

Signals from the neural crest regulate beta-cell mass in the pancreas

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Pancreatic islet cells and neurons share common functions and similar ontogenies, but originate in different germ layers. To determine whether ectoderm-derived cells contribute instructive signals to the developing endoderm-derived pancreas, we defined the chronology of migration and differentiation of neural crest cells in the pancreas, and tested their role in the development of the islets. The homeodomain transcription factor *Phox2b* marks the neural precursors from the neural crest that colonize the gut to form the enteric nervous system. In the embryonic mouse pancreas, we found *Phox2b* expressed briefly together with *Sox10* along the epithelial-mesenchymal border at E12.5 in cells derived from the neural crest. Downregulation of *Phox2b* shortly thereafter was dependent upon *Nkx2.2* expressed in the adjacent pancreatic epithelium. In *Phox2b*^{−/−} embryos, neurons and glia did not develop in the pancreas, and *Nkx2.2* expression was markedly upregulated in the epithelium. In addition, the number and replication rate of insulin-expressing beta-cells increased in the *Phox2b*^{−/−} mice. We conclude that, during pancreatic development, *Phox2b* and *Nkx2.2* form a non-cell-autonomous feedback loop that links the neural crest with the pancreatic epithelium, regulates the size of the beta-cell population, and thereby impacts insulin-secretory capacity and energy homeostasis.

KEY WORDS: Islet, *Nkx2.2*, *Pdx1*, *Phox2b*, *Sox10*

INTRODUCTION

Similarities between the pancreatic endocrine cells that form the islets of Langerhans and the neurons and endocrine cells that originate from the ectoderm germ layer via the neural crest previously led to the postulation that pancreatic islet cells also derive from the neural crest (Pearse and Polak, 1971). However, a classic series of experiments using quail-chick chimaeras established that the endocrine cells of the pancreas, along with the pancreatic duct and exocrine cells, as well as the endocrine cells of the gut, all originate from the endoderm germ layer, and not from the neural crest or other ectoderm (Andrew, 1976; Fontaine and Le Douarin, 1977; Pictet et al., 1976). Subsequent experiments further demonstrated that mesoderm-derived tissues, specifically notochord and vascular endothelium, provide signals that direct the differentiation of the pancreatic endoderm (Kim et al., 1997; Lammert et al., 2001). The potential role of ectoderm-derived cells in providing instructive signals to pancreatic endoderm has not been explored previously.

During embryonic development, the endocrine cells of the pancreas and gut differentiate from a layer of epithelial cells of endoderm origin that line the early gut lumen and pancreatic ducts. In the pancreas, endocrine differentiation occurs in parallel with the growth of the pancreatic buds, the first of which grows from the dorsal aspect of the proximal midgut at embryonic day 9.5 (E9.5) in the mouse. Endocrine differentiation depends on the inactivation of Notch signaling, which allows the transient expression of the basic helix-loop-helix transcription factor neurogenin 3. Neurogenin 3 triggers a cascade of genes, including the gene encoding the homeodomain transcription factor *Nkx2.2*,

that drive islet cell differentiation. *Nkx2.2* in turn drives the expression of the homeodomain transcription factor *Nkx6.1*, which, together with other factors, leads to the differentiation of the insulin-producing beta-cells (Murtaugh, 2007; Wilson et al., 2003).

At the same time that the first pancreatic cells start to bud from the dorsal gut endoderm, cells from the neural crest begin to arrive at the rostral foregut in a rostral-to-caudal migratory wave that eventually populates the entire gut with progenitors of neural and glial cells (Young and Newgreen, 2001). All of these cells initially express the HMG box transcription factor *Sox10* and its downstream target homeodomain transcription factor *Phox2b* (Kim et al., 2003; Young et al., 2003). *Sox10* persists in differentiated glia, and *Phox2b* persists in differentiated neurons, but both are required for the formation of neurons and glia in the gut (Herbarth et al., 1998; Pattyn et al., 1999; Southard-Smith et al., 1998; Young et al., 2003).

Little is known about the timing and pattern of migration of, and the gene expression in, neural crest cells in the pancreas, although *Sox10* has been detected in the early pancreatic buds (Lioubinski et al., 2003; Wilson et al., 2005). Interestingly, the expression of *Phox2b* overlaps with that of *Nkx2.2* and *Nkx6.1* in a subset of neural precursors in the central nervous system (CNS), where *Nkx2.2*-dependent downregulation of *Phox2b* expression is required for the differentiation of serotonergic neurons (Cordes, 2005). However, the expression and function of *Phox2b* in the pancreas, and the role of the neural crest cells in the development of the pancreatic endoderm has not been explored.

To explore the role of neural crest cells in pancreatic development, we defined the expression of *Phox2b* and *Sox10* in the embryonic pancreas, determined the origin and fates of the *Phox2b*-expressing cells, and tested their role in endocrine cell development.

MATERIALS AND METHODS

Mice

The *Phox2b*^{lacZ} mouse line and rescue experiments have been described previously (Pattyn et al., 2000; Pattyn et al., 1999). The *lacZ*-*cre* reporter mouse line *R26R* (Soriano, 1999), and the *Wnt1*-*cre* mouse line (Danielian

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et al., 1998) were acquired from The Jackson Laboratory (Bar Harbor, Maine). The *Pdx1-cre* mouse line was generously provided by Douglas Melton (Gu et al., 2002). All mice were backcrossed into and maintained in the C57BL/6 background.

Mice were housed on a 12-hour light-dark cycle in a controlled climate. Timed matings were carried out with E0.5 being set as midday of the day of discovery of a vaginal plug. All studies involving mice were approved by the UCSF Institutional Animal Care and Use Committee.

Immunohistochemistry

Harvested embryos were processed for whole-mount immunohistochemistry until E12.5. After that stage, only the gastrointestinal portion was processed. Tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline without calcium and magnesium ions (PBS) at 4°C, then washed three times in PBS alone. Tissue was then immersed into 30% sucrose in PBS and gently rotated overnight at 4°C. The next day, the sucrose solution was replaced by a series of embedding medium Tissue-Tek O.C.T. (Electron Microscopy Sciences, Hatfield, PA) dilutions in 30% sucrose. Tissue was incubated for 1 hour in each of the 25, 50 and 75% OCT solutions. Tissue was then immersed into blocks containing 100% OCT and frozen on dry ice. Frozen sections (5–10 µm) on slides were air-dried, washed once in cold PBS and antigens retrieved by boiling in Antigen Retrieval Solution (BioGenex, San Ramon, CA) for 10 minutes. Slides were then cooled down and washed in water and then in PBS. For cell counting experiments, the antigen retrieval step was omitted.

For immunofluorescence, slides were transferred into humidified chambers and sections were blocked with 5% normal goat serum (NGS) in PBS and goat anti-mouse IgG (MP Biomedicals, Aurora, OH) diluted 1:30 in 5% NGS, for 30 minutes and 1 hour at room temperature (RT), respectively. Slides were then washed three times in PBS and sections incubated with one or two primary antibodies (see Table 1), diluted in 5% NGS, overnight at 4°C. The next morning, slides were washed three times in PBS and sections incubated with the appropriate FITC- and/or Cy3-conjugated secondary IgG antibodies (The Jackson Laboratory), diluted 1:200 or 1:800 in 5% NGS, respectively, for 1 hour at RT and in the dark. After three more washes in PBS, sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and analyzed with epifluorescence microscopy. For DBA staining, the biotinylated lectin Dolichos biflorus agglutinin (DBA; Vector Laboratories), diluted 1:200 in PBS, was used in place of a primary antibody and was visualized using Alexa Fluor 546-conjugated streptavidin at a dilution of 1:200 (Molecular Probes, Eugene, OR). For triple staining (see Fig. S1B-D in the supplementary material), conjugated secondary antibodies anti-rabbit Alexa Fluor 633 and anti-mouse Alexa Fluor 568 (Molecular Probes) were used at a dilution of 1:500, and slides were analyzed and photographed by confocal microscopy.

For peroxidase staining, endogenous peroxidases were quenched by incubation in 30% H₂O₂ solution in methanol, for 30 minutes at RT. Slides were then placed in humidified chambers and washed in water and then in PBS. Sections were blocked in 5% NGS, for 30 minutes at RT, and incubated in primary antibody, diluted in 5% NGS, overnight at 4°C. The next morning, slides were washed three times in PBS and sections incubated with biotinylated secondary antibody, diluted 1:200 in 5% NGS, for 1 hour at RT. After three washes in PBS, sections were incubated in ABC solution (Vector Laboratories) for 30 minutes at RT. Slides were washed twice in PBS and once in 0.1 M Tris (pH 7.4). Sections were incubated with DAB (Sigma Aldrich, St Louis, MO) solution and the peroxidase reaction monitored under the light microscope. Reactions were stopped by placing slides in water, followed by dehydration in 95% and then 100% ethanol. Slides were then immersed into xylene, mounted in xylene-based mounting media and analyzed under the light microscope when dry.

For all histology studies, at least three embryos were examined, and representative examples are shown.

β-galactosidase detection

Whole-mount embryos were fixed in 4% paraformaldehyde in PBS without ions for 30 minutes at 4°C and then washed three times in PBS. Fixed and washed embryos were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution overnight at room temperature. Embryos were then washed with ice-cold PBS, post-fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C, washed again and then processed for frozen sections. Frozen sections (10 µm) were then used for peroxidase staining.

RT-PCR

Total RNA was isolated from pancreatic buds at different stages of development using the RNeasy Kit (Qiagen, Valencia, CA) and treated with Turbo DNase (Ambion, Austin, TX). cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), then 25 ng was used per PCR reaction (33 cycles for *sox10*, 21 cycles for β-actin, and 35 cycles for *phox2b*, using standard conditions).

For real-time quantitative PCR (TaqMan), probes were 5' FAM + 3' TAMRA fluorescently labeled. TaqMan was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) via a 2-step non-multiplexed assay. Calibration curves were generated prior to analysis using primer/probe sets for each transcript. Gene expression levels of the assayed genes were normalized to the expression levels of mouse β-glucuronidase (mGUS). All PCR primer and TaqMan probe sequences are listed in Table 2.

Cell counting

To count insulin- and glucagon-positive cells and proliferating insulin-positive cells, we used the pancreas from five *Phox2b*^{+/+} and five *Phox2b*^{-/-} E17.5 embryos. Whole blocks of frozen tissue were sectioned into 5 µm sections and each tenth section was stained and the cells counted under a

Table 1. Primary antibodies

Antibody	Species	Dilution	Staining	Source (catalog number)
Anti-amylase	Rabbit	1:1000	IF	Sigma, St Louis, MO (#8273)
Anti-β-galactosidase	Rabbit	1:2000	IF	MP Biomedicals, Solon, OH (#55976)
Anti-BLBP/FABP7	Rabbit	1:1000	IF	Chemicon International, Temecula, CA (#AB9558)
Anti-caspase3	Rabbit	1:150	IF	Cell Signaling Technology, Danvers, MA (#9661)
Anti-ghrelin (C-18)	Goat	1:2000	IF	Santa Cruz Biotechnology, Santa Cruz, CA (#sc-10368)
Anti-glucagon	Guinea pig	1:1000	P	Linco Research, St Charles, MO (#4031-01F)
Anti-glucagon	Mouse	1:8000	IF	Sigma-Aldrich, St Louis, MO (#G2654)
Anti-HuCD	Mouse	1:25	IF	Molecular Probes, Eugene, OR (#A-21271)
Anti-insulin	Guinea pig	1:2000	IF	Linco Research, St Charles, MO (#4011-01F)
Anti-Isl1	Mouse	1:100	IF	Developmental Studies Hybridoma Bank, Iowa City, IA (39.4D5)
Anti-Ki67	Mouse	1:100	IF	BD Biosciences Pharmingen, San Jose, CA (#556003)
Anti-Nkx2.2	Mouse	1:25	IF	Developmental Studies Hybridoma Bank, Iowa City, IA (74.5A5)
Anti-Pdx1	Guinea pig	1:4000	P	(Schwitzgebel et al., 2000)
Anti-Pgp9.5	Rabbit	1:1000	IF	Accurate Chemical and Scientific, Westbury, NY
Anti-Phox2b	Rabbit	1:1000	P, IF	Gift of J. F. Brunet, Paris, France (Pattyn et al., 1997)
Anti-Sox10	Guinea pig	1:500	IF	Gift of M. Wegner, Erlangen, Germany (Stolt et al., 2003)

P, peroxidase; IF, immunofluorescence.

Table 2. Sequences of primers and probes

Primer name	Primer sequence
RT-PCR-Phox2b-F	5'-TTCCTGCTGCTACGAGTCTCG-3'
RT-PCR-Phox2b-R	5'-CTCAGCGAAGACCTCTCCA-3'
RT-PCR-Sox10-F	5'-GCTGCTGCTATTACAGGCTCAC-3'
RT-PCR-Sox10-R	5'-CGTAGCCAGCTGCCGAGTAG-3'
RT-PCR-βactin-F	5'-TGAGAGGGAAATCGTGCGTG-3'
RT-PCR-βactin-R	5'-TGCTTGCTGATCCACATCTGC-3'
TaqMan-Nkx2.2-F	5'-GAACAGCAAGCTAGCCGAGG-3'
TaqMan-Nkx2.2-R	5'-CGATCAGTCCATATAAGGCTGG-3'
TaqMan-Ngn3-F	5'-ATCATCACTTTTCCAGGGTG-3'
TaqMan-Ngn3-R	5'-TCATCTATGGGCAAGAGCTG-3'
TaqMan-Phox2b-F	5'-CTGCAGCCAGGCCAGTG-3'
TaqMan-Phox2b-R	5'-GGACGGGCAACCGGAC-3'
TaqMan-Insulin(I+II)-F	5'-AGCGTGGCTTCTTACACACC-3'
TaqMan-Insulin(I+II)-R	5'-CCAGCTCCAGTTGTGCCACT-3'
TaqMan-Glucagon-F	5'-AGGAATTCATTGCGTGGCTG-3'
TaqMan-Glucagon-R	5'-CAATGGCGACTTCTCTGGG-3'
Probe name	Probe sequence
TaqMan-Nkx2.2	5'-CGCCGCACTCCAGGTTCTGTA-3'
TaqMan-Ngn3	5'-AATCCAGTGTGCGTCTTACCTC- ACTGGC-3'
TaqMan-Phox2b	5'-TCCAGTATAACCCGATAAGGAC- CACTTTGGG-3'
TaqMan-Insulin(I+II)	5'-TGGGTCTCCACTTCACGGCG-3'
TaqMan-Glucagon	5'-AGTCTCGCCTTCTCGGCTTTCA-3'

fluorescent microscope. Between 25 and 35 sections per block were counted. The pancreatic area was measured under the light microscope using OpenLab software. Cell number was expressed as the total number of cells per total pancreatic area.

RESULTS

Phox2b expression in the embryonic pancreas

To assess the expression of Phox2b in the developing pancreas, we measured the levels of its transcript in embryonic mouse pancreas by TaqMan real-time RT-PCR. *Phox2b* mRNA was below the limit of detection at embryonic day 10.5 (E10.5, Fig. 1A; see also Fig. S1A in the supplementary material), but peaked dramatically at E12.5, decreased again to lower levels at E13.5, and fell back to, or

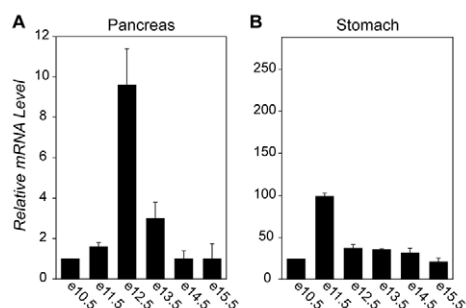


Fig. 1. The temporal pattern of Phox2b expression in the embryonic pancreas and stomach. *Phox2b* mRNA levels were determined by real-time RT-PCR (TaqMan) of RNA isolated from the pancreas (A) and stomach (B) of mouse embryos from E10.5 to E15.5. All values are expressed relative to the level in pancreatic RNA at E10.5 and represent the mean value of three independent experiments performed in triplicate \pm s.e.m. In A, mRNA values that are below or at the limit of detection (E10.5, E14.5 and E15.5) are displayed at the value for the limit of detection.

below, the limit of detection at later times (Fig. 1A). Note that mRNA values that do not exceed the limit of detection (E10.5, E14.5 and E15.5) are displayed at the value for the limit of detection in Fig. 1A. By contrast, the stomach robustly expressed *Phox2b* mRNA as early as E10.5, and reached levels up to two orders of magnitude higher than the pancreas. Although there was an expression peak at E11.5, high-level expression persisted in the stomach at later stages of embryonic development (Fig. 1B).

Immunohistochemical staining readily detected Phox2b protein expression in mouse embryonic pancreas and gut at E12.5 (Fig. 2A). In both the pancreas and the gut, Phox2b-expressing cells were located along the border between the mesenchyme and the epithelium, but did not co-stain with markers of pancreatic epithelium (Pdx1, Fig. 2B; Nkx2.2, see Fig. S1B-D in the supplementary material) or mesenchyme (Fig. 2C,D). Similar staining from earlier and later developmental stages detected few or no Phox2b-expressing cells in the pancreas, but abundant Phox2b expression throughout the gut, in agreement with the TaqMan data shown in Fig. 1 (Fig. 5D,F; data not shown).

Origins of the Phox2b-expressing cells in the pancreas

During pancreatic development, the endocrine cells originate in the pancreatic epithelium, then delaminate and migrate into the mesenchyme as they differentiate. To test the possibility that the Phox2b-expressing cells in the pancreatic mesenchyme similarly derive from the pancreatic epithelium, we performed a lineage tracing experiment (Fig. 3A,B). Initially in pancreatic development, all epithelial cells express the homeodomain transcription factor Pdx1 (Ahlgren et al., 1996; Offield et al., 1996), and therefore *cre* recombinase driven by the *Pdx1* gene promoter marks all cells derived from the pancreatic epithelium when combined with the marker gene *ROSA26 loxP-stop-loxP lacZ* (*R26R*) in transgenic mice (Gu et al., 2002; Heiser et al., 2006; Soriano, 1999). In *Pdx1-cre/R26R* embryos, β -galactosidase activity colocalized with immunohistochemical staining for Pdx1 (Fig. 3A) but not for Phox2b (Fig. 3B), demonstrating that the Phox2b-expressing cells did not derive from the pancreatic epithelium.

As the Phox2b-expressing cells of the pancreas did not originate from Pdx1-expressing pancreatic epithelium and did not co-stain with markers of the pancreatic mesenchyme (Fig. 2C,D), we hypothesized that these cells arose in the neural crest. Phox2b marks the migrating neural crest cells that colonize the gut tube and eventually form the enteric nervous system (Young et al., 1998). Although Phox2b expression appeared later in the developing pancreas than in the stomach (Fig. 1), this timing is consistent with the arrival of migrating neural crest cells that colonize more distal segments of the gut (Young et al., 1998).

To test definitively whether the Phox2b-expressing cells in the pancreas originate from the neural crest, we used the *Wnt1-cre* transgene to mark all cells of neural crest origin (Danielian et al., 1998) (Fig. 3C-E). In both the stomach and pancreas of *Wnt1-cre/R26R* embryos, β -galactosidase activity did not overlap with Pdx1 expression (Fig. 3C), but did completely overlap with immunohistochemical staining for Phox2b (Fig. 3D,E), demonstrating that the Phox2b-expressing cells in the pancreas originate in the neural crest. As the pancreas of the *Wnt1-cre/R26R* mouse matures, β -galactosidase activity uniquely marks the differentiating neurons and glial cells (see Fig. S2A-E in the supplementary material; data not shown).

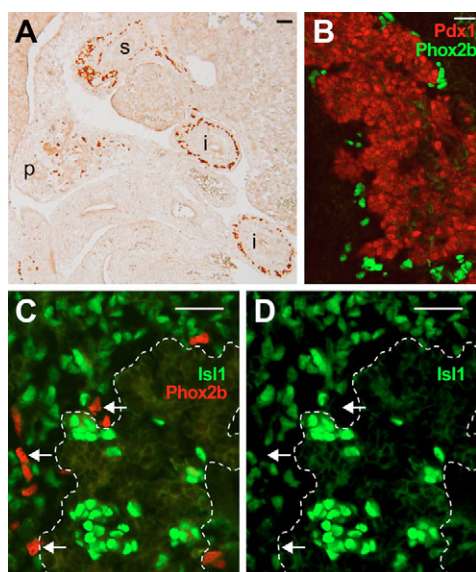


Fig. 2. The pattern of Phox2b expression in embryonic pancreas and gut. (A–D) Mouse embryos harvested at E12.5 were stained by immunohistochemistry with peroxidase (A) for Phox2b (brown) or by immunofluorescence (B–D) for Phox2b (FITC, green in B; Cy3, red in C), Pdx1 (Cy3, red in B) and Isl1 (FITC, green in C,D). Dashed line outlines the pancreatic epithelium. Arrows indicate representative nuclei with Phox2b staining. Scale bars: 50 μ m. p, pancreas; s, stomach; i, intestine.

Sox10 is co-expressed with Phox2b in the pancreas

The Sry-HMG box transcription factor Sox10 functions upstream of Phox2b in the migrating neural crest cells that colonize the gut, and is co-expressed by a subset of Phox2b-positive cells in the developing gut tube (Kim et al., 2003; Young et al., 2003). We found that *Sox10* mRNA expression in the pancreas paralleled *Phox2b* expression with a similar peak at E12.5 (Fig. 4A). Co-staining of Sox10 and Phox2b by immunofluorescence at E12.5 revealed a partial overlap in the expression of the two factors in the stomach (Fig. 4B–E), and a complete overlap in the pancreas (Fig. 4F–I; see

also Fig. S1B–D in the supplementary material). These data provide further support for the neural crest origin of pancreatic Phox2b-positive cells.

Regulation of Phox2b in the pancreas by Nkx2.2

In developing serotonergic neurons, the homeodomain transcription factor Nkx2.2 is required to inactivate Phox2b expression (Pattyn et al., 2003). To investigate whether pancreatic cells expressing Phox2b also express Nkx2.2, we co-stained for the two factors in the pancreas at E12.5. Consistent with the endodermal rather than neural crest origins of the Nkx2.2-expressing cells of the pancreas (Sussel et al., 1998), and with the lack of Phox2b co-staining with Pdx1 (Fig. 2), no cells in the pancreas co-expressed Phox2b and Nkx2.2 (Fig. 5A), and no Nkx2.2-expressing cells were marked by *Wnt1-cre*-mediated activation of *R26R* (Fig. 5B). In addition, lineage tracing with *Nkx2.2-cre* mice did not mark the Phox2b-expressing cells at E12.5 (F. Lynn, N.N. and M.S.G., unpublished). However, the ectoderm-derived Phox2b-positive cells lay in close proximity to, or direct contact with, the endoderm-derived Nkx2.2-positive cells (Fig. 5A,B).

To test whether Nkx2.2 expression influenced the nearby Phox2b-expressing cells, we examined mice with a targeted deletion of the *Nkx2.2* gene (Sussel et al., 1998). In contrast to the wild-type pancreas, in which *Phox2b* mRNA is downregulated at E13.5 (Fig. 1A), mRNA quantification by TaqMan revealed a threefold and a sixfold increase in *Phox2b* mRNA from E13.5 pancreas from *Nkx2.2*^{+/-} and *Nkx2.2*^{-/-} embryos, respectively, relative to their *Nkx2.2*^{+/-} littermates (Fig. 5C). Immunofluorescent staining for Phox2b confirmed the failure to downregulate Phox2b at E13.5 in pancreas from embryos lacking Nkx2.2: in the absence of functional Nkx2.2 protein, clusters of Phox2b-positive cells remained in the pancreas at E13.5 (Fig. 5E) and beyond (Fig. 6D–F; see also Fig. S1G,H in the supplementary material). By contrast, Phox2b expression in the stomach was not altered in the absence of Nkx2.2 (Fig. 5C,F,G).

Despite the persistence of Phox2b expression, the differentiation of neural and glial cells was not altered significantly in the *Nkx2.2* null pancreas (see Fig. S2F–I in the supplementary material). Furthermore, although a marked increase in ghrelin-expressing cells has been observed previously in the pancreas of *Nkx2.2* null embryos (Prado et al., 2004), these ghrelin-expressing cells did not express Phox2b in either wild-type or *Nkx2.2* null embryos, and

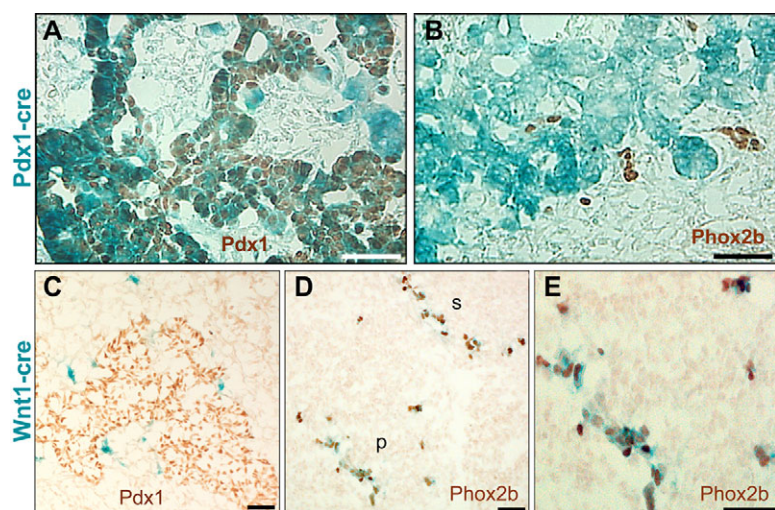


Fig. 3. Lineage tracing of Phox2b-expressing cells.

Lineage tracing was performed by crossing *R26R* mice with either *Pdx1-cre* (A,B) or *Wnt1-cre* (C–E) mouse lines. Mouse embryos harvested at E12.5 were stained for β -galactosidase activity with X-gal (blue), and stained for Pdx1 (A,C) or Phox2b (B,D,E) by immunohistochemistry with peroxidase labeling (brown). Scale bars: 50 μ m.

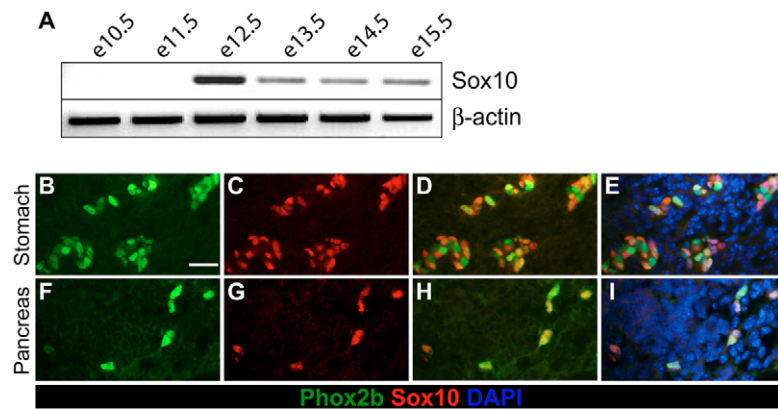


Fig. 4. Sox10 expression in the pancreas. (A) *Sox10* mRNA levels were determined by RT-PCR of RNA isolated from the pancreas of mouse embryos from E10.5 to E15.5 and compared with β-actin mRNA levels. Stomach (B-E) and pancreas (F-I) from mouse embryos harvested at E12.5 were stained by immunofluorescence for Sox10 (Cy3, red) and Phox2b (FITC, green), and co-stained with nuclear stain DAPI (blue). Scale bar: 25 μm.

were not marked by *Wnt1-cre*, indicating that they do not derive from Phox2b-expressing neural crest cells (see Fig. S1E-H in the supplementary material).

The persistence of Phox2b-expressing cells in the pancreas of mice lacking Nkx2.2 could result from a reduction in the apoptosis of Phox2b-expressing cells, an increase in their proliferation, or simply a failure to shut off *Phox2b* gene expression in the differentiating neural crest-derived pancreatic cells. We directly tested the first two possibilities by assessing rates of apoptosis and proliferation, respectively. Apoptotic cells, as detected by staining for cleaved caspase-3, were extremely rare at E12.5 in the pancreas (Fig. 6A), especially along the epithelial/mesenchymal border. There was no increase in apoptosis at E13.5 (Fig. 6B) and no difference in the animals lacking Nkx2.2 (Fig. 6C). In addition, we found that the Phox2b-expressing cells that persist in the absence of Nkx2.2 had extremely low rates of proliferation (Fig. 6D-F). These data support the conclusion that the Phox2b-expressing cells detected in the pancreas at E12.5 subsequently extinguish *Phox2b* gene expression but persist in the pancreas, and that this inactivation of *Phox2b* gene expression is dependent on the expression of Nkx2.2 in the nearby pancreatic endocrine cells.

Fate of Phox2b-expressing cells in the pancreas

Having shown that the expression of Nkx2.2 in the pancreatic epithelial cells impacts the development of the adjacent Phox2b-expressing cells, we investigated whether the Phox2b expression in these neural-crest-derived cells impacts, in turn, the development of the pancreas. To test this possibility, we analyzed *Phox2b*^{-/-} mice, in which the coding sequence of the *Phox2b* gene has been replaced by *lacZ* (Pattyn et al., 1999).

In the absence of Phox2b, a decreased number of neural crest cells migrate as far as the foregut, but they fail to differentiate, undergo apoptosis and are lost by E13.5 (Pattyn et al., 1999). We also observed a complete loss of neurons, as determined by staining for the neural marker Pgp9.5 (Uchl1 – Mouse Genome Informatics), an ubiquitin hydroxylase, at both E13.5 and E17.5 in the stomach of *Phox2b*^{-/-} mice (compare Fig. 7A with 7E, and 7B with 7F). Very few Pgp9.5-positive cells were detected in the pancreas of wild-type mice at E13.5, presumably because of the later migration of the neural crest cells into the pancreas. At E17.5, however, Pgp9.5-positive neurons could be detected in the wild-type pancreas in close proximity to, but not overlapping with, Pdx1-positive cells (Fig. 7C). Similar to the stomach, at E17.5 the pancreas of *Phox2b*^{-/-} embryos lacked any Pgp9.5-positive neurons (Fig. 7G). It should be noted that Pgp9.5 immunoreactivity has been reported in the pancreatic epithelium and early endocrine cells in fetal rodent pancreas

(Bouwens, 2004; Kent and Rowe, 1992; Yokoyama-Hayashi et al., 2002); however, we detected staining for Pgp9.5 in the pancreatic epithelium only before E13, and at a lower intensity. This early staining was observed in both *Phox2b*^{+/+} and *Phox2b*^{-/-} pancreatic tissue. The strong Pgp9.5 staining seen at E17.5 in wild-type pancreas colocalized with the neural marker HuC/D (D'Autreaux et al., 2007) (see Fig. S2C-E in the supplementary material), and not with Pdx1 or islet hormones (data not shown).

Phox2b-positive neural crest cells also give rise to the glial lineage. At E18.5 in wild-type embryos, Fabp7-positive glial cells surrounded the clusters of endocrine cells, including the insulin-expressing beta-cells, that organize into islets at this stage (Fig. 7D), and also surrounded the differentiating neurons (see Fig. S2F-I in the supplementary material). By contrast, the glial staining is lost in *Phox2b*^{-/-} embryos (Fig. 7H). These data support the conclusion that the neural and glial cells in the pancreas derive from Phox2b-expressing neural crest progenitors, and demonstrate that neural-crest-derived cells in the pancreas require Phox2b to develop into differentiated neurons and glia.

The role of Phox2b in development of the endocrine pancreas

To test the role of the Phox2b-expressing cells in endocrine development, we stained pancreas from *Phox2b*^{+/+} and *Phox2b*^{-/-} embryos at E17.5 for the four islet hormones. The pattern and intensity of staining for glucagon, somatostatin and pancreatic polypeptide were unchanged, but insulin staining was increased in *Phox2b*^{-/-} pancreas (Fig. 8A,B; data not shown). Cell counts revealed no difference in the number of glucagon-positive cells, but a 20-40% increase in the number of beta-cells in the *Phox2b*^{-/-} embryos (Fig. 8C). This increase in insulin was confirmed by TaqMan RT-PCR quantification of insulin mRNA (Fig. 8D). By contrast, the acinar and ductal compartments, and the overall size of the pancreas were not altered (see Fig. S3 in the supplementary material; data not shown).

The increase in the population of beta-cells could have resulted from a decrease in the apoptosis or an increase in the proliferation of beta-cells, or from an increase in the generation of beta-cells from neurogenin 3-expressing precursors. The rates of apoptosis are too low at this stage to affect significantly the size of the beta-cell population (Sander et al., 2000). However, the rate of beta-cell proliferation was significantly increased in pancreas from *Phox2b*^{-/-} embryos, as gauged by the percentage of insulin-positive cells co-staining with the proliferation marker Ki67 (Fig. 8F). This increase in proliferation could easily account for the increase in beta-cells in the *Phox2b*^{-/-} embryos.

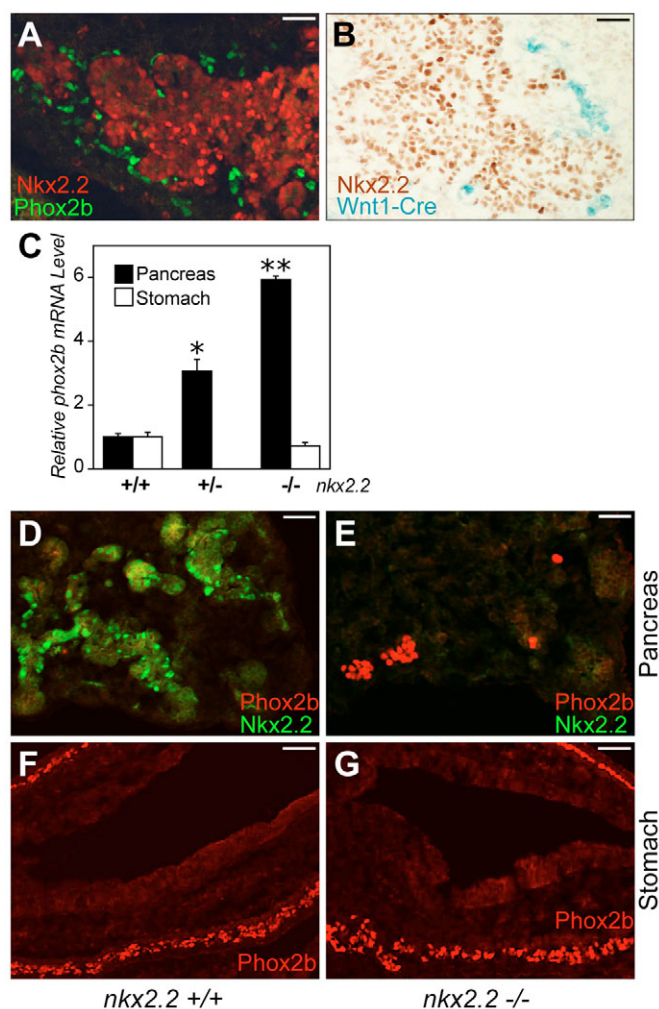


Fig. 5. *Nkx2.2* regulation of *Phox2b*. (A) Pancreas from a mouse embryo harvested at E12.5 stained by immunofluorescence for *Nkx2.2* (Cy3, red) and *Phox2b* (FITC, green). (B) Lineage tracing was performed by crossing *R26R* mice with *Wnt1-cre* mice. Mouse embryos harvested at E12.5 were stained for β -galactosidase activity with X-gal (blue), and stained for *Nkx2.2* by immunohistochemistry with peroxidase labeling (brown). (C) *Phox2b* mRNA levels were determined by real-time RT-PCR (TaqMan) with RNA isolated from pancreas (black bars) and stomach (white bars) from E13.5 embryos with the *Nkx2.2* genotypes shown. Values for both pancreas and stomach are expressed separately relative to the levels of each in *Nkx2.2*^{+/+} embryos. The *Phox2b* mRNA level in the stomach was not tested for the *Nkx2.2*^{+/-} embryos. Data represent the mean values of four independent experiments performed in triplicate \pm s.e.m. (D–G) The pancreas (D,E) and stomach (F,G) of *Nkx2.2*^{+/+} (D,F) and *Nkx2.2*^{-/-} (E,G) embryos at E13.5 were stained by immunofluorescence for *Phox2b* (Cy3, red) and *Nkx2.2* (FITC, green). An increase in *Phox2b* staining was observed in the pancreas from *Nkx2.2*^{-/-} embryos (compare D and E) but not in the stomach (compare F and G). * $P < 0.01$; ** $P < 0.001$, compared with *Nkx2.2*^{+/+} embryos by Student's *t*-test. Scale bars: 50 μ m.

Finally, we assessed the expression of *Nkx2.2* in the *Phox2b*^{-/-} embryos and found a marked increase in *Nkx2.2* mRNA relative to that in wild-type littermates at both E15.5 (Fig. 8G) and E17.5 (data not shown). By contrast, the levels of neurogenin 3 mRNA stayed at similar levels (Fig. 8G), further supporting the conclusion that the

increase in insulin-positive cells in the *Phox2b*^{-/-} embryos arose from increased proliferation, and not from an increased neogenesis, of beta-cells.

DISCUSSION

We provide here the first description of the timing and pattern of neural crest cell migration into the mouse pancreas, as well as of the gene expression programs and fates of the pancreatic neural crest cells, and thereby add to the overwhelming evidence that neural crest cells do not differentiate into pancreatic islet cells (Andrew et al., 1998). These neural crest cells do contribute indirectly to islet development, however, and we describe a novel non-cell-autonomous negative-feedback interaction between the ectoderm-derived neural crest cells and the endoderm-derived pancreatic cells that impacts gene expression in both cell populations and controls the size of the beta-cell population.

Phox2b marks the cells that migrate from the neural crest to the gut and form the enteric nervous system (Young et al., 1998; Young and Newgreen, 2001). We found that *Phox2b*-expressing cells appeared in the pancreas shortly after they do in the stomach, and lineage tracing demonstrated that these cells originated from the neural crest, and not the endoderm-derived pancreatic epithelium. Most likely, the neural crest cells that populate the pancreas follow a similar migration pattern to those that populate the gut, delaminating from neural ectoderm and tracking through the embryo to the developing pancreas; however, the difference in timing suggests that the pancreatic neural crest cells may originate from a temporally discrete wave of cells.

In many ways, the gut and pancreatic neural crest cells develop in parallel, differentiating through a similar series of gene expression changes. For example, *Sox10* marks the early neural crest cells as they migrate into both organs. *Sox10* precedes and overlaps *Phox2b* expression in the gut neural crest lineage, and then shuts off in differentiating neurons but persists in mature glial cells (Young et al., 2003). The extended wave of neural crest cells that migrates into the gut results in overlapping populations of cells with differing degrees of maturity and, thus, differing combinations of gene expression along the length of the gut (Young et al., 1999). We observed a similar, but more complete, coincidence of *Sox10* and *Phox2b* in the pancreas, possibly reflecting a more synchronous arrival and differentiation of neural crest cells in the pancreas. As in the gut (D'Auteaux et al., 2007), markers of differentiated neurons and glia then appeared shortly thereafter, along with lineage-specific markers, such as the bHLH transcription factor *Hand2* (data not shown).

In contrast to the gut, however, *Phox2b* expression in the pancreas is much more transient, and largely disappears within 24 hours. The decrease in *Phox2b* mRNA correlated with a marked drop in the number of cells that stained positive for the protein; but the absence of apoptosis and the subsequent appearance of large numbers of Pgp9.5-positive neurons and Fabp7-positive glia suggest that the neural-crest derived cells persisted, but silenced *Phox2b* gene expression. In the fetal mouse intestine, the differentiating neural and glial cells continue to express *Phox2b*, although it is eventually downregulated in the mature glia after birth (Young et al., 2003). Clearly, *Phox2b* silencing occurs much earlier and is much more common in the pancreas than in the gut.

This difference in *Phox2b* silencing could reflect intrinsic differences in the neural crest populations that migrate to each organ, or differences in the signals received after their arrival. The data from *Nkx2.2*^{-/-} embryos suggest that extrinsic signals play a crucial

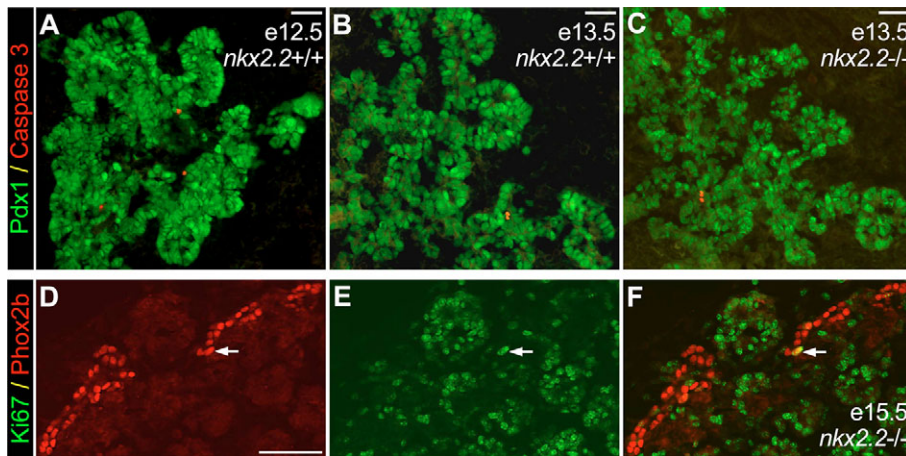


Fig. 6. Turnover of Phox2b-expressing cells. Pancreas from *Nkx2.2*^{+/+} (A,B) and *Nkx2.2*^{-/-} (C-F) mouse embryos at E12.5 (A), E13.5 (B,C), and E15.5 (D-F) were co-stained by immunofluorescence for Pdx1 (FITC, green) and the apoptosis marker cleaved caspase-3 (Cy3, red; A-C), or for Phox2b (Cy3, red; D,F) and the replication marker Ki67 (FITC, green; E,F). Scale bars: 50 μ m.

role, as, in the absence of *Nkx2.2* in the pancreatic epithelium, *Phox2b* persisted in the neural crest cells in the pancreas, whereas *Phox2b* expression remained unchanged in the stomach. Our data demonstrate that the increase in *Phox2b*-expressing cells in *Nkx2.2*^{-/-} embryos does not result from an increased proliferation or decreased apoptosis of these cells. We cannot rule out the possibility that the additional *Phox2b*-expressing cells might result from increased migration of neural crest cells into the *Nkx2.2*^{-/-} pancreas, but this explanation seems unlikely as we do not see an increase in *Sox10*-expressing cells (data not shown), or in neuronal or glial cells later in pancreatic development.

Lineage tracing with *Nkx2.2-cre* mice did not mark the *Phox2b*-expressing cells in the pancreas. Although we cannot absolutely rule out the possibility that a small population of early *Nkx2.2*-expressing neural progenitors was not marked in this lineage tracing experiment, we never saw *Nkx2.2* co-expressed with *Phox2b* in the pancreatic neural crest lineage. Thus, most likely, signals from the endoderm-derived cells that express *Nkx2.2* in the pancreatic epithelium silence the expression of *Phox2b* in the neural-crest-derived cells as they migrate into the pancreas (Fig. 9).

In addition to the peripheral nervous system, a subset of cells in the developing CNS expresses *Phox2b*. *Phox2b* expression marks a subset of neural progenitors in the embryonic hindbrain, from which

both motoneurons and serotonergic neurons are generated (Pattyn et al., 2003). *Phox2b* must be downregulated prior to differentiation of the serotonergic neurons, and, similar to the situation in the pancreas, this downregulation is dependent on *Nkx2.2*. The downregulation of *Phox2b* in the pancreas may play a similar role, permitting the differentiation of neural crest cells into specific cell types that contribute to pancreatic physiology.

Unlike the neural crest cells in the pancreas, however, the *Phox2b*-expressing neural progenitors in the embryonic hindbrain co-express *Nkx2.2* and *Nkx6.1* (Pattyn et al., 2003). Therefore, in the precursors of serotonergic neurons, unlike in the neural crest cells in the pancreas, *Nkx2.2* could directly downregulate *Phox2b* in a cell-autonomous fashion. In other cells in the embryonic hindbrain, however, co-expression of *Nkx2.2* and *Phox2b* persists, demonstrating that other signals are required for *Phox2b* silencing, and suggesting that, in the embryonic hindbrain, as in the pancreas, *Phox2b* silencing by *Nkx2.2* may proceed via a non-cell-autonomous signaling pathway.

Any of several signaling pathways could mediate non-cell-autonomous silencing of *Phox2b* by *Nkx2.2*-expressing cells. *Nkx2.2* regulates the expression of a broad set of signaling molecules in the pancreas, including several hormones (Prado et al., 2004; Sussel et al., 1998). In the absence of *Nkx2.2*, the pancreas

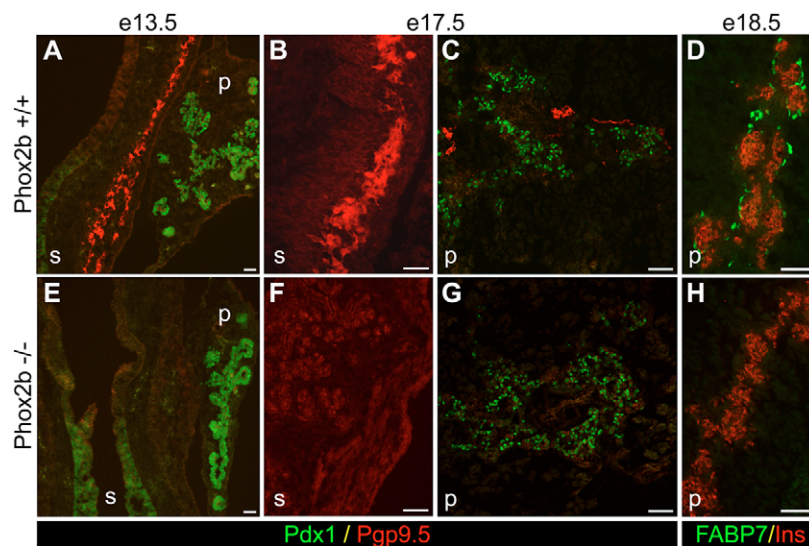
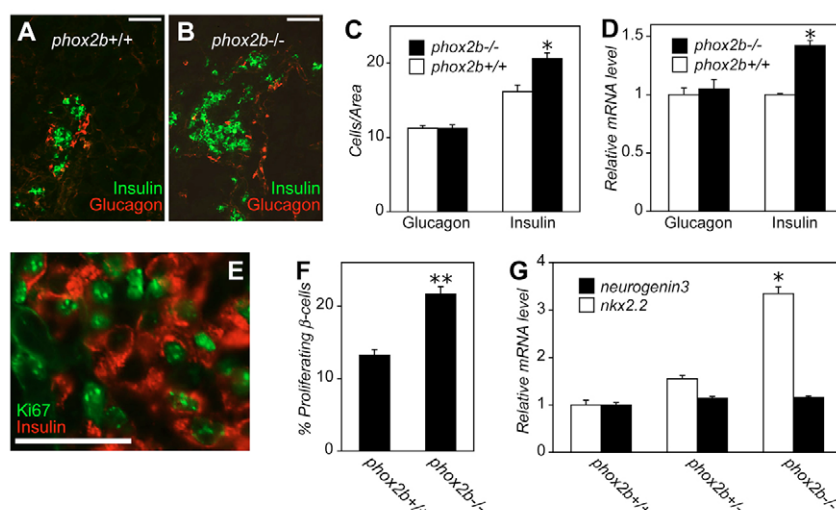


Fig. 7. Differentiated neural crest cells in pancreas and stomach. Pancreas and stomach from *Phox2b*^{+/+} (A-C) and *Phox2b*^{-/-} (E-G) mouse embryos at E13.5 (A,E) and E17.5 (B,C,F,G) were co-stained by immunofluorescence for Pdx1 (FITC, green) and the neural marker Pgp9.5 (Cy3, red). (D,H) Pancreas from *Phox2b*^{+/+} (D) and *Phox2b*^{-/-} (H) embryos at E18.5 were co-stained by immunofluorescence for insulin (Cy3, red) and the glial marker Fabp7 (FITC, green). s, stomach; p, pancreas. Scale bars: 50 μ m.

Fig. 8. Non-cell autonomous regulation of islet cells by *Phox2b*. Pancreas from *Phox2b*^{+/+} (A) and *Phox2b*^{-/-} (B) mouse embryos at E17.5 co-stained by immunofluorescence for insulin (FITC, green) and glucagon (Cy3, red). (C) Insulin- and glucagon-positive cells from *Phox2b*^{+/+} (white bars) and *phox2b*^{-/-} (black bars) embryos counted and expressed as the total number of cells per total pancreatic area in mm². Each data point represents the mean of five embryos \pm s.e.m. (D) Insulin and glucagon mRNA levels measured by real-time RT-PCR (TaqMan) from RNA from pancreas of *Phox2b*^{+/+} (white bars) and *Phox2b*^{-/-} (black bars) embryos at E17.5. Each data point represents the mean of four independent litters \pm s.e.m. (E) Pancreas from a *Phox2b*^{-/-} mouse embryo at E17.5 co-stained by immunofluorescence for Ki67 (FITC, green) and insulin (Cy3, red). (F) The percentage of beta-cells replicating at E17.5 in embryos with the genotypes shown assessed by counting the number of cells co-staining for insulin and Ki67 and dividing by the total number of cells staining for insulin. Each data point represents the mean of five pancreas \pm s.e.m. (G) *Nkx2.2* (white bars) and neurogenin 3 (black bars) mRNA levels determined by real-time RT-PCR (TaqMan) with RNA isolated from pancreas from E15.5 embryos with the *Phox2b* genotypes shown. Data represent the mean values of three independent experiments performed in triplicate \pm s.e.m. **P*<0.01, ***P*<0.001, by Student's *t*-test. Scale bars: 50 μ m.



does not produce insulin, the expression of proglucagon, islet amyloid polypeptide and pancreatic polypeptide decreases, and the expression of ghrelin markedly rises. Other signaling molecules may be affected as well, including molecules classically involved in neural and glial differentiation, such as the neurotrophins, some of which are expressed in the pancreas (Chalazonitis, 2004; Scharfmann, 1997). The molecules that guide neural and axonal migration could also affect *Phox2b* expression by impacting neural integration and signaling (Young et al., 2004). Of interest in this regard, we have identified netrin 1 in a screen for targets of *Nkx2.2* in the pancreas (N.N. and M.S.G., unpublished).

Nkx2.2-dependent downregulation of *Phox2b* might influence the cell types generated from neural crest cells in the pancreas, in the same way that it allows the generation of serotonergic neurons in the embryonic hindbrain. Neural crest derivatives in the pancreas eventually differentiate into the glial Schwann cells that surround the mature pancreatic islets (Smith, 1975), and into sympathetic, parasympathetic and sensory neurons (Ahren, 2000). Both Fabp7-positive glial cells and Pgp9.5/HuC/D-positive neurons were detected in *Nkx2.2*^{-/-} pancreas (see Fig. S2F-I in the supplementary material). Unfortunately, *Nkx2.2*^{-/-} mice die shortly after birth with severe diabetes (Sussel et al., 1998), making it difficult to establish whether any specific neural subtypes fail to differentiate when *Phox2b* is not downregulated.

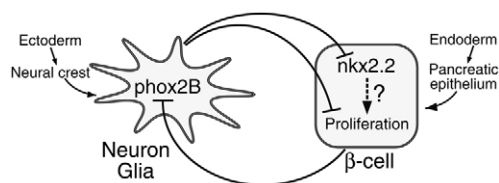


Fig. 9. Proposed model for the non-cell-autonomous interactions between *Phox2b* and *Nkx2.2* in the developing pancreas.

Just as the *Nkx2.2*^{-/-} embryos demonstrated that *Nkx2.2*-expressing cells inhibit *Phox2b* expression in the neural crest cells as they migrate into the pancreas, the *Phox2b*^{-/-} embryos demonstrated that neural crest cells, in turn, inhibit *Nkx2.2* expression in the pancreatic islet cells. In the absence of *Phox2b*, neural crest cells are lost from the gastrointestinal tract (Pattyn et al., 1999), and, in the present study, we confirmed that the pancreas of *Phox2b*^{-/-} embryos also lacks neural crest derivatives. Therefore, we can conclude that the neural crest cells generate an inhibitory signal, and that the signal is lost in the *Phox2b*^{-/-} pancreas, thereby releasing *Nkx2.2* expression in the pancreatic endocrine cells from inhibition (Fig. 9). Similar to the pancreatic signals that inhibit *Phox2b* expression, the identity of the neural crest signals that regulate *Nkx2.2* expression remains unknown.

The loss of neural crest cells in *Phox2b*^{-/-} pancreas also increased the proliferation of the *Nkx2.2*-expressing pancreatic beta-cells. Therefore, the neural crest cells in the pancreas inhibit both *Nkx2.2* expression and beta-cell proliferation. The inhibition of proliferation may be secondary to the inhibition of *Nkx2.2*, as the targets of *Nkx2.2* in the beta-cell include regulators of the cell cycle (Prado et al., 2004) (N.N. and M.S.G., unpublished); or, alternatively, signals from the neural crest cells might independently inhibit *Nkx2.2* expression and beta-cell proliferation in parallel.

After E16.5, the generation of new beta-cells from progenitor cells declines, and proliferation becomes the main source of new beta-cells and drives a rapid increase in the beta-cell population during the perinatal period (Finegood et al., 1995; Sander et al., 2000). The studies presented here demonstrate that cells derived from the neural crest play a role in modulating this wave of proliferation, and thus help to determine the size of the beta-cell population during this critical period. It is interesting to speculate whether the signaling loop between neural crest cells and pancreatic islet cells persists beyond embryonic development. The autonomic nervous system, both sympathetic and parasympathetic, is a well-documented regulator of adult islet function, including insulin and

glucagon secretion (Ahren, 2000), but its role in regulating gene expression and proliferation in normal islet cells has not been previously explored (Kiba, 2004).

The CNS can be viewed as an integrator of information regarding energy supplies. This information is then relayed to other tissues that acquire, store and use energy. For example, signals from the CNS via the autonomic nervous system regulate glucose production by the liver (Tiniakos et al., 1996) and the turnover of fatty acids in adipocytes (Turtzo and Lane, 2002), both directly and indirectly by regulating insulin and glucagon secretion from the islets. From this perspective, the islets form part of an integrated network of interacting sensors, regulators, producers and effectors of energy metabolism. To fulfill this role, the number of beta-cells must fluctuate in response to long-term changes in energy balance, and any breakdown in the signals that regulate the size of the beta-cell population will impact energy balance and may result in diabetes. The CNS may play an essential role in informing the islets of the overall energy state, and in regulating the size of the beta-cell population.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/12/2151/DC1>

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