**INTRODUCTION**

The mammalian pancreas is densely innervated by fibers of the parasympathetic (vagus nerve), sympathetic, and sensory (splanchnic nerve) systems. In addition to nerve fibers, clusters of neural cell bodies (intrapancreatic ganglia) are also present throughout the adult pancreas (Salvioli et al., 2002). The importance of both sympathetic and parasympathetic innervation in the physiology of the endocrine and exocrine pancreas is well established (Brunicardi et al., 1995; Ahren, 2000; Gilon and Henquin, 2001). The study of pancreatic innervation has gained renewed interest due to reports indicating that the proliferation of adult endocrine β-cells is controlled by neural stimuli (Imai et al., 2008; Lausier et al., 2010; Grouwels et al., 2012). Moreover, it has been suggested that embryonic β-cell formation is regulated by signals provided by the neural crest (NC) cells that populate the pancreas during development (Nekrep et al., 2008; Plank et al., 2011).

In mice, NC cells from the vagal region enter the developing foregut around embryonic day (E) 9.5 and then migrate rostrocaudally to completely colonize the gut by E14.5 (Young et al., 1998; Young and Newgreen, 2001), giving rise to neuronal and glial cells. Earlier work established that neural precursors that have previously colonized the gut migrate to the pancreas to form the intrapancreatic ganglia (Kirchgesner et al., 1992). More recently, two studies have characterized the timing of the migration and differentiation of NC cells in the pancreas (Nekrep et al., 2008; Plank et al., 2011). The arrival of NC cells at the developing pancreas coincides with the formation of the pancreatic bud (~E10.5-11.5 in mice), suggesting a close relationship between these processes. During late pancreas development, NC-derived cells are found primarily associated with islets (Burris and Hebrok, 2007; Plank et al., 2011), consistent with an important role of neural components in the regulation of hormone secretion. Although our knowledge of the colonization of the pancreas by neural precursors from the gut has advanced significantly in recent years, the cues that guide the migration of these cells into the pancreas remain poorly defined.

One of the most important factors for the formation of gut innervation during embryonic development is glial cell line-derived neurotrophic factor (GDNF) as a key player in this process. GDNF displays a dynamic expression pattern during embryonic development that parallels the chronology of migration and differentiation of neural crest derivatives in the pancreas. Conditional inactivation of Gdnf in the pancreatic epithelium results in a dramatic loss of neuronal and glial cells and in reduced parasympathetic innervation in the pancreas. Importantly, the innervation of other regions of the gut remains unaffected. Analysis of Gdnf mutant mouse embryos and ex vivo experiments indicate that GDNF produced in the pancreas acts as a neurotrophic factor for gut-resident neural progenitor cells. Our data further show that exogenous GDNF promotes the proliferation of pancreatic progenitor cells in organ culture. In summary, our results point to GDNF as crucial for the development of the intrinsic innervation of the pancreas.

**SUMMARY**

The mammalian pancreas is densely innervated by both the sympathetic and parasympathetic nervous systems, which control exocrine and endocrine secretion. During embryonic development, neural crest cells migrating in a rostrocaudal direction populate the gut, giving rise to neural progenitor cells. Recent studies in mice have shown that neural crest cells enter the pancreatic epithelium at E11.5. However, the cues that guide the migration of neural progenitors into the pancreas are poorly defined. In this study we identify glial cell line-derived neurotrophic factor (GDNF) as a key player in this process. GDNF displays a dynamic expression pattern during embryonic development that parallels the chronology of migration and differentiation of neural crest derivatives in the pancreas. Conditional inactivation of Gdnf in the pancreatic epithelium results in a dramatic loss of neuronal and glial cells and in reduced parasympathetic innervation in the pancreas. Importantly, the innervation of other regions of the gut remains unaffected. Analysis of Gdnf mutant mouse embryos and ex vivo experiments indicate that GDNF produced in the pancreas acts as a neurotrophic factor for gut-resident neural progenitor cells. Our data further show that exogenous GDNF promotes the proliferation of pancreatic progenitor cells in organ culture. In summary, our results point to GDNF as crucial for the development of the intrinsic innervation of the pancreas.

**KEY WORDS:** GDNF, Pancreas development, Innervation, Mouse

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recent generation of mice with a conditional allele for Gdnf now facilitates tissue-specific analysis of the role of GDNF in embryonic development as well as in adult physiological processes (Pascual et al., 2008).

It has recently been reported that activation of the GDNF-RET signaling system influences pancreatic β-cell formation. Transgenic mice overexpressing Gdnf under the control of the glial fibrillary acidic protein (Gfap) promoter display increased β-cell mass and improved glucose tolerance (Mwangi et al., 2008; Mwangi et al., 2010). However, the expression and function of endogenous Gdnf in the pancreas have not been explored. To investigate the possible function of GDNF signaling in the development of pancreas innervation, we performed expression and loss-of-function studies of Gdnf in the developing mouse pancreas.

**MATERIALS AND METHODS**

**Generation and maintenance of transgenic mice**

Mice used in this study were maintained at the IBIS/HUVR mouse facility according to institutional guidelines and animal protocols approved by the Ethics Committee of the University Hospital Virgen del Rocio. Pdx-1-Cre, Gdnf lox/lox and Gdnf ΔCΔT mice have been previously described (Sánchez et al., 1996; Gu et al., 2002; Pascual et al., 2008).

**Genomic DNA excision**

Quantification of the excision levels of the Gdnflox allele was performed as previously described (Pascual et al., 2008).

**Quantitative RT-PCR**

Total RNA was isolated using the RNeasy Kit (Qiagen). cDNA was synthesized using Omniscript reverse transcriptase (Qiagen). Real-time quantitative PCR was performed with SYBR Green PCR Master Mix using a 7900HT real-time PCR system (Applied Biosystems). RNA expression of target genes was normalized to that of cyclophilin A (peptidylprolyl isomerase A – Mouse Genome Informatics). Changes in gene expression levels were calculated using the ΔΔCt method. Gdnf expression was analyzed by semi-quantitative RT-PCR with cyclophilin A as the internal control. Primer sequences are listed in supplementary material Table S1.

**Tissue preparation, histology, immunohistochemistry and microscopy analysis**

For paraffin sections, whole embryos (up to E11.5) and dissected guts (E12.5-P0) were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 2 hours. Adult pancreata were fixed in 4% PFA at 4°C overnight. Tissues were processed for paraffin embedding in a Leica ASP200S tissue processor. For immunohistochemical analysis, paraffin sections were dewaxed, rehydrated, permeabilized in 0.2% Triton X-100 in PBS (PBT) and blocked in 3% donkey serum with 0.1% BSA in PBT (45 minutes at room temperature). Pancreatic sections were incubated with primary antibodies (4°C overnight), washed in PBS and incubated with appropriate secondary antibodies (45 minutes at room temperature). Primary antibodies are listed in supplementary material Table S2. When required, antigen retrieval was performed using a pressure cooker. Where indicated, sections were also stained with *Dolichos biflorus* agglutinin (DBA) lectin (Vector Laboratories). Secondary antibodies coupled to Alexa 488, Alexa 568 (Molecular Probes), FITC, Cy5 and Cy3 (Jackson ImmunoResearch) were used. For some primary antibodies, biotinylated secondary antibodies and FITC-/Cy3-conjugated streptavidin (Jackson ImmunoResearch) were used. When further amplification was required, tyramide signal amplification (Perkin Elmer) was performed. Staining for diaminobenzidine (DAB) was performed with the Elite ABC Kit (Vector Laboratories). Collagen-embedded cultured pancreatic rudiments were fixed in 4% PFA (30 minutes at room temperature), embedded in Tissue-Tek OCT Compound (Electron Microscopy Sciences) and stored at −80°C. Fluorescence was visualized and photographed with a BX-61 microscope (Olympus) or an LTC SP2 confocal microscope (Leica). All photomicrographs shown are representative of at least three independent samples of the indicated genotype. **β-galactosidase detection**

β-galactosidase detection was performed as previously described (Cervantes et al., 2010). Whole-mount staining images were captured using an SZX16 microscope (Olympus).

**Glucose tolerance test**

Glucose tolerance tests were performed after a 14-hour fast. Mice were injected intraperitoneally with glucose (2 g/kg body weight), and blood glucose levels were measured using a Roche Accu-Chek glucometer at the indicated times.

**Pancreas explants**

E11-12 dorsal pancreata were placed into rat-tail collagen type I (1 mg/ml; BD Biosciences) used as a three-dimensional gel in a cover glass in 24-well plates. After polymerization, OptiMEM medium (Life Technologies) with 100 ng/ml GDNF (Calbiochem), 100 ng/ml neurturin (NRTN; R&D Systems) or BSA (Sigma) was added to the explants and cultured for 3-4 days. For directed migration experiments, Cibacron Blue 3GA agarose beads (Sigma, C1285) were soaked with 10 µg/ml GDNF or BSA at 4°C for 1 hour as previously described (Heuckeroth et al., 1998; Iwashita et al., 2003), placed next to E11-12 pancreatic rudiments and cultured for 4 days.

**Quantitative analysis**

For β-cell area quantification, 11 sections (60 µm apart) from P0 and P21 pancreata were immunostained for insulin. ImageJ (NIH) was used to create thresholded binary images to calculate the endocrine area as the ratio of insulin area to total pancreatic area. The same process was used to measure the pancreatic neuronal area using HuC/D-immunostained P0 and P21 pancreatic sections.

To measure endocrine innervation, 11 sections (60 µm apart) from P0 pancreata were immunostained for Tuj1 and counterstained with DAPI. The Tubenbus plugin for ImageJ was used to detect neurites. Images were skeletonized to measure the total length of innervation. Results are presented as the ratio of nerve fiber area to endocrine area.

For measurement of sympathetic and parasympathetic innervation, 22 sections (30 µm apart) from P21 pancreata were immunostained for tyrosine hydroxylase (TH) or VACHT, respectively. Randomly chosen 45-50 islets were used for measurements. Sympathetic innervation was measured as the density of the TH+ area per endocrine area, whereas parasympathetic innervation was measured as the density of VACHT+ puncta per endocrine area.

β-cell proliferation was measured in 20-30 randomly chosen islets from 11 sections (60 µm apart) of P0 pancreas. Quantification of neural and pancreatic progenitor cell proliferation in cultured pancreas explants was analyzed in six sections (30 µm apart).

Pancreas explant size was measured by analyzing the total area occupied by the explant on days 1 and 4 of culture. The epithelial area of pancreas explants was measured by performing whole-mount immunostaining for PDX1 as previously described (Cano et al., 2006). z-stack images were captured every 1 µm using an LTC SP2 confocal microscope (Leica). The area covered by PDX1 staining was determined using ImageJ. For quantification of process outgrowth and cell migration, whole-mount immunostaining with HuC/D and Tuj1 was performed on pancreas explants. The area occupied by migrating cells (HuC/D+) and sprouting fibers (visualized by Tuj1 staining) were measured using ImageJ. For quantification of directed migration, double-immunostained pancreas explants were subdivided into quadrants proximal and distal to the agarose beads. The ratio of the area occupied by HuC/D+ cells in the quadrant proximal to the beads divided by the area occupied by cells in the opposite quadrant was used to calculate directed migration.

Significance was determined using two-tailed Student’s t-test and one-way ANOVA (post-hoc Tukey HSD test). P<0.05 was considered significant. Data are presented as mean ± s.e.m. unless stated otherwise.

**RESULTS**

Gdnf is transiently expressed during embryonic pancreas development

To investigate the possible role of Gdnf in the development of pancreatic innervation, we first analyzed its expression in the
Galactosidase (pancreas (supplementary material Fig. S1H). Interestingly, material Fig. S1F) and no expression was observed in the adult stomach and intestine (supplementary material Fig. S1I,J). Thus, in sharp contrast to the specific mesenchymal localization found in the expressed exclusively in the epithelial compartment of the pancreas, determined the pattern of no reliable antibodies for GDNF immunohistochemistry. To develop the developing pancreas. To the best of our knowledge, there are currently no reliable antibodies for GDNF immunohistochemistry. To determine the pattern of Gdnf expression, we performed enzymatic β-galactosidase (β-gal) detection on embryos of Gdnf\textsuperscript{lacZ/+} knock-in mice (Sánchez et al., 1996; Hidalgo-Figueroa et al., 2012). β-gal activity was first detected in pancreatic rudiments at E10.5 (Fig. 1A,B; supplementary material Fig. S1A). Gdnf was broadly expressed within the developing ductal epithelium at E12.5 (Fig. 1C; supplementary material Fig. S1B). At these stages, β-gal activity was extensively observed in progenitor cells (marked by PDX1 and DBA lectin reactivity) (Fig. 1C; supplementary material Fig. S1B,G) but not in differentiated endocrine cells (insulin- and glucagon-producing cells) (Fig. 1B; supplementary material Fig. S1A). At the onset of islet and acinar development, during the so-called secondary transition, Gdnf displayed a very specific expression pattern, being restricted to the pancreatic trunk epithelium that contains ductal-endocrine progenitor cells (Fig. 1D,E,G; supplementary material Fig. S1C-E). At this stage, β-gal activity was excluded from Cpa1 expressing exocrine cells (Fig. 1F,H). Gdnf expression rapidly declined in late embryogenesis. Only scattered cells in the ductal compartment remained β-gal\textsuperscript{−/−} at birth (Fig. 1I; supplementary material Fig. S1F). The expression was observed in the adult pancreas (supplementary material Fig. S1H). Interestingly, Gdnf is expressed exclusively in the epithelial compartment of the pancreas, in sharp contrast to the specific mesenchymal localization found in the stomach and intestine (supplementary material Fig. S1I,J). Thus, the Gdnf expression pattern in the embryonic pancreas parallels the chronology of migration and differentiation of NC derivatives in the pancreas (see below).

Neuronal and glial differentiation in the developing pancreas

Recent studies have characterized the chronology of migration of the NC cells that populate the pancreas during embryonic development (Nekrep et al., 2008; Plank et al., 2011). However, the process of neuronal and glial differentiation in the pancreas is not yet well defined. Before addressing the role of GDNF in pancreas innervation we examined when the differentiation of pancreatic neuronal and glial cells begins.

We analyzed NC cell differentiation in the pancreas by immunohistochemistry using progenitor markers (SOX10 and PHOX2B), neuronal markers [HuC/D (ELAVL3/4 – Mouse Genome Informatics) and TUJ1 (β-tubulin III, TUBB3)] and glial markers (GFAP). In agreement with genetic lineage-tracing studies, NC cells were found in the pancreatic mesenchyme at E10.5 (Fig. 2A) and reached the pancreatic epithelium at E11.5 (Fig. 2B). At E12.5, a few NC cells started to differentiate into neurons (Fig. 2C,F; supplementary material Fig. S3C). The expression of PHOX2B was maintained in these neuronal cells (Fig. 2E). GFAP\textsuperscript{+} glial cells were first observed at E13.5, although their numbers were low (Fig. 2D,F). GFAP\textsuperscript{+} cells expressed SOX10 (Fig. 2F). Between E13.5 and E15.5, the number of neuronal and glial cells dramatically increased (Fig. 2D; supplementary material Fig. S3G,I). Interestingly, most of the neuronal and glial cells were closely associated with endocrine cell clusters by E15.5 (supplementary material Fig. S3G,I). This close association between neurons, glial cells and islets persisted during postnatal life (Fig. 2I,J; see also Fig. 4A). Maturation of neuronal cells took place during the first weeks of postnatal life (Fig. 2G,H). By the third postnatal week, islets were highly innervated with sympathetic and parasympathetic fibers. However, innervation of the exocrine compartment was relatively scarce (Fig. 2I,J; see also Fig. 4D). As previously reported, intrapancreatic neurons expressed parasympathetic (VACH\textsuperscript{T}; SLC18A3 – Mouse Genome Informatics) but not sympathetic (TH) markers (Fig. 2L). To determine whether the components of the GDNF-RET signaling pathway are expressed in NC derivatives during pancreas development, we examined expression of the receptor tyrosine kinase RET and GFRα1 (the preferential co-receptor for GDNF) by immunohistochemistry. NC cells migrating into the pancreas expressed both RET and GFRα1 (Fig. 2K,L). Neuronal cells maintained the expression of RET and GFRα1 during later stages of pancreas development (Fig. 2M-P).

Pancreatic neurons and glia are absent in mice lacking GDNF

To analyze the role of GDNF in the development of pancreas innervation, Gdnf was specifically inactivated in pancreas by
crossing mice heterozygous for null and conditional alleles of Gdnf (hereafter Gdnflox/lacZ mice) to a transgenic mouse line that expresses Cre recombinase under the control of the pancreatic and duodenal homeobox 1 (Pdx1) promoter (Pdx1-Cre mice) (Gu et al., 2002). Pdx1 is expressed in all epithelial pancreatic cells early during embryonic development, thus enabling gene inactivation in the entire pancreas in Pdx1-Cre mice. Gdnflox/lacZ mice were used to ensure efficient Gdnf excision. Successful excision of the Gdnflox allele was confirmed by PCR of genomic DNA isolated from pancreas as early as E11.5 (Fig. 3A; supplementary material Fig. S2A,B). In addition, semi-quantitative RT-PCR analysis demonstrated significant reduction of Gdnf mRNA in embryonic mutant pancreas compared with control littermates (Fig. 3B). Pdx1-Cre;Gdnflox/lacZ mice were born normally and reached adulthood without any sign of compromised health.

To determine the role of GDNF in pancreatic innervation, pancreatic sections of newborn Pdx1-Cre;Gdnflox/lacZ mice were immunostained for neuronal and glial markers. The numbers of glial and neuronal cells were markedly decreased in Gdnf-deficient mice (Fig. 3C,D,I). The nerve fiber density of Pdx1-Cre;Gdnflox/lacZ islets was also significantly reduced compared with those of control mice (Fig. 3F,G,J). By contrast, innervation and neuronal cells in other regions of the gut, such as the stomach and intestine, were unaffected in Pdx1-Cre;Gdnflox/lacZ mice (supplementary material Fig. S2C,D; data not shown). For comparison purposes, pancreatic innervation in Gdnf null (conventional) mice was also analyzed. In Gdnf null mice, enteric NC cells fail to populate the gastrointestinal tract during early gut development (Pichel et al., 1996; Sánchez et al., 1996) (supplementary material Fig. S2E). GdnflacZ/lacZ pancreata were virtually devoid of neurons and glial cells (Fig. 3E,I). Immunostaining for TUJ1 revealed a decrease in nerve fibers in GdnflacZ/lacZ pancreas, similar to the reduction observed in Pdx1-Cre;Gdnflox/lacZ mice (Fig. 3H,J). Of note, most of the remaining nerve fibers in both GdnflacZ/lacZ and Pdx1-Cre;Gdnflox/lacZ mice appeared to be associated with blood vessels (supplementary material Fig. S2F,G; data not shown).

Altogether, our results indicate that GDNF secreted by the pancreas is necessary for the development of pancreatic innervation.

Reduced parasympathetic innervation in Gdnf mutant mice
As described above, the innervation of the endocrine pancreas increases significantly during the first 3 weeks of life. Similar to our observations in newborn mice, 3-week-old Pdx1-Cre;Gdnflox/lacZ mice displayed a profound reduction in the number of neuronal and glial cells (Fig. 4A-C). A quantitative analysis of sympathetic and parasympathetic innervation revealed a 54% reduction in the parasympathetic innervation density, measured as V AChT+ puncta per islet area, in 3-week-old Pdx1-Cre;Gdnflox/lacZ mice (Fig. 4D-H). A mild, but statistically significant, reduction in parasympathetic innervation...
Interestingly, no differences in the density of TH+ sympathetic nerve fibers were found between Pdx1-Cre;Gdnflox/lacZ and control mice (Fig. 4D-I). Of note, the TH+ nerve fibers were found closely associated with blood vessels (supplementary material Fig. S2H-K).

Thus, pancreas-specific inactivation of Gdnf results in selective loss of parasympathetic innervation of the islets.

**GDNF promotes the differentiation and migration of pancreatic neural progenitors**

To obtain mechanistic information about the role of GDNF in the intrinsic innervation of the pancreas, neuronal and glial differentiation was examined in Pdx1-Cre;Gdnflox/lacZ embryos at various developmental stages. A marked reduction in Hu/C/D+ neurons and GFAP+ glial cells was observed in Pdx1-Cre;Gdnflox/lacZ mice at all embryonic stages analyzed (supplementary material Fig. S3; data not shown). Similarly, immunostaining for TUJ1 revealed a decrease in neuronal cell bodies and nerve fibers in Pdx1-Cre;Gdnflox/lacZ pancreas as early as E12.5 (supplementary material Fig. S3A,B).

GDNF promotes the migration, proliferation, survival and differentiation of multipotent enteric precursor cells in the gut (Heucker et al., 1998; Taraviras et al., 1999; Young et al., 2001; Natarajan et al., 2002; Iwashita et al., 2003). To distinguish between these roles in underlying the reduction in NC derivatives, we quantified the number of neural progenitor cells in the pancreas of Pdx1-Cre;Gdnflox/lacZ embryos. A dramatic decrease in the number of PHOX2B+ and SOX10+ cells was observed in Pdx1-Cre;Gdnflox/lacZ pancreas at E11.5 (Fig. 5A-C), indicating that loss of pancreatic GDNF compromises colonization of the pancreas by NC-derived cells. To determine whether GDNF can act as a chemoattractant for pancreatic NC cells, E11.5 wild-type pancreas explants were cultured with beads soaked in GDNF. GDNF induced a marked asymmetric pattern of neurite outgrowth towards the beads (Fig. 5D,E). In BSA-treated control explants, most Hu/C/D+ neurons remained in the close vicinity of the explants. In stark contrast, groups of Hu/C/D+ neurons were observed associated with the beads in the presence of exogenous GDNF (Fig. 5D-G). The clusters of Hu/C/D+ cells were usually found associated with bundles of neurites (Fig. 5E).

Gdnf inactivation in the pancreas blocks the migration of NC-derived cells to the pancreas thereby precluding in vivo study of the role of GDNF in the proliferation and differentiation of pancreatic NC-derived cells. To circumvent these limitations, we used pancreas explants from E11.5 wild-type embryos. Exogenous GDNF in pancreas explants induced a significant expansion of pancreatic neural progenitors (Fig. 5H-J), which was likely to be a consequence of the dramatic increase in proliferating progenitor cells (Fig. 5H,K). To assess neuronal differentiation, pancreas explants were stained for Hu/C/D to mark neurons and for TUJ1 to visualize outgrowing neurites. The addition of exogenous GDNF to the culture medium induced the extension of neurites from embryonic pancreas explants (Fig. 5M,O). Almost no neurite outgrowth was observed in the absence of GDNF (Fig. 5L,O). Neurite outgrowth was accompanied by robust neuronal differentiation in GDNF-treated pancreas explants, as revealed by Hu/C/D immunostaining (Fig. 5N,O). However, very rare Hu/C/D+ neurons were found in control pancreas explants (Fig. 5O; data not shown).

Altogether, these results indicate that GDNF induces the differentiation and migration of neural progenitors of the pancreas.

**Loss of GDNF does not affect islet formation and proliferation**

Recent studies have suggested that GDNF-RET signaling may influence pancreatic β-cell formation (Mwangi et al., 2008;
Mwangi et al., 2010). Histological and immunohistochemical analyses using markers of all pancreatic epithelial cell types (endocrine, acinar and ductal) did not reveal any apparent abnormalities in Pdx1-Cre;Gdnflox/lacZ pancreas (Fig. 6A-D; supplementary material Fig. S4A-D). To determine whether endogenous pancreatic Gdnf might play a role in β-cell formation we performed a detailed quantitative analysis of islet formation in Pdx1-Cre;Gdnflox/lacZ mice. No differences in islet area were found between Pdx1-Cre;Gdnflox/lacZ and control mice at postnatal day (P) 0 and at 3 weeks of age (Fig. 6E). Similarly, no changes in β-cell proliferation were observed between Pdx1-Cre;Gdnflox/lacZ and control pups (Fig. 6F). To rule out the possibility that the lack of islet phenotypes in Pdx1-Cre;Gdnflox/lacZ mice might be due to insufficient Gdnf excision, we measured islet area in conventional Gdnflox/lacZ P0 pups (Fig. 6E; data not shown). No differences in the expression of key transcription factors required for endocrine formation, such as Pdx1, neurogenin 3 (Ngn3) and Nkx2.2, were observed between control and mutant embryos (Fig. 6G). Finally, glucose tolerance tests indicated that endocrine cell function was unaffected in Pdx1-Cre;Gdnflox/lacZ mice (supplementary material Fig. S4E,F). In summary, these results indicate that endogenous GDNF activity is not required for β-cell formation.

GDNF directly induces the proliferation of pancreatic progenitor cells

Our results in mice deficient for Gdnf argue that endogenous pancreatic GDNF is dispensable for pancreas formation. However, transgenic mice overexpressing Gdnf display increased pancreatic endocrine mass (Mwangi et al., 2010). To determine whether activation of the GDNF-RET signaling system can influence pancreas formation, we analyzed pancreatic epithelium formation in GDNF-treated pancreas rudiments. Addition of GDNF to embryonic pancreatic rudiments in culture led to a significant increase in organ size after 4 days of culture (Fig. 7A,B,G). Immunostaining for PDX1, a marker that specifically labels the pancreatic epithelium, confirmed the expansion of pancreatic epithelial area in GDNF-treated explants compared with vehicle-treated controls (Fig. 7C,D,H).

To elucidate the mechanisms leading to increased pancreatic epithelial area, we assayed the proliferation of pancreatic cells by phospho-histone H3 (pHH3) immunostaining. Exogenous GDNF in pancreatic explants caused increased proliferation of Pdx1-expressing cells (Fig. 7E,F,I). Similar results were obtained with KI67 as a marker of cell proliferation (data not shown).

Since GRα1, the preferential co-receptor for GDNF, is expressed in both epithelial and neuronal cells in the embryonic pancreas (Fig. 7J, Fig. 2K,M), we performed a series of experiments...
to determine whether the effect of GDNF on the expansion of pancreatic epithelial cells is direct or indirect due to the increased neural cell numbers. First, we treated pancreas explants with neurturin (NRTN), another member of the GDNF ligand family. GFRα2, the preferential co-receptor for NRTN, is expressed in neuronal, but not epithelial, cells during pancreas development (supplementary material Fig. S5A). As with GDNF, NRTN stimulated robust neurite outgrowth and neuronal differentiation in pancreas explants (supplementary material Fig. S5B-D). However, no increase in organ size or pancreatic epithelial cell proliferation was observed in NRTN-treated pancreas explants (Fig. 7G,I).

Next, we examined the effect of GDNF in pancreas explants of Gdnf lacZ/lacZ embryos. Gdnf null mice show a complete loss of neural progenitor cells in embryonic pancreas due to defects in NC cell colonization of the gastrointestinal tract during early gut development. As expected, no significant increase in neurite outgrowth and neuronal differentiation was observed in Gdnf lacZ/lacZ pancreas explants treated with GDNF (supplementary material Fig. S5E-G). Notably, GDNF caused an increase in organ size and in Pdx1-expressing cell proliferation in Gdnf lacZ/lacZ pancreas explants to the same extent as observed in wild-type pancreas explants (Fig. 7G,I).
In summary, our results indicate that GDNF can act cell-autonomously to promote the proliferation of embryonic pancreatic progenitors.

**DISCUSSION**

By performing gene inactivation studies in mice and *ex vivo* experiments using cultures of pancreas explants, we show that GDNF is required for the intrinsic innervation of the pancreas. We propose a model in which GDNF secreted by the pancreatic epithelium attracts NC cells into the pancreas as these cells migrate along the gut during embryonic development. During later stages of pancreas development, GDNF might contribute to the successful colonization of the pancreas as well as to the efficient proliferation and differentiation of NC derivatives.

The positive migratory response of pancreatic neural progenitor cells to GDNF seems to be very similar to that reported in other neural-derived cells, such as enteric neurons (Young et al., 2001; Natarajan et al., 2002), cortical GABAergic neurons (Pozas and Ibáñez, 2005) and neural progenitors of the lung, which, like the pancreas, is a derivative of the foregut (Tollet et al., 2002). In addition to migration, our *ex vivo* studies indicate that exogenous GDNF promotes the proliferation and differentiation of neural progenitors in the pancreas. In agreement with our results, it has been reported that GDNF promotes the proliferation and differentiation of enteric NC cells in culture (Taraviras et al., 1999; Barlow et al., 2008). Temporal inactivation of *Gdnf* in mice will be required to conclusively demonstrate an *in vivo* role of GDNF during the later stages of pancreas development.

The impairment in the migration of NC cells to the pancreas in *Gdnf*−/− mice results in a dramatic reduction in intrapancreatic neurons as well as in intrinsic innervation of the islets during the postnatal period. Interestingly, *Gdnf* inactivation results in selective loss of parasympathetic innervation of the islets, which is reminiscent of that observed in *Gfra2* mutant mice (Rossi et al., 2005). These results indicate that the establishment of sympathetic innervation in the pancreas does not require GDNF-RET signaling. Most of the remaining TH+ nerve fibers in both *Gdnf*lacZ/lacZ and *Pdx1-Cre;Gdnf*lacZ/lacZ mice were found closely associated with blood vessels, in agreement with previous studies reporting that sympathetic fibers reach pancreatic islets following blood vessels (Cabrera-Vásquez et al., 2009; Rodriguez-Díaz et al., 2011) and that this process may be mediated by nerve growth factor (Cabrera-Vásquez et al., 2009).

Recent studies have shown that GDNF promotes the survival and proliferation of β-cells *in vitro* (Mwangi et al., 2008). In addition, transgenic mice that overexpress GDNF in glia exhibit increased β-cell mass and proliferation (Mwangi et al., 2010). In our studies, addition of GDNF to embryonic pancreas explants induced the expansion of the pancreatic epithelial area associated with increased progenitor cell proliferation. These results are in agreement with previous reports showing that exogenous GDNF stimulates the proliferation of epithelial cells in embryonic kidney organ culture (Vega et al., 1996; Pepicelli et al., 1997). However, our results in mice deficient for *Gdnf* argue that endogenous pancreatic GDNF is dispensable for pancreas formation. This discrepancy could be reconciled considering that pancreas explant experiments and transgenic overexpression approaches might not reflect the true physiological function of GDNF in pancreas development, but rather the effect of GDNF signaling hyperactivation. Our studies suggest that GDNF induces pancreatic epithelial cell proliferation in a cell-autonomous manner. Furthermore, this effect of GDNF is specific to *Cpa1*-expressing pancreatic cells. *Cpa1+* cells serve as...
multipotent pancreatic progenitor cells before E14.5 and, at these stages, CPA1+ cells express GFRα1, the co-receptor for GDNF. Thus, we suggest that manipulation of the GDNF-RET pathway might be a useful strategy for the expansion of pancreatic progenitor cells in vitro.

Glucose tolerance is not affected in Pdx1-Cre;Gdnflox/lacZ mice, indicating that parasympathetic innervation of the islets is not crucial for glucose homeostasis under normal conditions. These results are consistent with previous studies performed in Gfra2 mutant mice (Rossi et al., 2005). Whether Pdx1-Cre;Gdnflox/lacZ mice display abnormalities in glucose homeostasis under stress conditions remains to be determined. More recently, a role of the nervous system in embryonic and adult β-cell formation has been described. Ablation of NC cells leads to an increase in embryonic β-cell proliferation, while in adult mice parasympathetic denervation via vagotomy reduces islet proliferation (Nekrep et al., 2008; Lausier et al., 2010; Plank et al., 2011). How β-cell proliferation is regulated by neural input is far from being

**Fig. 7.** GDNF promotes the proliferation of pancreatic progenitor cells. (A,B) GDNF increases organ size in E12.5 pancreas explants after 4 days of culture. (C,D) Maximum projection confocal images of PDX1 whole-mount staining on pancreas explants treated with BSA (C) and GDNF (D) for 4 days. (E,F) Proliferation of Pdx1-expressing cells is increased in GDNF-treated pancreas explants as shown by pH3 immunofluorescence (arrowheads indicate proliferating cells). (G) Quantification of explant size at days 1 and 4 after treatment with GDNF and NRTN of Gdnf null and control pancreatic buds (as in A,B). Gdnf null, n=3; Gdnf wild-type, n=6-10 samples per group. (H) Quantification of PDX1-stained area after treatment with GDNF (as in C,D). n=5-7 samples per group. (I) Proliferation indexes of PDX1+ cells in control and Gdnf null pancreas explants treated with GDNF and control explants treated with NRTN. n=4-5 samples per group. (J) GFRα1 is expressed at the tips of the branching pancreas epithelium at E13.5. GFRα1 is also expressed by neural precursor cells (asterisks). J’-J” Expression of GFRα1 (J”) and the tip cell marker CPA1 (J’), overlap (J”, merge) in E13.5 pancreas epithelium. (K) GFRα1 is expressed by CPA1+ cells at E15.5. K’-K” Expression of GFRα1 (K”) and CPA1 (K’) overlap (K”, merged). (L,M) GDNF induces proliferation of CPA1+ cells as shown by Ki67 immunofluorescence. (N) Proliferation indexes of CPA1+ cells and CPA1− cells in GDNF- and BSA-treated pancreas explants after 4 days in culture. n=3 samples per group. (G,H,N) Data are mean ± s.e.m. *P<0.05, **P<0.01 (Student’s t-test in H,N, one-way ANOVA in G,J). Scale bars: 100 μm in A-F; 50 μm in J-M.
understood and further studies are needed to clarify the underlying mechanisms. In these studies it is crucial to manipulate innervation specifically in the pancreas without affecting other tissues, such as the gut, that might influence β-cell proliferation. The pancreas-specific Gdnf mutant mouse might provide a useful tool to study the role of intrinsic pancreatic innervation in pancreas development and to analyze neural-islet interactions in the adult endocrine pancreas without the potentially confounding effects of manipulating the innervation of extrapancreatic tissues.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
J.L-B. participated in the design of the study, performed the experiments, analyzed the data and commented on the manuscript. M.H.-F. aided in pancreatic bud ex vivo experiments. A.P. contributed to experimental design and discussion of the data. J.L.-B. provided reagents and contributed to discussion of the data. A.L.-C. contributed to discussion of the data. D.A.C. participated in the design of the study, supervised the project, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Supplementary material
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