the view that these cells have begun to differentiate toward an endocrine fate (Fig. 5G,H) (Tomita, 2002). As mature β-cells express both NKX6.1 and insulin (Sander et al., 2000), and insulin+ cell numbers were reduced in $Gdf11^{-/-}$ mice, these results raised the possibility that immature β-cells expressing NKX6.1 accumulate in $Gdf11^{-/-}$ mice.

To verify this possibility, we examined expression of insulin by NKX6.1+ cells in $Gdf11^{-/-}$ mice and controls. By E17.5, insulin was expressed by nearly all NKX6.1+ pancreatic cells in wild-type mice (Fig. 5I,K). By contrast, immunostainable insulin was undetectable in 20% of NKX6.1+ pancreatic cells in $Gdf11^{-/-}$ mice (Fig. 5J,K). In $Gdf11^{-/-}$ pancreata, we did not detect expression of glucagon in NKX6.1+ cells (Fig. 5L,M),

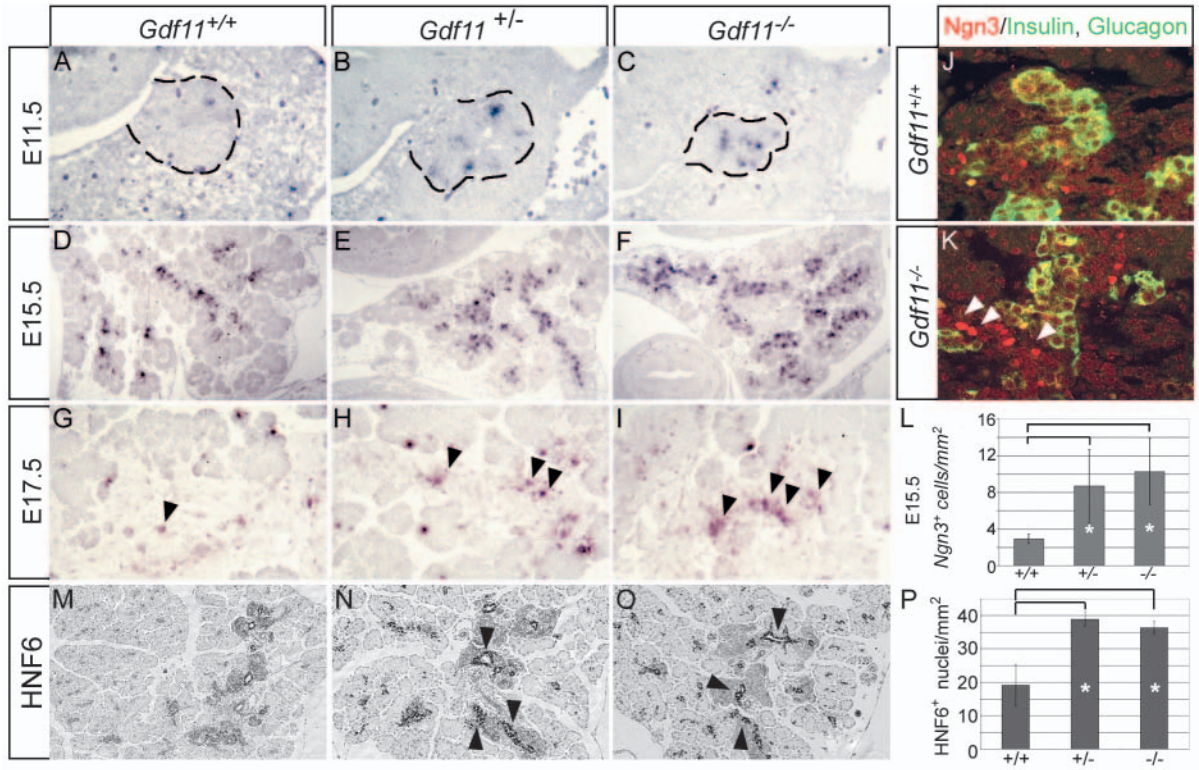

Fig. 4. Premature expansion and increased numbers of *Ngn3*$^{+}$ pancreatic cells in *Gdf11*-deficient mice. Pancreatic *Ngn3* expression (blue) detected by antisense RNA in-situ hybridization in *Gdf11*$^{+/+}$ (A,D,G), *Gdf11*$^{+/-}$ (B,E,H) and *Gdf11*$^{-/-}$ (C,F,I) embryos at the indicated embryonic stages. (G-I) *Ngn3*-expressing cells at E17.5 are marked by black arrowheads for clarity. (J,K) Pancreatic expression of insulin and glucagon (green) and *Ngn3* (red) at E17.5 in mice with indicated genotypes. White arrowheads (K) mark three of the numerous *Ngn3*$^{+}$ nuclei observed in *Gdf11*$^{-/-}$ mice. (L) Quantification of *Ngn3*$^{+}$ pancreatic nuclei per mm^2 tissue at E15.5 The increase of *Ngn3*$^{+}$ pancreatic cells in *Gdf1*$^{+/-}$ and *Gdf11*$^{-/-}$ embryos compared with wild-type littermates is significant (asterisks) at $P<0.05$. Data from three to four embryos per genotype are presented as the average ± standard error of the mean. (M-O) Expression of HNF6 (black nuclei) in *Gdf11*$^{+/+}$, *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice at E17.5. Black arrowheads in N and O mark a subset of ductal cells surrounding lumen that are heavily stained by anti-HES1 antisera. (P) Quantification of HNF6^{+} nuclei per mm^2 tissue at E17.5. Data from three to four embryos per genotype are presented as the average ± standard error of the mean. Asterisks indicate that the difference in numbers of HNF6^{+} nuclei in *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice compared with *Gdf11*$^{+/+}$ mice is significant at $P<0.05$.

nor was there increased production of PP^{+}, somatostatin^{+} (Fig. 3I-N). Recent studies suggest that mouse islets may also express the hormone ghrelin (Prado et al., 2004) but we did not detect increased numbers of ghrelin^{+} cells in *Gdf11*$^{-/-}$ pancreata (Fig. 5N,O). Thus our studies indicate that NKX6.1^{+} cells in *Gdf11*$^{-/-}$ mice have not acquired the ability to express hormones characteristic of other islet cells. Rather, these data, together with identification of a 3-fold excess of NKX6.1^{+} insulin^{-} cells, support the conclusion that *Gdf11* promotes insulin expression and terminal differentiation in developing β-cells.

Smad2 mutation leads to pancreatic defects similar to those in Gdf11-deficient mice

SMAD2 and SMAD3, also known as R-SMADs, are intracellular signal transduction proteins activated by ligand-bound activin receptors (reviewed by Massague and Chen, 2000), and both are expressed in the developing mouse pancreas (Brorson et al., 2001) (data not shown). Recent biochemical studies demonstrate that binding of GDF11 to type II activin receptors can activate SMAD2 (Oh et al., 2002). However, TGF-β signals may stimulate other signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade (reviewed by Massague, 1998). Moreover, Ogihara and co-workers recently provided evidence from in vitro studies that activin signaling regulates *Ngn3* expression in the tumor-derived AR42J cell line, and that SMAD-dependent signaling may not be required for this effect (Ogihara et al., 2003). Thus, it was unclear if perturbation of SMAD signaling in vivo would perturb pancreatic islet development.

We postulated that if GDF11 activates SMAD2 to regulate pancreas development, then loss of *Smad2* functions might phenocopy pancreatic defects observed in *Gdf11*-deficient mice. As *Smad2*$^{-/-}$ mice fail to form endoderm (Nomura and Li, 1998; Tremblay et al., 2000), we examined pancreas development in *Smad2*$^{+/-}$ mice. We observed increased numbers of *Ngn3*$^{+}$ cells and reduced β-cell mass in *Smad2*$^{+/-}$ mice (Fig. 6A,D,E,H). We also found increased numbers of NKX2.2^{+} and NKX6.1^{+} cells in the pancreas of *Smad2*$^{+/-}$ mice at E17.5 (Fig. 6B,C,F,G). Spleen and stomach development in *Smad2*$^{+/-}$ mice, by contrast, appeared normal (data not shown), suggesting that pancreas defects in *Smad2*$^{+/-}$ mice occurred in the absence of generalized foregut defects. Thus, our results

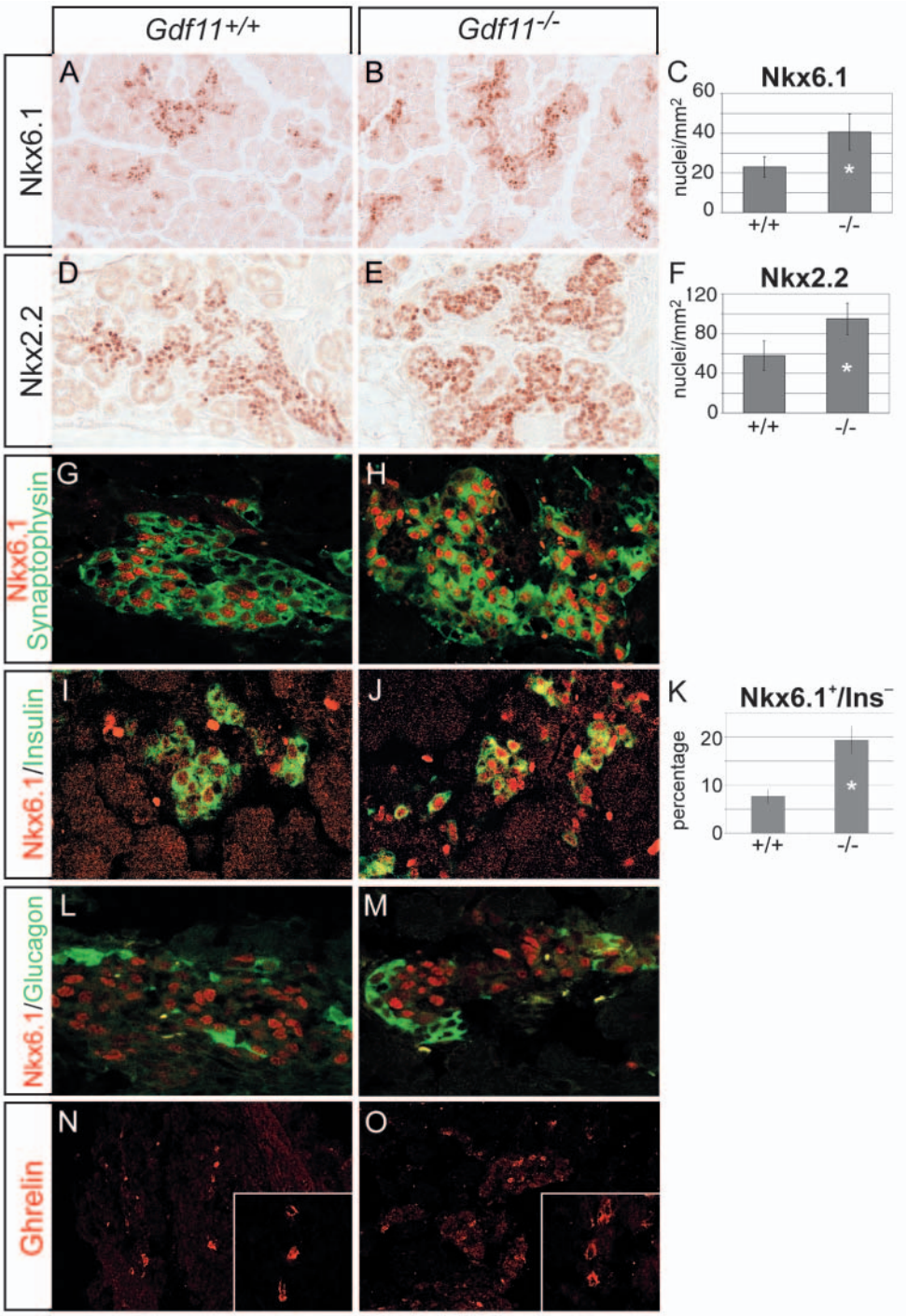

Fig. 5. Defects of β-cell maturation in *Gdf11*$^{-/-}$ mice at E17.5. (A-B) Accumulation of cells expressing NKX6.1 (brown nuclei) in *Gdf11*$^{-/-}$ mice. (C) Quantification of NKX6.1^{+} nuclei per mm^2 tissue. Data here and in panels F and K are shown as the average measurements ± standard error of the mean (n=3 mice per genotype). Asterisk indicates that the measured difference in NKX6.1^{+} nuclei in *Gdf11*$^{-/-}$ compared with *Gdf11*$^{+/+}$ mice is significant at P<0.05. (D,E) Accumulation of cells expressing NKX2.2 (brown nuclei) in *Gdf11*$^{-/-}$ mice. (F) Quantification of NKX2.2^{+} nuclei per mm^2 tissue. The asterisk indicates that the measured difference in NKX2.2^{+} nuclei in *Gdf11*$^{-/-}$ compared with *Gdf11*$^{+/+}$ mice is significant, at P<0.05. (G,H) NKX6.1 positive cells retain their neuroendocrine identity in *Gdf11*$^{-/-}$ mice at E17.5. NKX6.1 (red nuclear staining) is co-expressed with synaptophysin (green cytoplasmic staining) in both wild-type and null animals. (I,J) Absence of insulin expression (green) in a subset of NKX6.1^{+} cells (red nuclear staining) in *Gdf11*$^{-/-}$ mice at E17.5. (K) Quantification of the percentage of NKX6.1^{+} cells lacking immunostainable insulin in wild-type and *Gdf11*$^{-/-}$ mice. The asterisk indicates that the difference in values is significant, at P<0.05. (L,M) Absence of glucagon expression (green) in NKX6.1^{+} cells (red nuclear staining) in wild-type and *Gdf11*$^{-/-}$ mice at E17.5. (N,O) Normal numbers of ghrelin^{+} cells in wild-type and *Gdf11*$^{-/-}$ mice at E17.5 (red cytoplasmic staining). Panel N and O insets demonstrate cytoplasmic localization of ghrelin.

show that *Smad2* functions are essential for islet development, and suggest that *Smad3* functions are unable to compensate fully for loss of *Smad2* function in the embryonic pancreas. The similarity of pancreatic defects in mice deficient for *Smad2* or *Gdf11* also provides indirect evidence that GDF11 and SMAD2 function in the same signaling pathway to control accumulation and maturation of *Ngn3*-expressing cells in pancreas development. The phenotypes observed in mice haploinsufficient for *Smad2* or *Gdf11* suggest that pancreas cell fates are highly sensitive to loss-of-function mutations affecting this signaling pathway.

Evidence that *Gdf11* regulates pancreatic NGN3+ cell number in parallel to the Notch pathway

The Notch pathway regulates differentiation of NGN3^{+} cells in pancreas development (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Jensen et al., 2000a; Jensen et al., 2000b). In the pancreas and other organs, Notch signaling culminates in expression of *Hes1*, a transcriptional repressor of *Ngn3* (Jensen et al., 2000b; Lee et al., 2001). Perturbation of Notch signaling in the pancreas and other organs can lead to detectable changes in *Hes1* expression (Jarriault et al., 1995; Jensen et al., 2000b; Hald et al., 2003; Norgaard et al., 2003). Thus, to explore the

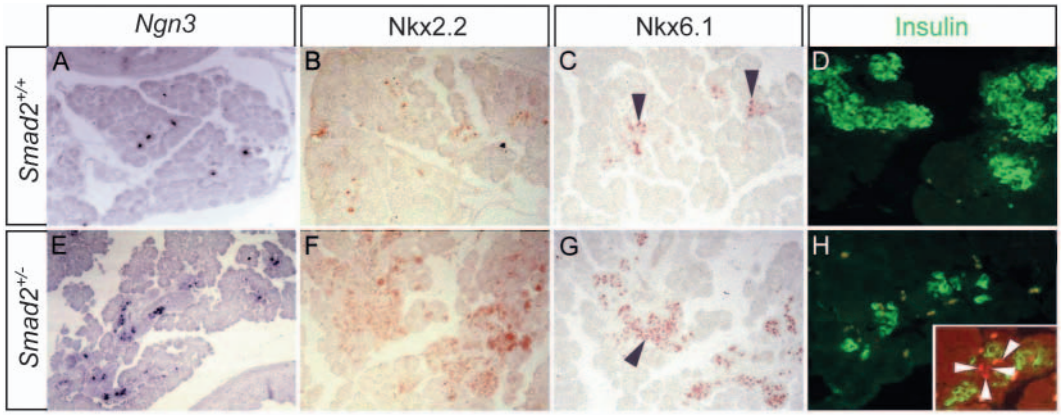

Fig. 6. Pancreatic defects in E17.5 *Smad2* mutant embryos. (A,E) *Ngn3* expression (blue) detected by antisense RNA in-situ hybridization. (B,F) NKX2.2 expression (brown nuclei) adjacent to ductal epithelium. (C,G) NKX6.1 expression (brown nuclei marked by arrows). (D,H) Insulin expression (green) detected by IHC and confocal microscopy. Inset, H: NGN3$^+$ nuclei (red) adjacent to insulin$^+$ cells (green). Images are representative of four or more animals per genotype.

possibility that increased *Ngn3* expression in *Gdf11*-deficient mice resulted from perturbed Notch signaling, we quantified HES1$^+$ pancreatic cells. Numbers of HES1$^+$ cells in *Gdf11*-deficient mice at E13.5, 15.5 and 17.5 were similar to those in wild-type littermates (Fig. 7A-D and data not shown). While further studies are required to exclude the possibility that pancreatic Notch signaling is perturbed in *Gdf11*-deficient mice, these data provide support for the view that GDF11 signaling pathways regulate development of pancreatic NGN3$^+$ cells in parallel or downstream of the Notch pathway.

To distinguish between these latter two possibilities, we examined *Gdf11* expression in mice lacking HES1 (Ishibashi et al., 1995). In-situ hybridization revealed that pancreatic *Gdf11* expression in *Hes1*-deficient and wild-type littermate mice was indistinguishable (Fig. 7E,F). Although it remains possible that translation or post-translational modification of GDF11 protein may be affected by *Hes1*-deficiency, these results suggest that signaling pathways regulated by GDF11 control the development of pancreatic NGN3$^+$ cells in parallel with the Notch signaling pathway.

Discussion

GDF11 signaling is required for morphogenesis of foregut-derived organs

Mice lacking GDF11 manifest gross defects in the development of the stomach, pancreas and spleen. GDF11, like Sonic hedgehog (Apelqvist et al., 1997; Hebrok et al., 1998; Harmon et al., 2002) and retinoids (Stafford and Prince, 2002; Kumar et al., 2003), is therefore an essential signal for establishing normal foregut development. The foregut phenotypes observed in *Gdf11*$^{-/-}$ mice are similar to those we previously described in mice deficient for type IIB activin receptor (Kim et al., 2000), consistent with a recent study that demonstrated biochemical and genetic interactions between GDF11 and ACTRIIB (Oh et al., 2002). It is possible that the organ defects observed in *Gdf11*$^{-/-}$, and *ActRIIB*$^{-/-}$ mutants are secondary to changes in axial patterning. However, in *Gdf11*$^{+/-}$ and in *Smad2*$^{+/-}$ mutant mice, increased numbers of pancreatic cells expressing NGN3, HNF6 or glucagon were found in the absence of gross defects in stomach, spleen or other foregut-derived organs. Previously, we also reported that *ActRIIA*$^{+/-}$ *ActRIIB*$^{+/-}$ mice, which harbor *trans*-heterozygous mutations in the type II activin receptors A and B, develop hypoplastic pancreatic islets without detectable malformations in the stomach or spleen (Kim et al., 2000). Thus, while only a subset of pancreatic phenotypes show haploinsufficiency, together these observations indicate that the embryonic pancreas may be more sensitive than the stomach and spleen to disrupted TGF-β signaling.

GDF11 signaling regulates the number of pancreatic NGN3$^+$ islet progenitor cells

One of the principal findings in this study is that GDF11 modulates the number of NGN3$^+$ cells, a pancreatic progenitor cell population crucial for islet development. Our data support a model in which GDF11 negatively regulates the formation of NGN3$^+$ cells in the developing pancreas. *Smad2* is also required for this process, suggesting that *Gdf11* and *Smad2* function in the same pathway in pancreatic development. While there is good evidence that Notch signaling regulates production of these islet progenitors, to our knowledge this is the first demonstration that TGF-β signaling can impact the development of these cells. Supporting this view, we observed that mice deficient for *Gdf11* harbor defects in islet and exocrine cell fates similar to those observed in animals that mis-express *Ngn3*. For example, following forced expression of *Ngn3* in foregut endoderm, chick embryos produce increased numbers of glucagon$^+$ cells (Grapin-Botton et al., 2001). Likewise, in mice that prematurely express *Ngn3*, including mice lacking *Hes1* (Jensen et al., 2000b) or in transgenic mice expressing *Ngn3* from *Pdx1* (also known as *Ipf1*) regulatory elements (Apelqvist et al., 1999; Schwitzgebel et al., 2000), there is a relative increase in the number of glucagon$^+$ cells, accompanied by reductions in acinar and insulin$^+$ cell numbers. We suggest that increased production and accumulation of NGN3$^+$ cells in *Gdf11*$^{-/-}$ mice is also the basis for increased numbers of glucagon$^+$ cells, and reductions in insulin$^+$ and acinar cell numbers, observed in these mice.

While the cellular origin of NGN3$^+$ cells in the developing

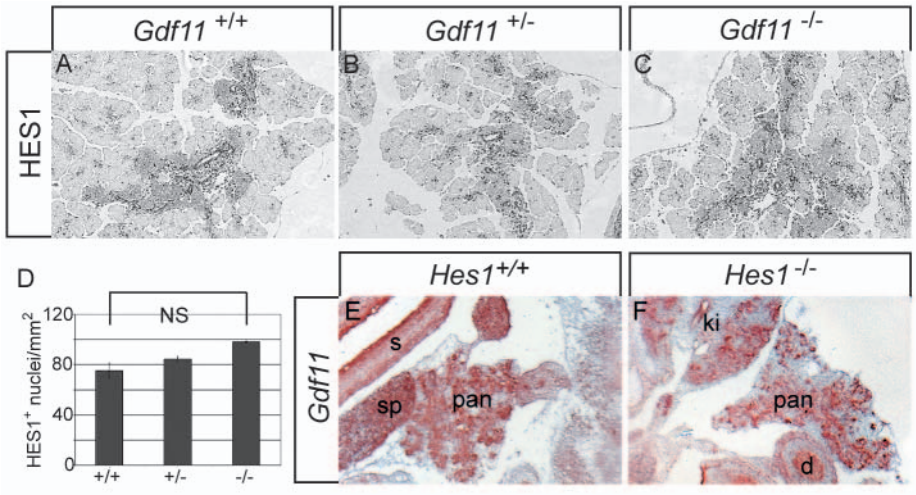

Fig. 7. Pancreatic expression of HES1 in *Gdf11*-deficient mice. (A-C) Expression of HES1 (black nuclei) in *Gdf11*$^{+/+}$, *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice at E17.5. (D) Quantification of HES1^{+} nuclei per mm^2 tissue. The difference in numbers of HES1^{+} nuclei in *Gdf11*$^{+/+}$, *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice is not significant (NS). (E,F) *Gdf11* expression (rust brown) detected by in-situ hybridization in *Hes1*$^{+/+}$ and *Hes1*$^{-/-}$ pancreata at E15.5.

pancreas remains the focus of investigation, results from several studies provide some evidence that embryonic NGN3^{+} cells derive from pancreatic ductal cells (Gradwohl et al., 2000; Jensen et al., 2000b; Schwitzgebel et al., 2000; Gu et al., 2002). Could the increased numbers of NGN3^{+} cells we detected in *Gdf11*-deficient pancreata reflect a global increase in this ductal cell compartment? To address this possibility, ductal cells were marked and quantified using previously described immunohistochemical methods (Reid and Harris, 1998; Kobayashi et al., 2002; Dor et al., 2004). These analyses demonstrated that ductal cell number was unchanged in *Gdf11*$^{-/-}$ and heterozygous *Gdf11*$^{+/-}$ mice. Thus, our results make it unlikely that a global expansion of ductal cell mass is the basis for increased numbers of NGN3^{+} cells observed in *Gdf11*-deficient mice.

Our observations further suggest that the increased number of NGN3^{+} cells in *Gdf11*-deficient mice is not solely an indirect consequence of defective β-cell maturation. The majority of islet cells are insulin^{+} β-cells, which derive from NGN3^{+} precursors (Gu et al., 2002), and we found evidence for impaired β-cell maturation in *Gdf11*$^{-/-}$ mice. In *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice, the increase in numbers of NGN3^{+} cells was similar. However, the β-cell mass in heterozygous *Gdf11*$^{+/-}$ and wild-type mice was indistinguishable. Thus, in *Gdf11*$^{+/-}$ mice, the increase of NGN3^{+} cells is genetically *uncoupled* from apparent defects in production of insulin^{+} cells. These observations support our view that the increased number of NGN3^{+} cells in mice lacking GDF11 or SMAD2 results from increased production of NGN3^{+} cells. We have tested purified GDF proteins for activity when added to E10.5 dorsal pancreatic rudiments grown in tissue culture. We have not yet detected significant activity of these factors in regulating formation of *Ngn3*-expressing cells (Å.A., E.H. and S.K., unpublished). Thus, our studies do not yet establish whether GDF11 is sufficient to regulate the development of endogenous pancreatic islet precursor cells.

GDF11 and Notch signaling: parallel negative regulators of islet progenitor cell development?

Our data suggest that GDF11 and SMAD2 signaling activity regulate the development of pancreatic NGN3^{+} pro-endocrine cells. Prior reports suggested a role for the Notch signaling pathway in regulating the expansion of the NGN3^{+} cell compartment in pancreas development (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Jensen et al., 2001a; Jensen et al., 2001b). In certain contexts, others have recently provided evidence for signaling interactions between the Notch and TGF-β pathways (Zavadil et al., 2001; Blokzijl et al., 2003). In our studies, the number of cells expressing Hes1, a transcriptional repressor of *Ngn3* (Jensen et al., 2000b), was unchanged in *Gdf11* deficient mice. While further studies are needed to exclude the possibility of other Notch signaling defects in *Gdf11*-deficient mice, these data suggest that GDF11 activity may not be required for pancreatic Notch signaling and production of HES1^{+} cells. Moreover, the number of pancreatic cells expressing *Gdf11* was similar in mice lacking *Hes1* and control mice. Regulation of TGF-β ligand activity is thought to occur at several stages, including transcription, post-translational processing, activation and inhibition (Massague and Chen, 2000; Attisano and Wrana, 2002). Thus, it remains possible that *Hes1*-deficiency or other Notch signaling defects may perturb *Gdf11* signaling pathways. These caveats notwithstanding, our results provide evidence for a model in which GDF11 and Notch signaling function in parallel pathways to negatively regulate production of pancreatic NGN3^{+} pro-endocrine cells.

Identification of unequivocal pancreatic endocrine defects arising from *Gdf11* or *Smad2* haploinsufficiency suggests that developing islet progenitor cells are particularly sensitive to perturbations in TGF-β signaling, and raises the possibility that subsets of familial pancreatic endocrine disorders could result from heterozygous mutation in *Smad2* or *Gdf11*. In mice deficient for *Gdf11*, we observed that some phenotypes, such as accumulation of excess NGN3^{+}, HNF6^{+} or glucagon^{+} cells, was similarly severe in both *Gdf11*$^{+/-}$ and *Gdf11*$^{-/-}$ mice, while others, such as reduction of β-cell mass, was observed only in *Gdf11*$^{-/-}$ mice. While the molecular basis for these gene-dosage effects requires further elucidation, we note that the correlation of phenotypic severity with *Gdf11* gene dosage is consistent with similar effects we previously reported for mice harboring one, two or three null alleles of the type 2 activin receptors *ActRIIA* and *ActRIIB* (Kim et al., 2000). Together, these observations suggest that the strength or duration of TGF-β signaling may be a crucial determinant of cell fates in the developing pancreas.

The preservation of some β-cell development in mice deficient for *Gdf11* or *Smad2* indicates that additional TGF-β ligands and intracellular SMADs may also regulate pancreatic islet development. Consistent with this possibility, prior work has shown that TGF-β ligands like BMP7, activins, TGF-β1, TGF-β2, and TGF-β3, and the R-SMAD members SMAD1, SMAD3, and SMAD5 are expressed in the developing pancreas (Lyons et al., 1995; Miralles et al., 1999; Brorson et al., 2001; Dichmann et al., 2003). Thus, redundant activities from these factors in the pancreas might mitigate the observed in vivo effects of *Gdf11* or *Smad2* loss of function. Further experiments, including studies of genetic interactions between *Gdf11* and *Smad2*, are required to determine effects of more severe disruption of TGF-β signaling in islet development.

GDF11 and SMAD2 activity regulate pancreatic β-cell maturation and number

The second principal finding in our study is that GDF11 may regulate the terminal differentiation of NKX6.1$^+$ cells into insulin-producing β-cells. While GDF11 has been shown previously to regulate the number of cellular progenitors in organs such as the olfactory primordium, there has been no prior evidence, to our knowledge, for GDF11 roles in maturation of cells toward a terminally differentiated fate. Thus, our study provides novel insights into the range of developmental roles for GDF11 in neuroendocrine cell differentiation.

Recent evidence suggests that GDF11 activity regulates neurogenesis in embryonic olfactory epithelium (Wu et al., 2003). In this study, Calof and colleagues described the number of *ngn1*$^+$ immediate neuronal precursors (INPs) in the olfactory epithelium of *Gdf11*$^{-/-}$ mice as being increased by 20%, with corresponding increases in the number of differentiated olfactory neurons (Wu et al., 2003). Thus, GDF11 negatively regulates formation of *ngn1*$^+$ INPs but is not essential for neuronal cell maturation or survival in the developing olfactory epithelium. In the pancreas of *Gdf11*$^{-/-}$ mice we observed a 300% increase of NGN3$^+$ cells, accumulation of immature endocrine cells, and reduced β-cell mass. Together, our data show that GDF11 activity regulates *both* the production of pancreatic islet progenitor cells and the maturation of these progenitor cells into insulin-producing β-cells. Further support for this view also comes from a recent study by Sarvetnick and colleagues, who show that TGF-β signaling may regulate endocrine cell maturation in the postnatal pancreas (Zhang et al., 2004). Future studies should allow us to determine whether GDF11 and SMAD2 signaling also serve to maintain the function and fate of fully differentiated endocrine cells in the adult pancreas.

Cell cycle regulators also probably control maturation of β-cells and other islet cells, as the majority of islet cells are post-mitotic, and increased hormone production is associated with growth arrest (Ptasznik et al., 1997). TGF-β signaling is an established regulator of the cell cycle, and in specific contexts TGF-β signals have been shown to control the expression of cell cycle components, including p15, p18, p21 and p27^{kip1}, members of the family of cyclin-dependent kinase inhibitors (Moustakas et al., 2002). In maturing neural progenitor cells, GDF11 activity increases expression of the cyclin-dependent kinase inhibitor *p27*^{kip1} (Wu et al., 2003), suggesting that disrupted endocrine cell maturation in *Gdf11*$^{-/-}$ mice might reflect an impairment of growth arrest. Indeed, we have also observed an overall reduction of pancreatic p27^{kip1} expression in *Gdf11*$^{-/-}$ mice (E. Harmon, PhD thesis, Stanford University, 2003), but it remains unclear from these and other studies whether p27^{kip1} expression is reduced specifically in pancreatic endocrine or β-cell precursors (N.S., Å.A., E.H. and S.K., unpublished). Thus, additional tests are required to clarify how GDF11 may promote β-cell maturation.

This study provides evidence that GDF11 is an endogenous inhibitor of endocrine progenitor cell formation in the embryonic pancreas. Our studies reveal that *Gdf11* is expressed throughout the pancreatic epithelial cell compartment when NGN3$^+$ endocrine progenitor cells first appear. Thus, we postulate that GDF11 is a crucial autocrine or paracrine regulator of islet progenitor cell formation. Similar short-range signaling roles have been proposed for regulation of myoblast development by GDF8, a TGF-β ligand highly homologous to GDF11, and for GDF11 in regulating neurogenesis (Lee and McPherron, 1999; Thomas et al., 2000; Wu et al., 2003). Identification of TGF-β signals that regulate pancreatic islet development may prove useful for generating strategies to regenerate or replace islets in patients with diabetes mellitus.

We thank Dr S.-J. Lee (John Hopkins University School of Medicine, Baltimore, MD) for *Gdf11* mutant mice; Drs R. Kageyama (Kyoto University, Kyoto, Japan) and A. Bhushan (Childrens' Hospital, Los Angeles) for help with *Hes1* mutant mice; Drs M. German and Y. Kosaka (Diabetes Center, University of California at San Francisco) for advice on synaptophysin detection; and Drs German, Bhushan, M. Hebrok and P. Serup for helpful discussions. We also thank J. Heit and Drs L. Selleri, J. Gilthorpe, T. Sugiyama, I. Rulifson and Y. Hori for critical reading of earlier versions of this manuscript. E.B.H. was supported by a National Institutes of Health (NIH) training grant; Å.A.A. was supported by the Swedish Foundation for International Cooperation in Research and Higher Learning (STINT); and N.G.S. was supported by a Ruth Kirschstein National Research Service Award Individual Fellowship from the NIH, a Digestive Diseases Endocrinology training grant from the NIH, and a Stanford Dean's postdoctoral fellowship. This work was supported by grants to S.K. from the NIH (RO1 DK56709), the American Diabetes Association, the Pew Charitable Trusts Biomedical Scholars Program, a gift through the Verto Institute from Dr Raymond Sackler and Beverly Sackler, and a Pilot Project award through the Stanford Digestive Disease Research Core Center (DK99017).

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