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

Nada Nekrep¹, Juehu Wang¹, Takeshi Miyatsuka¹ and Michael S. German¹-²-_shared

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 Phox2bLacZ 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 1:500. 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 fluoroscein 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 (inGUS). 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

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P, peroxidase; IF, immunofluorescence.
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 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 migratory 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).

Table 2. Sequences of primers and probes

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<th>Primer name</th>
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<td>RT-PCR-Sox10-F</td>
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<td>RT-PCR-Phox2b-R</td>
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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 ±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.
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 Phox2b-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...
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-c 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 immunoactivity 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’Autreux 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.
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’Autreaux 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 in the pancreas, possibly reflecting a more synchronous gene 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’Autreaux 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).

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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 role in regulating neural crest cell migration and differentiation.
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

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**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 μm.

**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 μm.
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 ±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 ±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 ±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 ±s.e.m. *P<0.01, **P<0.001, by Student’s t-test. Scale bars: 50 μm.

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, parasymphathetic 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 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.

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. S9). 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

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