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Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis

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SUMMARY

The importance of mesenchymal-epithelial interactions for the proper development of the pancreas has been acknowledged since the early 1960s, even though the molecule(s) mediating this process have remained unknown. We demonstrate here that *Fgf10*, a member of the fibroblast growth factor family (FGFs), plays an essential role in this process. We show that *Fgf10* is expressed in the mesenchyme directly adjacent to the early dorsal and ventral pancreatic epithelial buds. In *Fgf10*$^{-/-}$ mouse embryos, the evagination of the epithelium and the initial formation of the dorsal and ventral buds appear normal. However, the subsequent growth, differentiation and branching morphogenesis of the pancreatic epithelium are arrested; this is primarily due to a dramatic reduction in the proliferation of the epithelial progenitor cells marked by the production of the homeobox protein PDX1. Furthermore, FGF10 restores the population of PDX1-positive cells in organ cultures derived from *Fgf10*$^{-/-}$ embryos. These results indicate that *Fgf10* signalling is required for the normal development of the pancreas and should prove useful in devising methods to expand pancreatic progenitor cells.

Key words: FGF10, Pancreas, Progenitor cells, Mesenchymal-epithelial interactions, Proliferation, Mouse

INTRODUCTION

The pancreas originates from the dorsal and ventral regions of the foregut endoderm directly posterior to the stomach. The first indication of pancreatic morphogenesis occurs in mice at 15-25 somites or embryonic day (E) 8.5-9.5, when the endoderm evaginates to form buds (Golosow and Grobstein, 1962; Wessels and Cohen, 1967). Subsequently the mesenchyme condenses around the underlying endoderm and the epithelial buds grow in size accompanied by the differentiation of cell types that will constitute the functional units of the pancreas (Pictet et al., 1972); exocrine cells maintain epithelial characteristics and form branched ducts and acini; endocrine cells detach from the epithelial buds and migrate towards the mesenchyme and cluster together to eventually form islets (Slack, 1995). Cell marking and chimeric mouse studies have shown that these differentiated exocrine and endocrine cell types are not determined by lineage, but instead arise from a common population of progenitor endoderm cells (Deltour et al., 1991; Herrera, 2000; Jensen et al., 2000a; Percival and Slack, 1999). Several studies suggest that *Pdx1*, a homeodomain transcription factor expressed in all epithelial cells in early pancreatic buds, marks this pluripotent cell population (Gradwohl et al., 2000; Krapp et al., 1998). Consistent with this notion, evagination of the endoderm occurs in *Pdx1* null mice, however morphogenesis and differentiation of the pancreatic buds is arrested (Offield et al., 1996; Jonsson et al., 1994). Embryonic tissue recombination studies suggest a cell-autonomous role for *Pdx1* in the epithelium to impart competence to respond to mesenchymal signals (Ahlgren et al., 1996).

Inductive signals originating in the mesenchyme have been shown to play an essential role in the development of the pancreatic epithelium (Golosow and Grobstein, 1962; Wessels and Cohen, 1967). These classic studies use recombined embryonic tissues to show that pancreatic buds could develop in vitro, but that isolated epithelium would not undergo any growth or morphogenesis in the absence of mesenchyme. Recent studies suggest that the default state of the epithelium is to form islets, and mesenchyme provides an instructive signal for differentiation of exocrine cells (Gittes et al., 1996; Miralles et al., 1998). While these in vitro experiments have been useful in assessing the role of mesenchyme and various growth factors in pancreatic development, they do not allow for the identification of endogenous signalling molecules. In addition, in these experiments the epithelial tissue was isolated after having had extended contact with the mesenchyme, so there was the possibility of an early involvement of

mesenchyme in the formation and development of the pancreatic epithelial buds, which was not examined.

In this study, we used genetic methods to reveal an essential role for mesenchyme at the earliest stages of pancreatic bud outgrowth. We show that the secreted growth factor, *Fgf10* is expressed in the mesenchyme at stages that coincide with the rapid growth of epithelial buds. Using *Fgf10*$^{-/-}$ embryos, we provide evidence that *Fgf10* signalling regulates proliferation of, and, therefore, the size of, the epithelial progenitor cell population marked by PDX1. Loss of this pancreatic progenitor pool led to abnormal differentiation and morphogenesis of the pancreas.

MATERIALS AND METHODS

Generation and genotyping of *Fgf10* mutant mice

Targeted disruption of *Fgf10*, and genotyping of offspring by PCR analysis of genomic DNA have been described previously (Sekine et al., 1999). All mice were bred on a C57BL/6 genetic background.

RNA in situ and immunohistological analysis

Gastrointestinal tract that included the lung, stomach, pancreas and duodenum were dissected and fixed by immersion in either 4% neutral buffered paraformaldehyde (for RNA in situ analysis and TUNEL) or in Bouin's fixative (for immunohistochemistry and bromodeoxyuridine (BrdU) detection). Whole-mount in situ hybridization was performed using standard protocols. The *Pdx1* probe was provided by Dr C. Wright. Unless otherwise noted, gastrointestinal tracts were oriented so that sections were cut along the anterior to posterior axis. For general histology, sections were stained with Haematoxylin and Eosin. Immunofluorescence analysis was performed on 6 μm paraffin sections essentially as described previously (Miralles et al., 1998). The primary antibodies used in this assay were the following: guinea pig anti-insulin, diluted 1:200 (DAKO); mouse anti-glucagon, diluted 1:2000 (Sigma); rabbit anti-carboxypeptidaseA, diluted 1:200 (Biogenesis); mouse anti-pan-cytokeratin derived from the PCK-26 hybridoma, diluted 1:50 (Sigma); rabbit anti-PDX1, diluted 1:500 (gift from J. Habener); mouse anti-ISL1 (39.4D5) diluted 1:10 (Developmental Hybridoma Bank). The secondary antibodies used were diluted as follows: FITC-conjugated anti-rabbit, anti-guinea pig, diluted 1:200 (Jackson Laboratory); Rhodamine-conjugated anti-mouse, anti-rabbit, diluted 1:200 (Jackson Laboratory). TUNEL assay on paraffin sections was performed using a commercially available kit (Roche).

BrdU detection and cell counting

BrdU labelling was initiated by intraperitoneal injection (50 μg/g body weight) 30 minutes before sacrifice of the pregnant mother. Embryos were dissected and processed as described above. Double immunofluorescence analysis for PDX1/BrdU was performed; BrdU was revealed using anti-BrdU (Amersham). The number of PDX1-positive cells and PDX1-/BrdU-positive cells in the dorsal pancreatic buds were counted and the percentage of BrdU incorporated calculated (proliferative index). For cell quantification, four consecutive sections from each of four wild type and four *Fgf10* mutants were analyzed in this manner, giving a total of 32 data points. Statistical significance was determined using Student's *t*-test.

In vitro organ cultures

Gastrointestinal tract was dissected from E9.5 embryos and cultured in three-dimensional collagen gels with RPMI medium containing 1% calf serum. In some cases the medium was supplemented with 50 ng/ml human recombinant FGF10 (R&D) every 24 hours. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator. After 48 hours, the tissue was fixed in Bouin's fixative, embedded in paraffin wax and processed for immunohistology as described above.

RESULTS

In order to identify secreted growth factors expressed in the mesenchyme during the earliest stages of pancreatic development, E10.5 pancreatic mesenchyme was analyzed by RT-PCR. *Fgf10* was expressed at high levels, leading us to characterise its expression in detail by in situ hybridisation. *Fgf10* is expressed at E9.5 in two thin stripes in the posterior foregut, the area of the gut tube where the pancreatic buds form (Fig. 1A). Within the anterior foregut, expression is also observed where the lung buds form (Bellusci et al., 1997). In both regions, *Fgf10* expression was restricted to the mesenchyme that had condensed around the developing gut tube. At E10.5, *Fgf10* expression had expanded to a broad area within the mesenchyme surrounding the developing pancreatic buds and caudal stomach but not in the rostral duodenum (Fig. 1B). Examination of sections of E10.5 gut tube confirmed that the expression of *Fgf10* was restricted to the mesenchyme and surrounded both pancreatic epithelial buds (Fig. 1C,D). By E11.5, *Fgf10* was still expressed at high levels in the mesenchyme located directly above the growing dorsal epithelial bud, but only low levels were detected around the ventral bud (Fig. 1E). By E12.5, virtually no *Fgf10* expression was detected around either bud (not shown). Fgf10 expression was not detected in the pancreas during later stages of embryogenesis, although FGF10 expression has been reported in the adult islet cells (Hart et al., 2000). Thus during embryogenesis, *Fgf10* is transiently expressed in pancreas-associated mesenchyme during early stages of epithelial bud formation.

The spatially and temporally specific expression of *Fgf10* in the mesenchyme surrounding the growing epithelial buds led us to examine a role for *Fgf10* in the development of the pancreas by analyzing mice in which *Fgf10* had been inactivated by homologous recombination in ES cells (Min et al., 1998; Sekine et al., 1999). In E17.5 wild-type embryos, the pancreas consisted of prominent glandular tissue near the spleen that derives from the dorsal bud. The ventral bud contributed to the diffuse multilobulated tissue along the duodenal region (Jensen et al., 2000b) (Fig. 2B,C). Histological analysis of the pancreatic tissue at this stage allowed identification of exocrine acini cells arranged in grape-like clusters as well as some heavily nucleated spheroidal clusters of endocrine islets (Fig. 2D). These islet cells can be more clearly distinguished by immunostaining for endocrine hormones: a classic pattern of insulin-expressing cells at the center of the islet cluster surrounded by glucagon-expressing cells on the periphery of the islet structure (Fig. 2E). The acinar cells, in contrast, expressed carboxypeptidaseA, a marker of differentiated exocrine cells (Fig. 2F). E17.5 *Fgf10* mutant littermates had an apparently normal gastrointestinal tract except for the stomach and pancreatic areas, which were reduced in size (Fig. 2A,G). The spleen, an organ that derives from dorsal mesenchyme was present in mutant embryos (Fig. 2G). Analysis of the pancreas in the *Fgf10* mutant embryos revealed rudimentary glandular tissue in both splenic and duodenal regions, suggesting that the pancreatic tissue

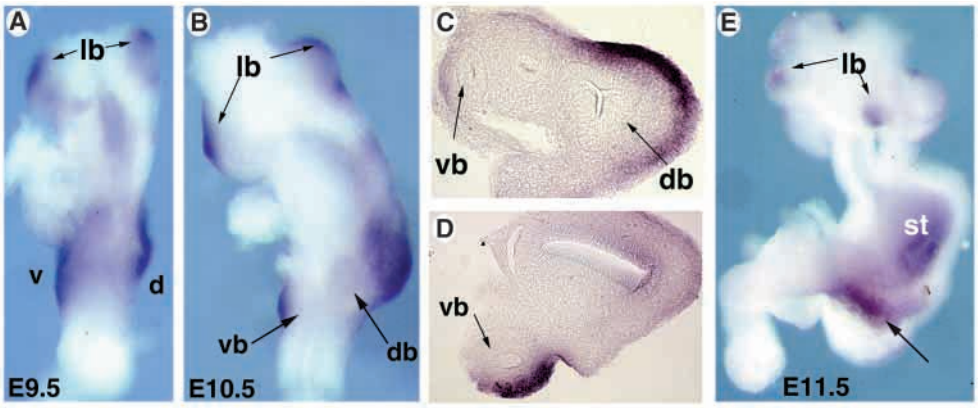

Fig. 1. *Fgf10* is expressed in the pancreatic mesenchyme during the early stages of pancreatic organogenesis. (A,B,E) Whole-mount in situ hybridisation on dissected gastrointestinal tracts (dorsal is to the right) showing (A) *Fgf10* expression in the gut dissected from an E9.5 embryo. The first signs of *Fgf10* expression in the posterior foregut were two distinct stripes where the dorsal and ventral pancreatic buds emerge. Expression of *Fgf10* in the foregut anterior to the pancreas was also observed in the lung buds. (B) By E10.5, the dorsal epithelial bud was clearly visible and *Fgf10* was expressed broadly in the surrounding mesenchyme. Expression of *Fgf10* also extended into the posterior stomach mesenchyme. (C,D) Vibratome sections (20 μm) of the gut from the E10.5 embryo shown in B demonstrates that *Fgf10* expression is confined to the mesenchyme adjacent to the dorsal (C) and ventral (D) bud. (E) *Fgf10* continued to be expressed at E11.5 in a restricted area in the dorsal mesenchyme (indicated by an arrow). The uniform weak staining observed in the stomach epithelium was due to the trapping of the in situ probe in the lumen of the stomach. d, dorsal; v, ventral; db, dorsal bud; vb, ventral bud; lb, lung bud; st, stomach.

had derived from both buds (Fig. 2H,I). The rudimentary pancreatic tissue was devoid of any spheroidal islet clusters, although some well-formed acinar tissue containing zymogen granules was clearly evident (Fig. 2J). Immunohistological analysis of the pancreatic tissue revealed a few scattered glucagon and insulin-positive cells confirming the lack of islet formation in the *Fgf10* mutant pancreas. The insulin-positive cells often co-stained for glucagon (Fig. 2K) suggesting that the few insulin cells that do differentiate are abnormal. The acinar tissue by contrast, stained for carboxypeptidaseA, indicating that exocrine cell differentiation and morphogenesis occur in the mutant pancreas, although in drastically reduced amount compared to the wild-type littermate (Fig. 2I).

The reduced size of the posterior foregut area where the pancreas forms suggests that early deficiencies in the formation of the foregut may account for the observed phenotype. To investigate the early stages in foregut epithelial development, we examined the expression of *Hnf3β*, a member of the winged helix family of transcription factors. *Hnf3β* is expressed in the developing gut tube endoderm and serves as a marker for all budding epithelial tissue (Ahlgren et al., 1996). Whole-mount in

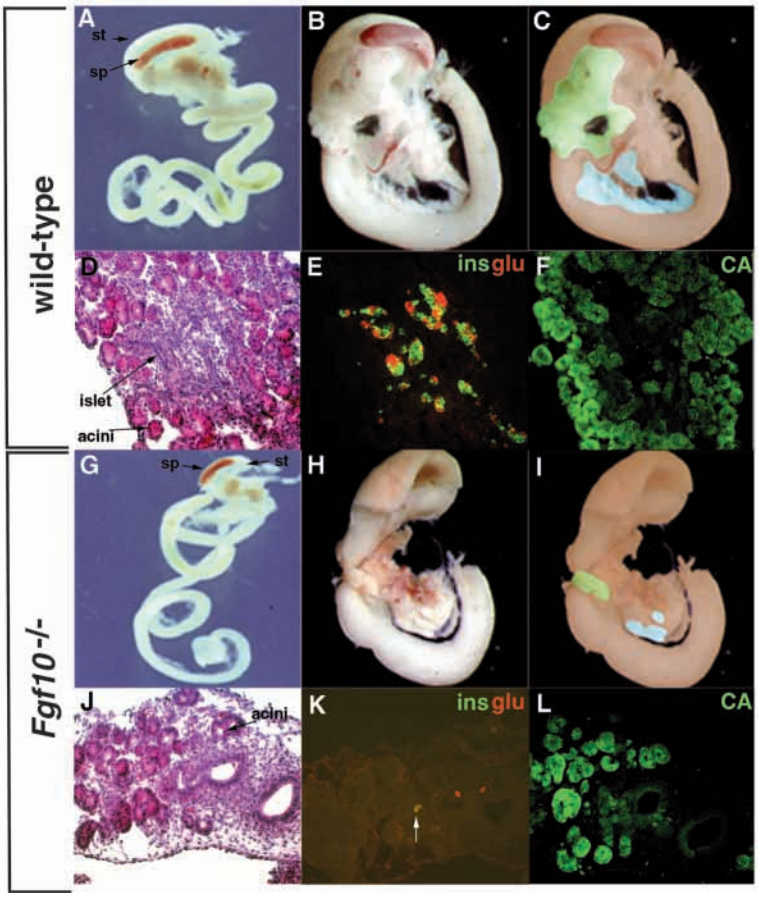

Fig. 2. Pancreatic hypoplasia and absence of islet cells in *Fgf10*$^{-/-}$embryos. (A) Gastrointestinal tract from an E17.5 wild-type embryo. (B) Gross appearance of pancreatic region dissected from an E17.5 wild-type embryo and (C) a schematic representation illustrating the pancreas. The pancreatic tissue at this stage is localised near the spleen, which derived from the dorsal bud (green), and along the duodenum, which derived from the ventral bud (blue). (D) Haematoxylin and Eosin staining of the pancreas tissue from an E17.5 wild-type embryo showing the presence of acini exocrine tissue and heavily nucleated clusters of islet cells. (E) The islet clusters express insulin (green) and glucagon (red). (F) Exocrine tissue expresses carboxypeptidaseA (green). (G) The gastrointestinal tract from E17.5 *Fgf10*$^{-/-}$ embryos were overtly similar to that of wild-type littermates except for a smaller stomach. (H) The pancreatic tissue in the mutant *Fgf10* embryos was drastically reduced (I) although present in both the splenic (green) and duodenal (blue) locations. (J) Haematoxylin and Eosin staining shows the presence of acinar tissue but no islet clusters are evident. (K) Scattered insulin (green) and glucagon-expressing (red) cells are present. Co-expression of insulin and glucagon indicates that these endocrine cells are immature (arrow). (L) The acini from the mutant *Fgf10* embryo stained for the exocrine marker, carboxypeptidaseA. CA, carboxypeptidaseA; ins, insulin; glu, glucagon; st, stomach; sp, spleen.

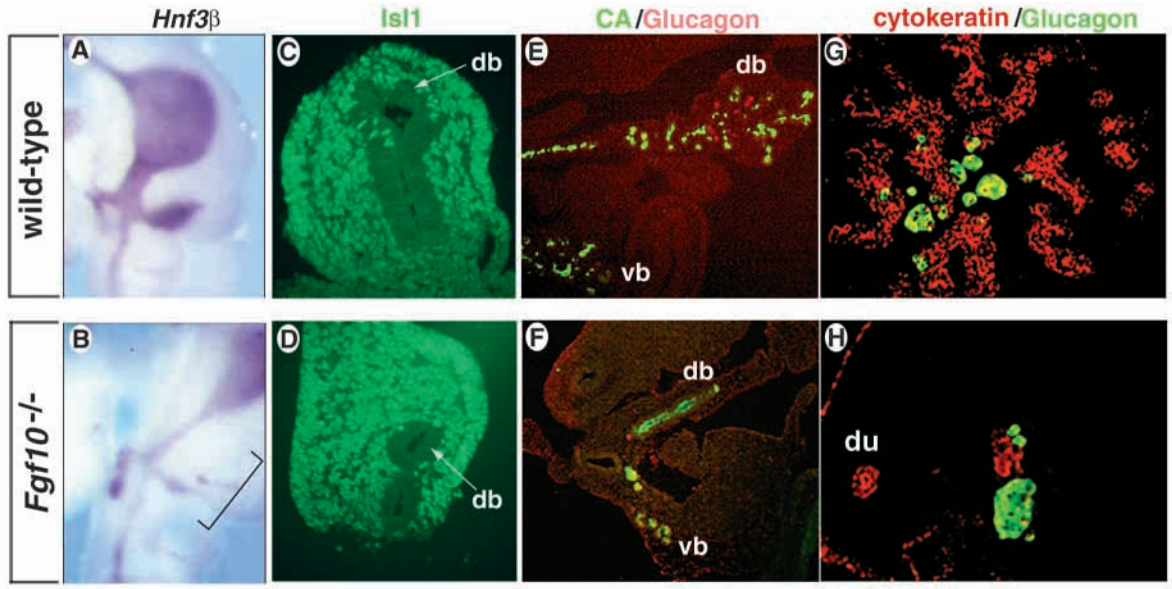

Fig. 3. The size of the pancreatic epithelium in *Fgf10*$^{-/-}$ embryos is greatly reduced. (A) *Hnf3β* whole-mount in situ hybridisation to the epithelium of the foregut of wild-type E11.5 embryos. (B) *Hnf3β* expression in the pancreatic region and posterior stomach was greatly reduced in the foregut of *Fgf10* mutant embryos. (C) ISL1 was expressed predominantly in the dorsal mesenchyme and a few differentiating endocrine cells in the pancreatic epithelium from E10.5 wild-type embryos. The dorsal bud is oriented to the top. (D) Strong expression of ISL1 was observed in the dorsal mesenchyme of *Fgf10*$^{-/-}$ embryos at E10.5, although very few scattered cells within the epithelium that expressed ISL1 were detected. (E) Sagittal sections of the dissected gut from wild-type embryo stained with carboxypeptidaseA (green) and glucagon (red). The branched morphology of both dorsal and ventral pancreatic buds is evident. (F) Sagittal section of dissected gut from an *Fgf10*$^{-/-}$ embryo shows that the formation of both dorsal and ventral buds occurred, however, no branching of the epithelium is apparent. (G) Transverse sections of the dorsal bud of E13.5 wild-type embryo stained for the pan-epithelial markers cytokeratin (red) and glucagon (green). The dorsal pancreatic bud of a wild-type embryo shows a characteristic highly branched epithelium. (H) The dorsal bud of the *Fgf10*$^{-/-}$ embryo has a small pancreatic epithelium and no branching of the epithelium is visible. Differentiation of early endocrine cells, as identified by glucagon staining, occurred in the mutant embryos and these cells are seen typically clustered together. db, dorsal pancreatic bud; vb, ventral pancreatic bud; ISL1, Islet1; CA, carboxypeptidaseA; du, duodenum.

situ hybridization on E11.5 gastrointestinal tracts confirmed that *Hnf3β* is expressed in the epithelial tissue of the stomach, pancreatic buds and the duodenum (Fig. 3A). In *Fgf10*$^{-/-}$ embryos, the epithelial tissue around a limited area of the posterior foregut, including the caudal portion of the stomach and the pancreatic buds, was greatly reduced; the epithelial tissue from other parts of the gut, such as anterior stomach and duodenum, appeared to form normally (Fig. 3B, shown partially). This area of reduced epithelial tissue in the mutant embryos corresponded to the domain of *Fgf10* expression in

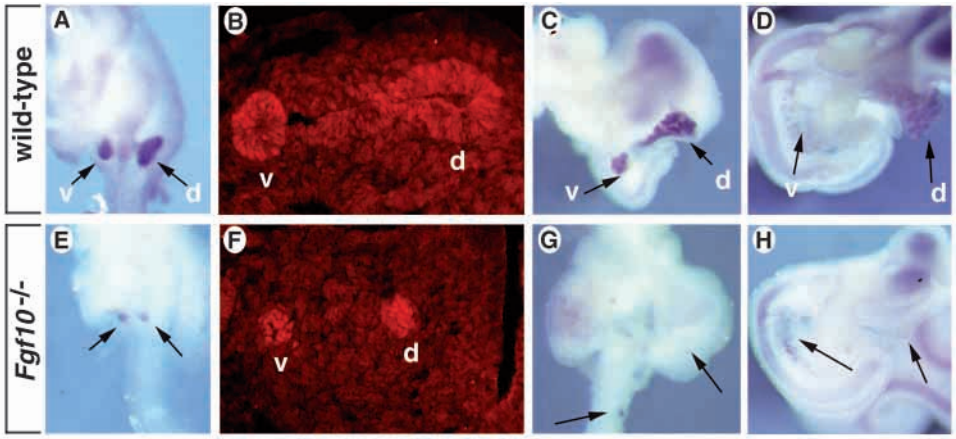

Fig. 4. *Fgf10*$^{-/-}$ embryos have a small pancreatic primordium because the *Pdx1*-expressing epithelial progenitor cell population is not maintained. Dorsal is to the right. (A) *Pdx1* is expressed uniformly in undifferentiated cells throughout the developing pancreatic buds in wild-type embryos at E10.5 (B) Immunofluorescence analysis of PDX1 expression in transverse section of an E10.5 wild-type embryo shows the buds emerging from the foregut. (C) By 12.5, *Pdx1* expression is no longer uniform (due to differentiation of precursor cells) and the branching of the epithelium is evident in the dorsal bud. (D) By E13.5, *Pdx1* expression is increasingly restricted within the epithelium and accentuates the lobulated structure of both the pancreatic buds. As compared to the wild-type littermates the *Pdx1* expression in the *Fgf10*$^{-/-}$ embryos at E10.5 (E) identifies the formation of two small but distinct pancreatic buds. (F) Immunofluorescence analysis confirmed the reduced expression of PDX1 in these two pancreatic buds in mutant embryos. *Pdx1* expression is no longer observed later in development at E12.5 (G) and E13.5 (H). Arrows indicate the area within the gut where the pancreatic buds normally form. Occasionally, some weak expression of *Pdx1* was observed in the ventral bud of mutant embryos (H). v, ventral; d, dorsal.

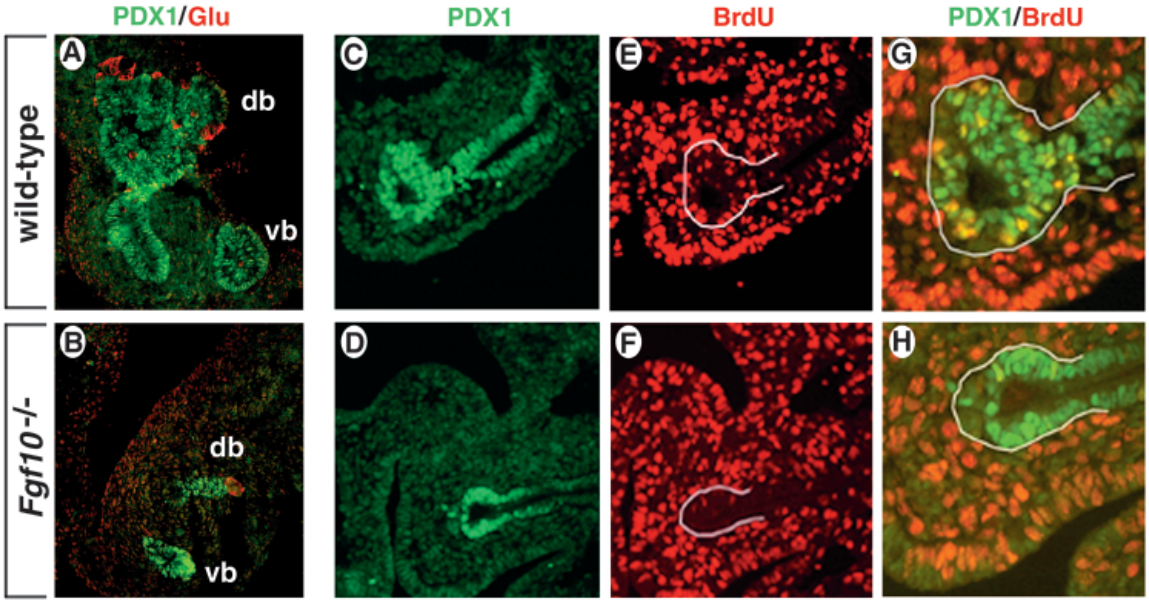

Fig. 5. The smaller pancreatic epithelium in the *Fgf10* mutants was primarily due to the decreased proliferation of progenitor cells that are marked by expression of PDX1. (A,B) Immunofluorescence analysis for the expression of PDX1 (green) and glucagon (red) in the pancreatic epithelium of E11.5; (A) wild-type littermate and (B) $Fgf10^{-/-}$ embryo. The pancreatic epithelium in the *Fgf10* mutant embryo is reduced in size, although no concomitant increase in glucagon expression is evident. In addition, no glucagon-positive cells are evident in the ventral bud of the $Fgf10^{-/-}$ embryo. (C-H) Analysis of proliferating precursor epithelial cells in the dorsal bud of $Fgf10^{-/-}$ (D,F,H) and wild-type (C,E,G) littermate embryos at E10.5. PDX1 labelling identifies the dorsal pancreatic epithelium, which is smaller in $Fgf10^{-/-}$ (D) compared to wild-type littermates (C). (F) BrdU labelling shows very few proliferating cells within the dorsal pancreatic epithelium of $Fgf10^{-/-}$ embryos. (E) In wild type a large fraction of dorsal pancreatic epithelial cells have gone through S-phase and stained BrdU positive. The outline designates the boundary of the dorsal epithelial bud. (G,H) Merged images show double labelling for PDX1 and BrdU and confirm that the proliferating epithelial cells also express PDX1 (magnified 2×). To quantify the difference in proliferation of precursor epithelial cells between $Fgf10^{-/-}$ embryos and their wild-type littermates, four consecutive sections from each of four wild-type and four *Fgf10* mutants were used to calculate the proliferative index ($BrdU^{+}/PDX1^{+}$)×100. Using such analysis, wild-type embryos displayed an average proliferative index of 50.4±3.8 (n=4) as compared with 15.1±2.9 (n=4) for $Fgf10^{-/-}$ embryos.

the surrounding mesenchyme observed in wild-type embryos. To examine the state of the mesenchyme surrounding the posterior foregut region, the expression of ISL1, a homeodomain protein and a dorsal mesenchyme marker, was analyzed at E10.5. At this stage, ISL1 is expressed throughout the dorsal mesenchyme of the posterior foregut and in a few endocrine cells within the pancreatic epithelium (Ahlgren et al., 1997) (Fig. 3C). The general architecture of the

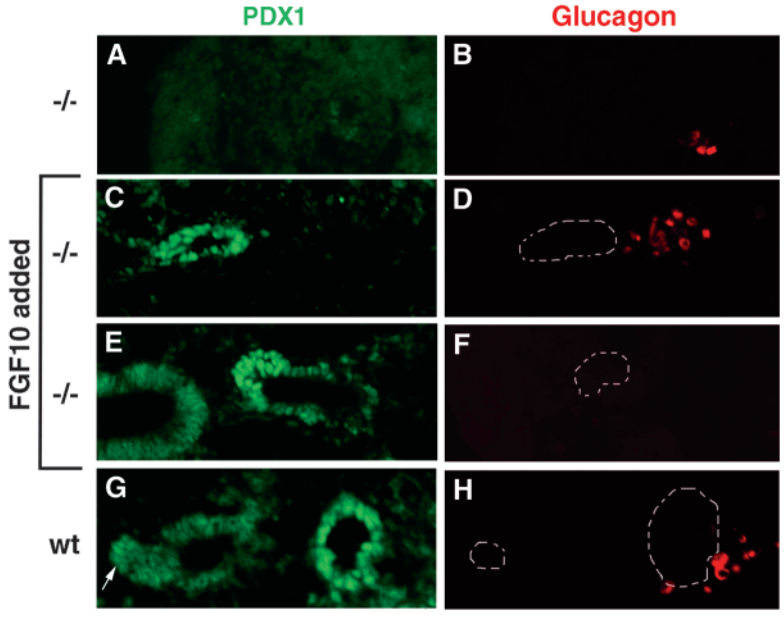

Fig. 6. Exogenously added FGF10 can rescue the PDX1-expressing pool of epithelial cells in cultured pancreas derived from *Fgf10* mutants. Consecutive sections of explanted cultured tissue were stained for PDX1 (green) and glucagon (red). (A) Cultured explanted tissue derived from an *Fgf10* mutant stained for PDX1. Very few stained cells were detected. (B) Section adjacent to A showing that glucagon-positive cells were present. (C) Explanted tissue derived from *Fgf10* mutants, cultured with 50 ng/ml FGF10 and stained for PDX1. The PDX1-positive cells are present in the bud outgrowth from the foregut epithelium. (D) Glucagon-expressing cells in an adjacent section to C. (E) An additional population of PDX1-positive cells detected in the explanted tissue from *Fgf10* mutants that was cultured with FGF10. (F) No glucagon-positive cells were detected in the section adjacent to that in E. (G) Cultured explanted tissue derived from a wild-type littermate and stained for PDX1. The arrow indicates an additional population of PDX1-stained cells. (H) Glucagon-positive cells in the section adjacent to that in G. The white dashed lines in D, F and H indicate the position of the PDX1-positive cells in C, E and G, respectively.

mesenchyme and the expression of ISL1 were unaffected in *Fgf10*$^{-/-}$ embryos (Fig. 3D). These results indicate that FGF10 signalling, which originates in the mesenchyme, targets the adjacent epithelium. This hypothesis is consistent with the epithelial localization of FGFR2b, a receptor that can mediate FGF10 signalling during early pancreatic development (Finch et al., 1995; Miralles et al., 1999; Orr-Urtreger et al., 1993) (and data not shown).

We next explored the morphology and the differentiation that occurred in the limited pancreatic epithelium that formed in *Fgf10*$^{-/-}$ embryos. By E13.5, in wild-type embryos, the epithelium undergoes morphogenetic movements referred to as branching morphogenesis that leads to the formation of a ductal network in the pancreas. Differentiation of exocrine cells and some endocrine cells is apparent at this stage although most mature endocrine cells appear after E14.5. We have utilised the expression of carboxypeptidaseA to identify differentiated exocrine cells and as a marker for the branching pattern of the epithelium within the pancreatic bud. Sagittal sections across both buds of wild-type littermates displayed the typical branching of the pancreatic epithelium (Fig. 3E). The staining pattern suggests that the epithelium initially elongates before undergoing repeated branching to form a larger network of branched epithelium. Similar sections from *Fgf10* null embryos showed that both dorsal and ventral buds had formed; the elongation of the epithelium was initiated, however this epithelium lacked branched structures (Fig. 3F). To further investigate the morphology of the pancreatic epithelium, we utilised a pan-epithelial marker, cytokeratin. Transverse sections of dissected E13.5 gastrointestinal tracts were analyzed for cytokeratin expression by immunofluorescence and co-stained with glucagon to localise the pancreatic epithelium (Fig. 3G,H). Dorsal buds of wild-type embryos had dispersed glucagon cells that had migrated away from the highly branched epithelium. In contrast, very little epithelium formed in the dorsal bud of *Fgf10* mutant embryos and this formed a single tube lacking any branched structure (Fig. 3H). Differentiated glucagon-positive endocrine cells were evident in the mutant pancreas, however, unlike in the wild type, these cells were typically clustered together. Thus, in the *Fgf10*$^{-/-}$ embryos, formation of dorsal and ventral buds are initiated, as is the differentiation of both endocrine and exocrine cells. However, the pancreatic epithelium is significantly reduced in size and fails to undergo branching.

The severe reduction of the pancreatic epithelium in the *Fgf10*$^{-/-}$ embryos suggests a defect in the formation of the epithelial progenitor cells. Epithelial progenitor cells can be distinguished by the expression of *Pdx1*, which is initiated when the foregut endoderm is committed to a pancreatic fate. *Pdx1* continues to be expressed uniformly in all epithelial cells of the early developing pancreatic buds (Ahlgren et al., 1996; Guz et al., 1995; Jonsson et al., 1994). At E10.5, *Pdx1* was expressed throughout the pancreatic epithelium in both buds: the dorsal bud was elongated and larger than ventral bud (Fig. 4A,B). At later stage (E12.5), *Pdx1* continued to be expressed in the pancreas when branching of the dorsal bud epithelium is discernible (Fig. 4C). Weaker *Pdx1* expression was also observed in the regions flanking the pancreatic buds, namely the caudal portions of the stomach and the rostral portion of the duodenum. By E13.5, *Pdx1* expression highlighted the lobulated structures of both dorsal and ventral pancreatic buds (Fig. 4d). In *Fgf10*$^{-/-}$ embryos, *Pdx1* expression at E10.5 was significantly reduced, nevertheless, distinct dorsal and ventral sites of expression were clearly observable (Fig. 4E). Transverse sections of E10.5 embryos analyzed for PDX1 expression confirmed the formation of two buds that contained far fewer PDX1-positive cells compared to the wild-type littermates' pancreas (Fig. 4F). At later stages in *Fgf10*$^{-/-}$ embryos, *Pdx1* expression was no longer observed in the dorsal pancreatic region, although very weak expression of *Pdx1* was observable in the ventral pancreas (Fig. 4G,H). Thus the specification of PDX1-positive cells occurred in the absence of *Fgf10*. However, the maintenance of PDX1-positive cells was clearly dependent on FGF10 signalling from the mesenchyme. These results imply a requirement for FGF10 signalling in maintaining/expanding the progenitor cell population marked by PDX1 during the early stages of pancreatic organogenesis.

We investigated a number of possibilities by which FGF10 could maintain/expand the pancreatic epithelial progenitor population. FGF10 could act as a factor required to keep the epithelial progenitors in an undifferentiated state, as a survival factor required to inhibit apoptosis or as a mitogenic factor required to simulate proliferation. To investigate these possibilities, we first assessed whether FGF10 keeps the epithelial progenitors in an undifferentiated state. If this were the case, absence of FGF10 would result in premature differentiation of the precursor epithelial cells that would deplete the progenitor pool of cells. The earliest differentiated cells to emerge during normal pancreatic development are glucagon-expressing cell (Pictet and Rutter, 1972) and increased early differentiation of precursor epithelial cells would result in an excessive number of glucagon-positive cells at the expense of PDX1-expressing cells (Apelqvist et al., 1999; Jensen et al., 2000b). While glucagon-positive cells during normal pancreatic development are readily discernible in the dorsal bud as early as E9, ventral bud expression begins only around E11. To look for premature differentiation in both pancreatic buds, we analyzed E11.5 embryos for the expression of glucagon and PDX1 expression (Fig. 5A). As already shown (Fig. 4), few PDX1-positive cells were observed in the dorsal buds of the *Fgf10*$^{-/-}$ embryos, however, no concomitant increase in glucagon-positive cells was evident (Fig. 5B). Significantly, no precocious glucagon-positive cells were seen in the ventral bud of the *Fgf10*$^{-/-}$ embryo (Fig. 5B). Furthermore, no excessive expression of other early markers for endocrine cells, ISL1 and neurogenin 3 (Apelqvist et al., 1999; Jensen et al., 2000a; Schwitzgebel et al., 2000) were observed within the *Fgf10* mutant pancreatic epithelium consistent with absence of premature endocrine cell differentiation (Fig. 3D and data not shown). These observations indicate that premature differentiation of endocrine cells is unlikely to be the reason for the decreased number of PDX1-positive cells. These results argue against a role for FGF10 in inhibiting differentiation within the pancreas. We next asked whether FGF10 is required for pancreatic epithelial cell survival. Apoptosis could lead to depletion of cells, however, no increase in apoptosis was observed within the E10.5 *Fgf10*$^{-/-}$ pancreatic epithelium as determined by Tdl-mediated dUTP nick end labelling (TUNEL) assay (data not shown), indicating that progenitor cells, at least at this stage, were not lost by apoptosis.

We next examined whether FGF10 is required for the

proliferation of the epithelial progenitor cells by determining the extent of cell proliferation within the population of PDX1-positive cells. To assess proliferation levels, we assayed DNA synthesis in the pancreatic epithelium of E10.5 embryos by BrdU incorporation. In pancreatic epithelium from wild-type embryos, a high percentage of PDX1-labelled cells along the rim of the growing bud adjacent to the mesenchyme had gone through S-phase and were, therefore, BrdU positive (Fig. 5C,E,G). In contrast, very few PDX1-positive cells had incorporated BrdU in the pancreatic epithelium from *Fgf10*$^{-/-}$ mutant embryos (Fig. 5D,F,H). This defect in proliferation was restricted to the epithelium since BrdU incorporation in the mesenchyme of *Fgf10*$^{-/-}$ embryos did not appear to be affected (Fig. 5E,F). To quantify the proportion of proliferating pancreatic epithelial cells, the number of nuclei, out of all PDX1-positive cells, that had incorporated BrdU were calculated (see Materials and Methods). Calculations were based on four wild-type and four *Fgf10* mutant embryos, scoring four sections per embryo. We observed a 70% reduction in the proportion of proliferative PDX1-positive cells within the pancreatic epithelium of *Fgf10*$^{-/-}$ embryos as compared to wild-type littermates (Student's *t*-test, $P<0.0001$). Thus while we failed to observe an increase in apoptosis or any accelerated differentiation of pancreatic cells, we did detect a significant decrease in pancreatic progenitor cell proliferation, labelled by PDX1. This suggests that the reduction in the pancreatic epithelium observed in the *Fgf10* mutant embryos is likely to be a result of underproliferation of pancreatic epithelial cells.

Our results indicate that FGF10-driven proliferation is required to generate a quantitatively normal pool of epithelial progenitor cells and in the absence of *Fgf10*, PDX1-positive progenitor cells are lost. We investigated whether exogenously supplied FGF10 could rescue this pool of progenitor epithelial cells in *Fgf10* mutant embryos. The gastrointestinal tracts from E9.5 wild-type littermates and *Fgf10* mutant embryos were isolated and cultured in collagen gel with or without addition of recombinant FGF10 to the culture medium. After 2 days, the explanted cultured tissue were fixed, embedded in paraffin wax and processed for immunohistology for PDX1 and glucagon. No PDX1-positive cells were detected in cultured explants derived from *Fgf10* mutant embryos, although glucagon-positive cells were readily observed (Fig. 6A,B). However, a significant number of PDX1-positive cells was observed in explants derived from *Fgf10* mutant embryos cultured in the presence of FGF10. These PDX1-labelled cells formed an epithelial bud on one side of the duodenum (Fig. 6C); an adjacent section showed that glucagon-positive cells were present in the mesenchyme adjacent to the bud containing the PDX1-positive cells (Fig. 6D). An additional set of PDX1-positive cells was detected on the opposite side of the duodenum in these explanted tissue (Fig. 6E); however, no glucagon-positive cells were detected adjacent to these PDX1-positive cells (Fig. 6F). Cultured explants derived from wild-type embryos displayed similar PDX1-positive epithelial buds (Fig. 6G,H). Since differentiation of glucagon-expressing cells normally occurs in the dorsal bud approximately 2 days before it does in the ventral bud, the two populations of PDX1-positive cells we observe in vitro could represent the two different buds. These results demonstrate that in organ cultures, soluble FGF10 is capable of rescuing the epithelial progenitor cells in the *Fgf10* mutant embryos.

DISCUSSION

The mechanisms involved in regulating the development and function of the pancreas have garnered renewed attention in the last few years. Mice with targeted disruptions of genes involved in transcriptional regulation and cell-cell interactions within the endoderm have provided insight on cell fate determination within the pancreas (Edlund, 1998; Edlund, 1999). More recently, several studies have advanced our understanding of the role of inductive signalling between mesoderm-derived tissues in regulating pancreatic endoderm (Kim and Hebrok, 2001). These studies have primarily focused on the relatively early role of notochord and cardiac tissue that regulate the competence of the endoderm to form pancreas and specify its stereotyped position along the foregut (Deutsch et al., 2001; Kim et al., 1997). These events occur before the mesenchyme condenses around the gut tube and although several classic studies pointed to the importance of mesenchyme-epithelial signalling for the development of the pancreas, the molecular characterization of these signalling events has not been done.

We have presented evidence here that FGF10 is a key signalling molecule involved in the mesenchymal-epithelial interaction that is required during the early stages of pancreatic development. In *Fgf10* null mutants, the initiation of both dorsal and ventral pancreatic buds, as indicated by expression of PDX1, occurs normally. This is consistent with the fact that the onset of PDX1 expression and the evagination of the epithelium occurs prior to the existence of any condensed mesenchyme (Kim et al., 1997; Slack, 1995; Wessels and Cohen, 1967). Our data demonstrate that *Fgf10* is required to promote the proliferation of this initial population of PDX1-labelled progenitor cells within the developing buds. In the *Fgf10* mutants, these cells within the pancreatic buds show a marked decrease in proliferation, which is likely to account for the failure in the growth of the pancreatic bud. While fewer differentiated cells were observed in the *Fgf10* mutants, the expression of endocrine and exocrine markers indicates that the differentiation of the progenitor cells is not directly affected. This is consistent with the idea that cell-cell interactions within the endoderm control cell fate determination and suggests that these signals are still active in the *Fgf10* mutants (Apelqvist et al., 1999). In the absence of the expansion of the progenitor pool in the *Fgf10* mutants, this differentiation would lead to a progressive reduction in the number of progenitor cells available for differentiation and ultimately resulting in insufficient number of cells to generate the pancreas.

Our results suggest that the primary role of *Fgf10* during the formation of the pancreas is to regulate the proliferation of the pancreatic epithelial progenitor cells and hence the size of the pancreatic primordium. The size of the pancreatic primordium would affect subsequent events such as the morphogenesis and differentiation of the pancreatic epithelium. Several studies suggest that the emergence of differentiated cell types from the epithelial primordium may be spatially and temporally regulated. The endocrine cells that differentiate early do not contribute to islet structures and differ in nature from the later differentiating endocrine cells (Jensen et al., 2000a; Schwitzgebel et al., 2000). This phenomenon, referred to as 'secondary transition', occurs around E14 in mouse when the mature endocrine cells that form α and β cells, begin to emerge (Pictet and Rutter, 1972). In *Fgf10* mutants, the pancreatic

progenitor epithelial cells are depleted before secondary transition occurs and this may explain the lack of islet cells in the rudimentary pancreatic tissue of these mice. However, the few exocrine cells that differentiate from the limited progenitor epithelial cells are sufficient to form acinar structures in the mutant embryos. The exocrine cells are capable of proliferating even after differentiation and this may also account for the formation of acinar tissue (Pictet et al., 1972). Although we focused on the development of the pancreas, the expression of *Fgf10* in the mesenchymal layer adjacent to the posterior stomach suggests that *Fgf10* could play a similar role in the formation of the stomach. In fact, stomach mesenchyme was shown to have the same trophic effects as pancreatic mesenchyme (Percival and Slack, 1999). In stomachs of *Fgf10* mutants, columnar epithelium with mucin-negative vacuoles that are normally observed in the posterior stomach were absent (data not shown) indicating that FGF10 may also regulate the proliferation of these cells.

Until now our knowledge of the role of mesenchymal factors in the development of the pancreas, has been primarily based on in vitro manipulations by physically separating mesenchyme and epithelial layers. These experiments have shown that the mesenchyme can affect the cell fate choice of epithelial cells. Such analysis have led to the proposal that the default pathway for the embryonic pancreatic epithelium is to form islets and the mesenchyme is required for the formation of acinar structures (Gittes et al., 1996; Miralles et al., 1998). These studies describe a role of the mesenchyme that is distinct from the role of FGF10 signalling described here. In vivo, FGF10 signalling from the mesenchyme would occur when the mesenchyme first contacts the foregut epithelium and induces the expansion of the pancreatic epithelial bud. The in vitro approaches examine the role of the mesenchyme after the formation and expansion of the epithelium bud has occurred. Thus the *Fgf10* signalling from the mesenchyme would represent an early role for mesenchyme in the formation and development of the pancreatic epithelial buds that was not explored with the in vitro assays. Our results also seem to indicate that the mesenchyme is capable of affecting different facets of pancreatic development at different times. We speculate that the ability of the mesenchyme to elicit different responses from the epithelium relies on the temporally distinct expression of different signalling molecules in the mesenchyme.

Perturbation of FGF receptor activity can also provide important clues about the role of FGF signalling in the development of the pancreas. Mice deficient in FgfR2-IIIb have been reported to display dysgenesis of the pancreas (De Moerlooze et al., 2000). While detailed analysis of the pancreas was not reported, the gross morphological characterization suggests that the pancreatic phenotype of these embryos is milder than *Fgf10* mutants (Bradley Spencer-Dene and Clive Dickson, personal communication). FGF ligands and receptors are also expressed in the adult mouse pancreas and localised to the β-cells. Transgenic mice that express soluble dominant-negative forms of FGF receptors have been used to show the importance of FGF signalling for β-cell function (Hart et al., 2000). Interestingly, the FGF signalling components are dependent on the expression of *Pdx1* in these adult β-cells. However, the expression pattern and the role of *Pdx1* during early embryogenesis is quite distinct from its role in adult β-cells (Ohlsson et al., 1993), thus limiting our ability to draw simple parallels with the role of FGF10 in the early development of the pancreas.

Studies of the development of the respiratory system in the mouse have shown that *Fgf10* is reiteratively used to pattern successive rounds of branching (Hogan, 1999; Metzger and Krasnow, 1999; Warburton et al., 2000). This repeated use of *Fgf10* in early lung development is reflected in its highly dynamic expression pattern in the mesenchyme near the position where bronchi bud (Bellusci et al., 1997) indicating that during lung development, the location of *Fgf10* influences the branching pattern of the epithelium and the final shape of the organ. Unlike the lung, *Fgf10* is expressed along the entire mesenchyme layer surrounding the early pancreatic epithelium buds. This suggests that *Fgf10* primarily controls the physical dimensions of the pancreas by regulating the early expansion of the pancreatic epithelium. Thus *Fgf10* appears to be utilised to achieve different effects in the development of the lung and pancreas. However, the local regulation of proliferation may be a general role for FGF10 signalling for the bud outgrowth occurring early in the development of these foregut-derived organs. Reaction-diffusion patterning models invoke counteracting mechanisms that specifically inhibit the proliferation activity to control the size and shape of the bud outgrowth (Meinhardt, 1996; Wolpert, 1998). This negative signalling could originate in the epithelium and act as a feedback mechanism. Several studies suggest that during lung development, sonic hedgehog (SHH) produced by the endoderm could down-regulate *Fgf10* in the mesenchyme (Bellusci et al., 1997; Litingtung et al., 1998; Pepicelli et al., 1998). *Shh* expression is absent in the foregut region that gives rise to the pancreatic buds (Apelqvist et al., 1997; Hebrok et al., 2000) and this expression patterns of *Shh* is consistent with the possibility that SHH could limit the extent of FGF10 signalling and control the growth of pancreatic tissue. Indeed, analysis of mice with targeted inactivation in *Shh* show a threefold increase in pancreatic mass (Hebrok et al., 2000). Whether hedgehog signalling acts to control growth by counteracting FGF10 remains to be determined. Another potentially important negative signalling in bud outgrowth may involve the *sprouty* gene family (Hacohen et al., 1998). Whether *Sprouty* can serve to restrict the proliferative response of FGF10 during early pancreatic development is currently being pursued.

Mesenchymal-epithelial interactions controlling pancreatic development have eluded molecular characterization (Wells and Melton, 1999). The results presented here identify a mesenchymal secreted factor, FGF10 and provide evidence for its role in the proliferation of epithelial progenitor cells. Our results will also be useful in establishing ways to amplify pancreatic cells in vivo or in vitro to develop therapeutic approaches to manage type I and type II diabetes.

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