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GDNF is required for neural colonization of the pancreas

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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

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; Ahrén, 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 (Kirchgessner 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). GDNF belongs to a family of neurotrophic factors that also includes neurturin, persephin and artemin. GDNF family ligands are involved in the development and maintenance of sensory, enteric, sympathetic and parasympathetic neurons. GDNF family members are also important for development outside the nervous system, especially kidney morphogenesis (Airaksinen and Saarma, 2002). GDNF binds to the GDNF family receptor α1 (GFRα1), which forms a co-receptor complex with the transmembrane tyrosine kinase receptor RET. The GDNF-RET signaling pathway plays multiples roles in the formation of the enteric nervous system by regulating the migration, proliferation, differentiation and survival of enteric NC cells (Enomoto et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Young et al., 2001; Natarajan et al., 2002; Iwashita et al., 2003). Mutations in components of the GDNF-RET pathway cause Hirschsprung's disease, a congenital abnormality characterized by the absence of enteric ganglia in the hind gut (Amiel and Lyonnet, 2001). *Gdnf* null (conventional) mice lack enteric neurons throughout most of the gastrointestinal gut, demonstrating the essential role of GDNF in the development of the enteric nervous system (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). Total inactivation of *Gdnf* in mice results in perinatal lethality due to defects in kidney morphogenesis, precluding the study of the physiological role of GDNF-RET signaling in adult mice. The

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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. *Pdx1-Cre*, $Gdnf^{lox/lox}$ and $Gdnf^{lacZ/+}$ mice have been described previously (Sánchez et al., 1996; Gu et al., 2002; Pascual et al., 2008).

Genomic DNA excision

Quantification of the excision levels of the $Gdnf^{lox}$ 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 Tubeness 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

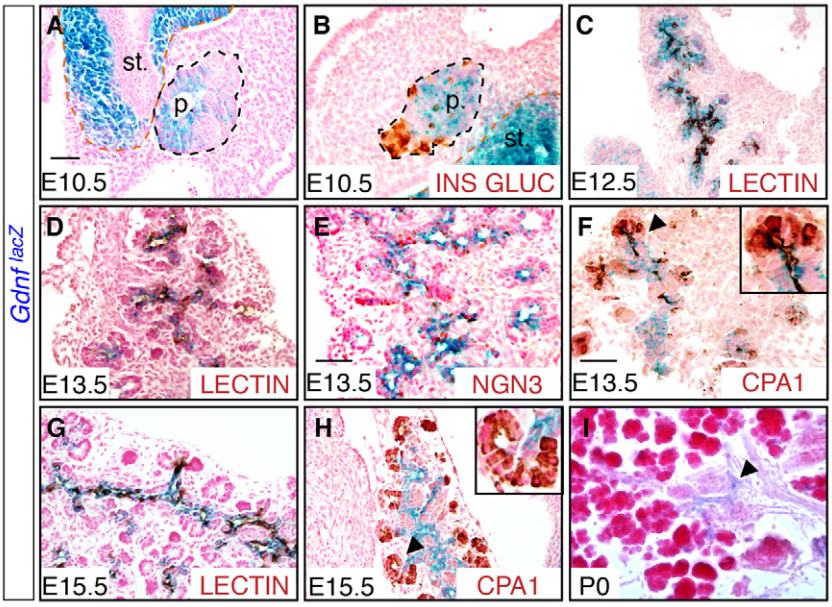

Fig. 1. *Gdnf* is expressed in the epithelium of the embryonic pancreas. (**A**) Analysis of β-galactosidase activity in $Gdnf^{lacZ/+}$ mice reveals expression in the pancreatic epithelium at E10.5. (**B**) Almost no β-galactosidase activity (blue) is found in differentiated insulin- and glucagon-expressing cells at E10.5. In A and B, pancreatic epithelium (p.; black dashed line) and stomach (st.; red dashed line) are outlined. (**C**) Broad *Gdnf* expression is observed in the pancreatic epithelium at E12.5. (**D-H**) *Gdnf*-expressing cells are found within the ductal/endocrine progenitor domain (marked by expression of lectin and *Ngn3*, respectively) during the secondary transition at E13.5 (D,E) and E15.5 (G), whereas no *Gdnf* expression is observed in exocrine cells (F,H). Arrowheads in F and H indicate the regions enlarged in the insets. (**I**) Faint β-galactosidase activity is observed in ducts (arrowhead) at P0. Scale bars: 50 μm (in A for A-D,G-I).

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 $Gdnf^{lacZ/+}$ 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 $\beta\text{-gal}^+$ at birth (Fig. 1I; supplementary material Fig. S1F) and no 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,E; supplementary material Fig. S3C). The expression of PHOX2B was maintained in these neuronal cells (Fig. 2E). $GFAP^+$ glial cells were first observed at E13.5, although their numbers were low (Fig. 2D,F). $GFAP^+$ 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 (VAChT; SLC18A3 – Mouse Genome Informatics) but not sympathetic (TH) markers (Fig. 2I,J).

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

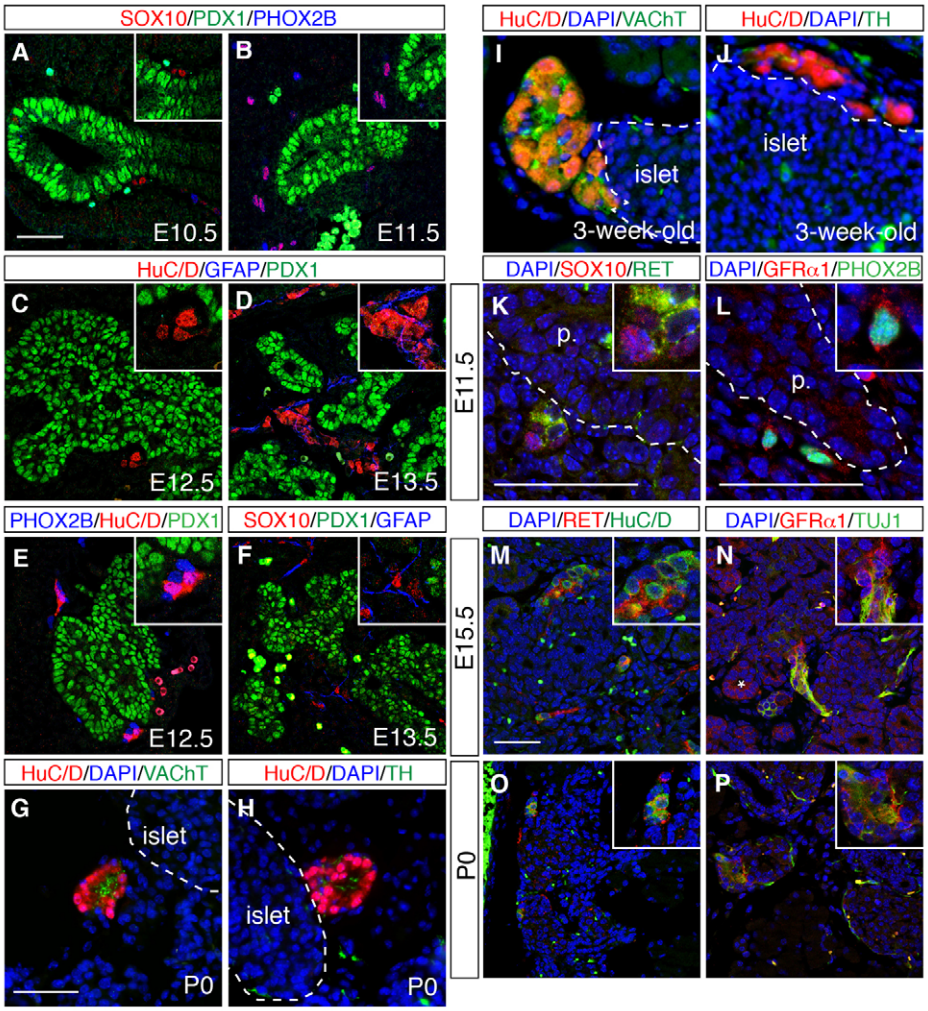

Fig. 2. Differentiation of NC cells and their derivatives in the pancreas. (**A**,**B**) Immunostaining on sections of wild-type embryonic mouse pancreas demonstrate that NC cells expressing the progenitor markers PHOX2B and SOX10 are found in the pancreatic rudiment at E10.5 (A) and E11.5 (B). (**C**) HuC/D$^+$ neuronal, but not GFAP$^+$ glial, cells are found in the pancreas at E12.5. (**D**) GFAP$^+$ glial cells are first observed at E13.5. (**E**) HuC/D$^+$ neuronal cells express PHOX2B. (**F**) GFAP$^+$ glial cells express SOX10. (**G**,**H**) Paucity of islet innervation at P0. (**I**,**J**) Neurons in intrapancreatic ganglia express parasympathetic, but not sympathetic, markers at 3 weeks of age. (G-J) Islets are outlined. (**K**,**L**) Neural precursor cells at E11.5 express the GDNF receptor RET (K) and co-receptor GFRα1 (L). Pancreatic epithelium (p.) is outlined. (**M**,**N**) At E15.5, both GDNF-RET components are strongly expressed in neuronal cells. (**O**,**P**) After birth, expression of GFRα1 decreases (O), but RET expression is maintained in neuronal cells (P). Scale bars: 50 μm (in A for A-F; in G for G-J; in M for M-P).

crossing mice heterozygous for null and conditional alleles of *Gdnf* (hereafter $Gdnf^{lox/lacZ}$ 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. $Gdnf^{lox/lacZ}$ mice were used to ensure efficient *Gdnf* excision. Successful excision of the $Gdnf^{lox}$ 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*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ 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). $Gdnf^{lacZ/lacZ}$ pancreata were virtually devoid of neurons and glial cells (Fig. 3E,I). Immunostaining for TUJ1 revealed a decrease in nerve fibers in $Gdnf^{lacZ/lacZ}$ pancreas, similar to the reduction observed in *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ mice (Fig. 3H,J). Of note, most of the remaining nerve fibers in both $Gdnf^{lacZ/lacZ}$ and *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ 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 VAChT$^+$ puncta per islet area, in 3-week-old *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ mice (Fig. 4D-H). A mild, but statistically significant, reduction in parasympathetic

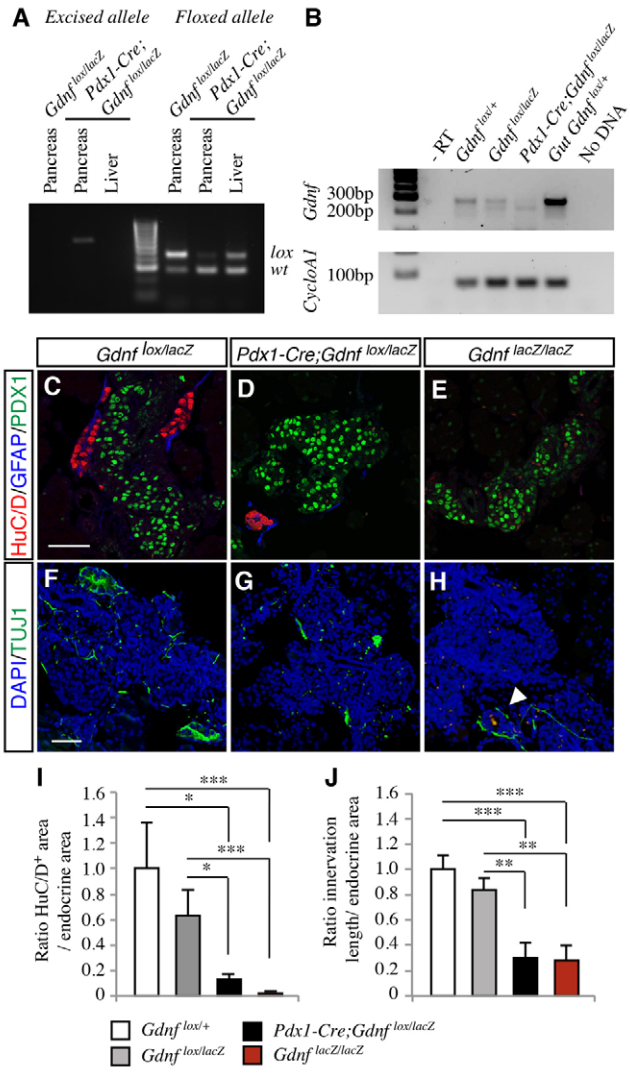

Fig. 3. Decreased number of NC derivatives and islet innervation in P0 *Gdnf* mutant mice. (**A**) PCR analysis on genomic DNA from transgenic pancreas demonstrates excision of the *Gdnf* allele (left) and the reduction in the $Gdnf^{lox}$ allele in pancreas but not in liver (right). DNA was isolated from 3-week-old mice. (**B**) Semi-quantitative RT-PCR analysis of *Gdnf* and cyclophilin A (*CycloA*) mRNA expression levels in E15.5 pancreas. (**C**,**D**) Glial and neuronal cells are decreased in *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ compared with control pancreas. (**E**) Glial and neuronal cells are absent from $Gdnf^{lacZ/lacZ}$ pancreas. (**F-H**) TUJ1 staining reveals reduced islet innervation in *Pdx1-Cre;* $Gdnf^{lox/lacZ}$ (G) and $Gdnf^{lacZ/lacZ}$ (H) compared with control (F) mice. Note that innervation is reduced but not completely absent (arrowhead) in $Gdnf^{lacZ/lacZ}$ mice. (**I**) Quantitative analysis reveals a marked decrease in neuronal area in *Pdx1-Cre;* $Gdnf^{lox/lacZ}$ compared with control pancreas. (**J**) Total endocrine innervation, measured as total length of $TUJ1^+$ fibers relative to endocrine area, is reduced in *Pdx1-Cre;* $Gdnf^{lox/lacZ}$ and $Gdnf^{lacZ/lacZ}$ compared with control mice. (I,J) Data are mean ± s.e.m.; n=3 samples per group. Wild-type ($Gdnf^{lox/+}$) values are adjusted to 1 to facilitate comparison. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA). Scale bars: 50 μm (in C for C-E; in F for F-H).

innervation was also observed in $Gdnf^{lox/lacZ}$ heterozygous mice (Fig. 4H), in agreement with studies indicating that gut innervation is sensitive to decreased *Gdnf* gene dosage (Shen et al., 2002). Interestingly, no differences in the density of TH^+ sympathetic nerve fibers were found between *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ embryos at various developmental stages. A marked reduction in HuC/D^+ neurons and $GFAP^+$ glial cells was observed in *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ 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*;$Gdnf^{lox/lacZ}$ 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 (Heuckeroth 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*;$Gdnf^{lox/lacZ}$ embryos. A dramatic decrease in the number of $PHOX2B^+$ and $SOX10^+$ cells was observed in *Pdx1-Cre*;$Gdnf^{lox/lacZ}$ 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 HuC/D^+ neurons remained in the close vicinity of the explants. In stark contrast, groups of HuC/D^+ neurons were observed associated with the beads in the presence of exogenous GDNF (Fig. 5D-G). The clusters of HuC/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,I,K). To assess neuronal differentiation, pancreas explants were stained for HuC/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 HuC/D immunostaining (Fig. 5N,O). However, very rare HuC/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;

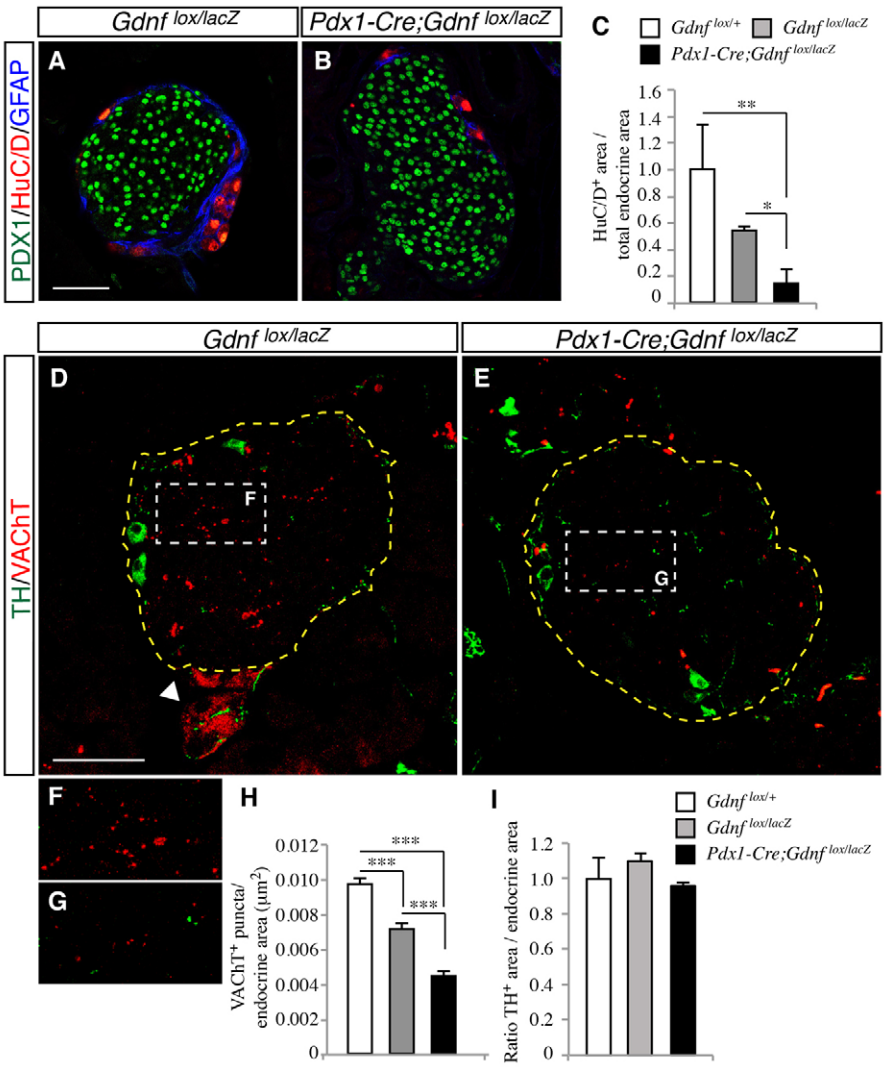

Fig. 4. Loss of endocrine parasympathetic innervation in *Gdnf*-deficient mice.
(**A**,**B**) Decrease of neuronal and glial cells in 3-week-old *Pdx1-Cre; Gdnf*$^{lox/lacZ}$ compared with control pancreas. (**C**) Quantification of neuronal area. Control values are adjusted to 1 to facilitate comparison. (**D-G**) Confocal images from control (D) and *Pdx1-Cre; Gdnf*$^{lox/lacZ}$ (E) islets immunostained for parasympathetic (VAChT) and sympathetic (TH) markers. Islets are outlined in yellow. Arrowhead (D) indicates intrapancreatic ganglia. The boxed areas are shown at higher magnification in F and G. (**H**) Quantification of VAChT$^+$ synaptic puncta in endocrine pancreas reveals a decrease in *Pdx1-Cre; Gdnf*$^{lox/lacZ}$ compared with control mice. A statistically significant decrease in VAChT$^+$ synaptic puncta is also observed in heterozygous *Gdnf*$^{lox/lacZ}$ mice. (**I**) No differences were observed in TH$^+$ area in endocrine pancreas between mutant and control mice. Data are mean ± s.e.m.; n=3 samples per group. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA). Scale bars: 50 μm (in A for A,B; in D for D,E).

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*;*Gdnf*$^{lox/lacZ}$ 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*;*Gdnf*$^{lox/lacZ}$ mice. No differences in islet area were found between *Pdx1-Cre*;*Gdnf*$^{lox/lacZ}$ 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*;*Gdnf*$^{lox/lacZ}$ and control pups (Fig. 6F). To rule out the possibility that the lack of islet phenotypes in *Pdx1-Cre*;*Gdnf*$^{lox/lacZ}$ mice might be due to insufficient *Gdnf* excision, we measured islet area in conventional *Gdnf*$^{lacZ/lacZ}$ mice. Islet area and architecture were also unaffected in *Gdnf*$^{lacZ/lacZ}$ 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*;*Gdnf*$^{lox/lacZ}$ 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 explants. 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 pancreas 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 GFRα1, 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

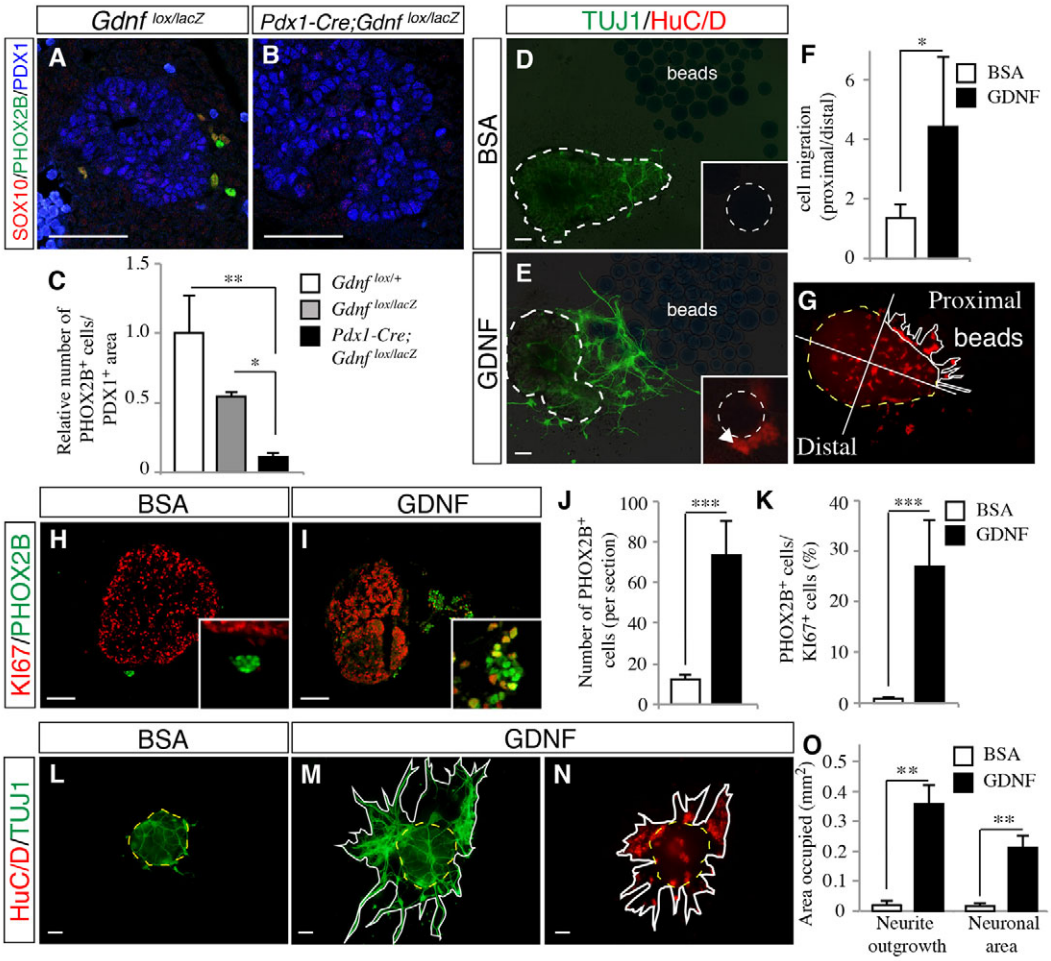

Fig. 5. GDNF promotes the migration, proliferation and differentiation of neural progenitor cells of the pancreas. (**A**,**B**) PHOX2B- and SOX10-expressing cells are reduced in *Pdx1-Cre; Gdnf*$^{lox/lacZ}$ (B) compared with control (A) pancreas at E11.5. (**C**) Quantification of PHOX2B$^+$ cells at E11.5. Control values are adjusted to 1 to facilitate comparison. *n*=3 samples per group. (**D**,**E**) Whole-mount TUJ1 immunostaining of E12.5 pancreas explants (outlined) cultured for 4 days with agarose beads soaked in BSA (D) or GDNF (E). Insets show high-magnifications of the same pancreas explants immunostained for HuC/D. No HuC/D$^+$ cells are found close to control BSA-soaked beads (circular outlines). A large number of HuC/D$^+$ cells (arrowhead) originating in the pancreas have migrated towards the GDNF-soaked beads. (**F**) Quantification of neuronal cell migration in cultured pancreas explants. *n*=5 samples per group. (**G**) Neuronal cell migration is measured as the ratio of the area occupied by HuC/D$^+$ cells of the pancreas explant in the quadrant proximal to beads (outlined in white) divided by the area occupied by cells in the opposite quadrant. The pancreatic epithelium is outlined in yellow. (**H**,**I**) Sections of E11.5 pancreas explants cultured for 3 days with BSA (H) or GDNF (I) and immunostained for the neural progenitor marker PHOX2B and the proliferation marker KI67. (**J**) Quantification of the number of PHOX2B-expressing cells per section of pancreas explant. (**K**) Quantification of the number of proliferating neural progenitor cells in pancreas explants. (J,K) *n*=3 samples per group. (**L-N**) E11.5 wild-type pancreas explants cultured with GDNF exhibit increased neurite outgrowth (M) and neuronal differentiation (N) compared with BSA-treated explants (L). Pancreatic epithelium is outlined in yellow. (**O**) Quantification of neuronal cell area and neurite outgrowth in pancreas explants. Neurite outgrowth was measured as the area occupied by TUJ1$^+$ fibers (as shown in M, outlined in white). Neuronal area was calculated as the area occupied by HuC/D$^+$ cells (as shown in N, outlined in white). *n*=5 samples per group. (C,F,J,K,O) Data are mean ± s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001 (Student's *t*-test, except one-way ANOVA in C). Scale bars: 100 µm.

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).

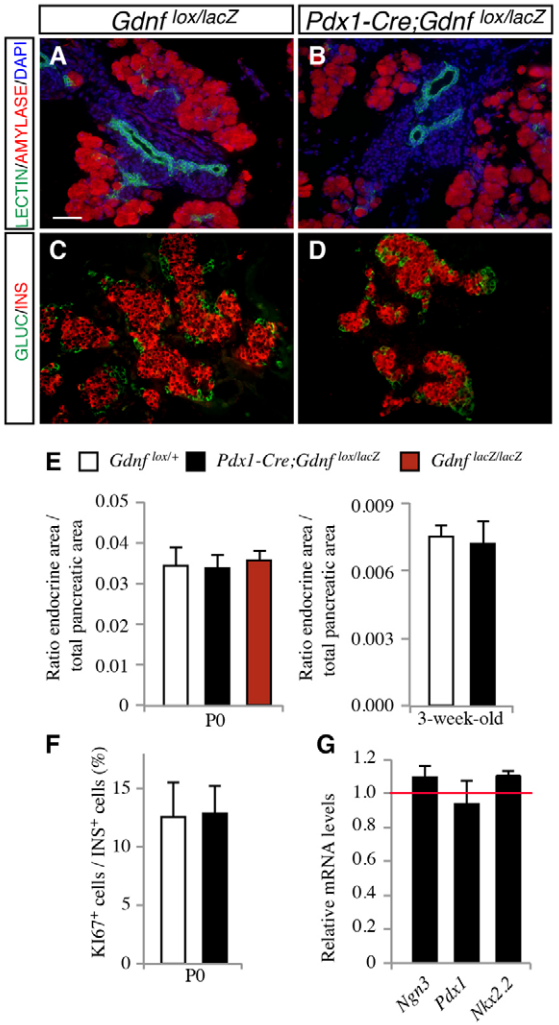

Fig. 6. *Gdnf* is dispensable for pancreas formation. (**A-D**) Normal exocrine (A,B) and endocrine (C,D) formation in *Gdnf* mutant mice at P0. (**E**) No differences in islet area between control and *Gdnf* mutant mice were found at P0 and at 3 weeks of age. (**F**) β-cell proliferation is unaffected in *Gdnf* mutant mice at P0. (E,F) Data are mean ± s.e.m.; *n*=3 and *n*=5 samples per group for P0 and 3-week measurements, respectively. (**G**) Quantitative RT-PCR analysis of the expression of endocrine transcription factors in E15.5 pancreas. Results show relative expression levels as ratios of normalized mean gene expression in *Pdx1-Cre; Gdnf*$^{lox/lacZ}$ mice compared with *Gdnf*$^{lacZ/+}$ heterozygous mice (*n*=3 animals from each genotype). Data are mean ± s.e.m. Control values are adjusted to 1 to facilitate comparison. Scale bar: 50 μm (in A for A-D).

We then examined whether GDNF induced proliferation in a distinct population of cells within the embryonic pancreas epithelium. *Gfra1* appears to be preferentially expressed at the tips of the branching pancreatic epithelium (Fig. 7J). Further colocalization analysis showed substantial overlap between GFRα1 and the 'tip cell' marker CPA1 during embryonic pancreas formation (Fig. 7J,K). We then examined whether GDNF differentially regulated the proliferation of *Cpa1*-expressing cells. Consistent with previous *in vivo* studies, we observed a higher proliferation rate in CPA1$^+$ cells compared with CPA1$^-$ cells in pancreas explants (Fig. 7N). GDNF induced a marked increase in the proliferation of CPA1$^+$ cells, whereas it had no effect in CPA1$^-$ cells (Fig. 7L-N).

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*$^{lox/lacZ}$ 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-Diaz 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

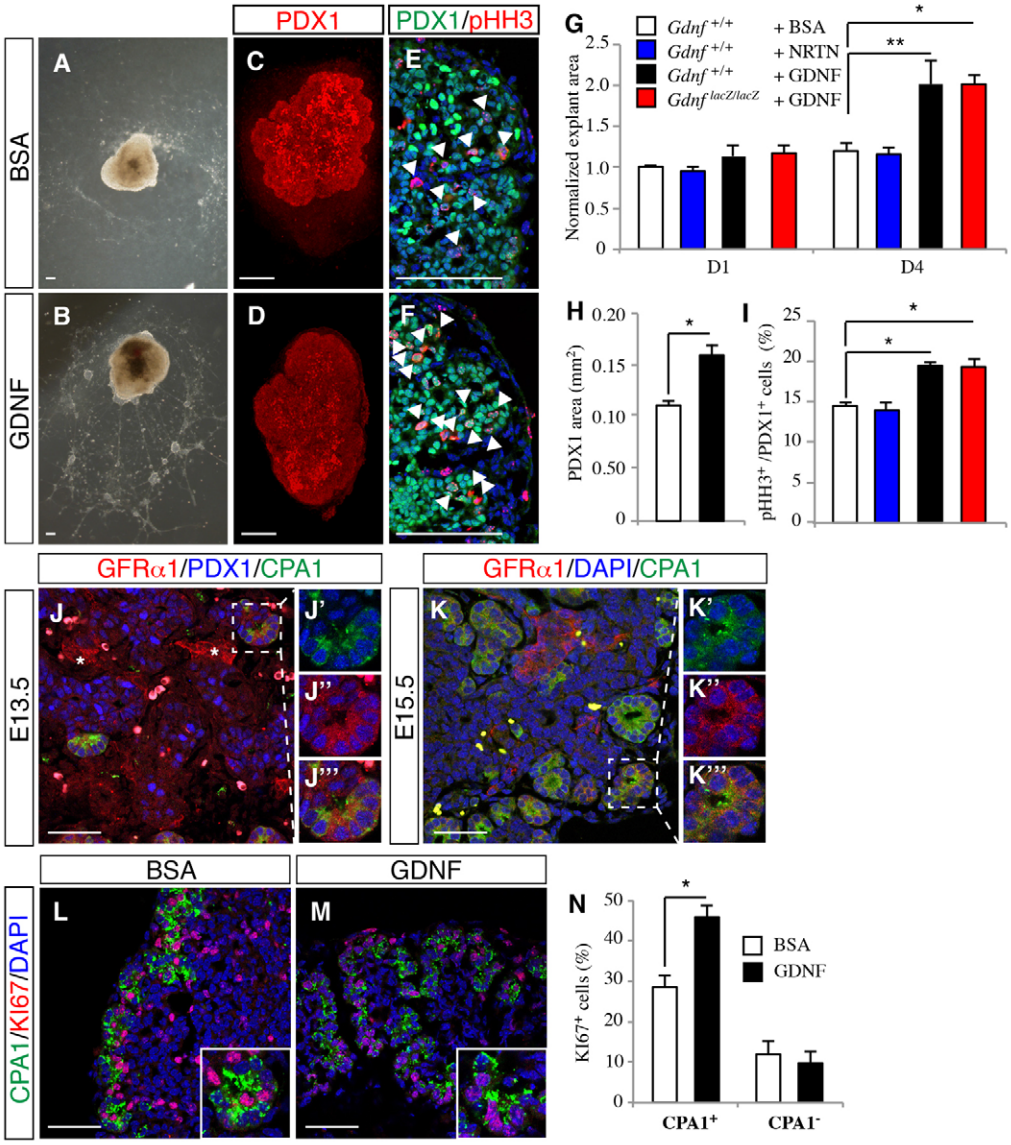

Fig. 7. GDNF promotes the proliferation of pancreatic progenitor cells. (**A**,**B**) GDNF increases organ size in E12.5 pancreas explants after 4 days of culture. (**C**,**D**) Maximum projection confocal images of PDX1 whole-mount staining on pancreas explants treated with BSA (C) and GDNF (D) for 4 days. (**E**,**F**) Proliferation of *Pdx1*-expressing cells is increased in GDNF-treated pancreas explants as shown by pHH3 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,I). Scale bars: 100 µm in A-F; 50 µm in J-M.

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*;*Gdnf*$^{lox/lacZ}$ 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*;*Gdnf*$^{lox/lacZ}$ 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

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.M.-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 dicussion 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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