

FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development

Gitte Anker Norgaard, Jan Nygaard Jensen, and Jan Jensen*

Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, CO 80262, USA

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Abstract

FGF10 plays an important role in the morphogenesis of several tissues by control of mesenchymal-to-epithelial signaling. In the pancreas, mesenchymal FGF10 is required to maintain the Pdx1-expressing epithelial progenitor cell population, and in the absence of FGF10 signaling, these cells fail to proliferate. Ectopic expression of FGF10 in the pancreatic epithelium caused increased proliferation of pancreatic progenitor cells and abrogation of pancreatic cell differentiation of all cell types. A hyperplastic pancreas consisting of undifferentiated cells expressing Pdx1, Nkx6.1, and cell adhesion markers normally characterizing early pancreatic progenitor cells resulted. Differentiation was attenuated even as proliferation of the pancreatic cells slowed during late gestation, suggesting that the trophic effect of FGF10 was independent of its effects upon cell differentiation. The FGF10-positive pancreatic cells expressed *Notch1* and *Notch2*, the Notch-ligand genes *Jagged1* and *Jagged2*, as well as the Notch target gene *Hes1*. This activation of Notch is distinct from the previously recognized mechanism of lateral inhibition. These data suggest that FGF10 signaling serves to integrate cell growth and terminal differentiation at the level of Notch activation, revealing a novel second role of this key signaling system during pancreatic development. © 2003 Elsevier Inc. All rights reserved.

Keywords: Pancreas development; FGF10; Notch; Jagged; Insulin

Introduction

One of the less understood areas of pancreatic development is how morphogenesis and cytodifferentiation are connected, and what genetic networks are in effect to control these processes. Geometrically, the pancreatic structure is attained by branching morphogenesis of two independent pancreatic buds, dorsally and ventrally localized (Edlund, 2002). This process is regulated by the interplay of the pancreatic mesenchyme and an expanding endodermally derived epithelium. Mechanistically, branching morphogenesis is presently best understood in vertebrate lung development. Here, spatially controlled signaling among endodermally secreted morphogens, including sonic hedgehog (Bellusci et al., 1997a) and BMP4 (Bone Morphogenetic Protein 4) (Weaver et al., 1999; Bellusci et al., 1996) as well as FGF10 produced by the mesenchyme (Bellusci et

al., 1997b), is pivotal in determining position of new branches, epithelial outgrowth, and tip arrest. These processes are generally mediated by adjustments to the proliferative capacity and chemotaxis of the epithelial cells (Hogan and Kolodziej, 2002).

Although fundamental differences in tubulogenesis exist between pancreas and lung (Hogan and Kolodziej, 2002), a common observation is that as long as branching morphogenesis ensues, differentiation is suppressed, allowing for the expansion of the progenitor cells. Therefore, the genetic program for the branching morphogenesis might also play a role connecting it to the programs controlling cytodifferentiation. Early embryological studies have noted a connection between morphogenesis and cytodifferentiation in the pancreas. Wessels and Cohen (1967) investigated how pancreatic cell differentiation in the rat was affected by alterations in the mesenchymal/epithelial ratio and found that cytodifferentiation was accelerated if pancreatic mesenchyme was depleted. Later studies revealed that removal of pancreatic mesenchyme would allow for an increased ratio

* Corresponding author. Fax: +1-303-315-4892.

E-mail address: jan.jensen@uchsc.edu (J. Jensen).

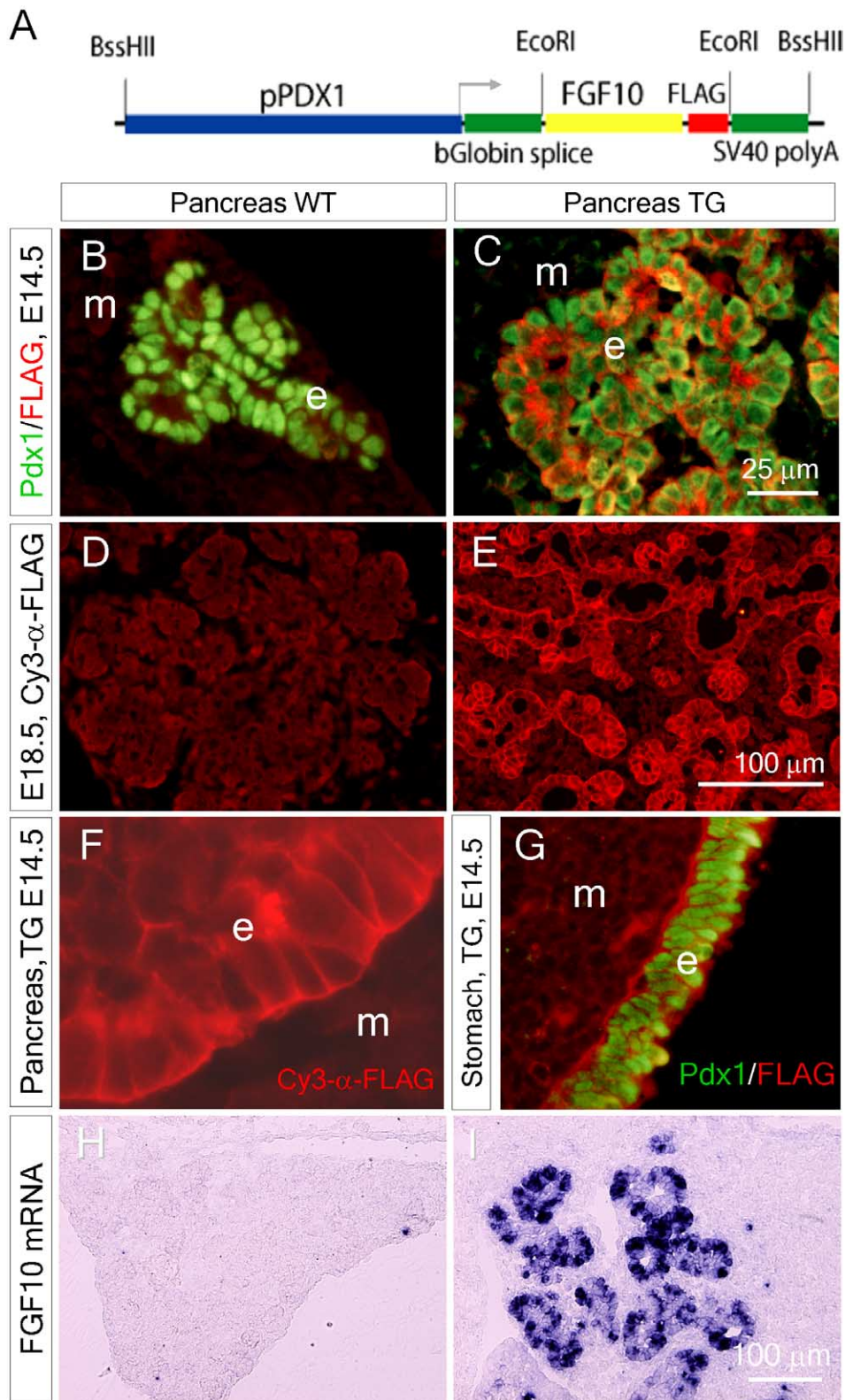


Fig. 1. Expression of ectopic FGF10^{FLAG}. (A) Schematic picture of the TG pPDX1-FGF10^{FLAG} construct. (B) Costaining of Pdx1 and FLAG of the WT pancreas (E14.5) (C). Same, E14.5 TG pancreas, homogenous FGF10-FLAG staining is observed in the Pdx1⁺ cells. (D,E) Anti-FLAG staining of WT (E) and TG (F) E18.5 pancreas epithelium express FGF10-^{FLAG}. Only TG cells are stained. (F) Closer inspection of the FGF10-^{FLAG} staining pattern of the E14.5 TG embryo reveals that a significant portion of the FLAG-immunoreactivity is localized to the plasma membrane of the epithelial cells. (G) FLAG-immunoreactivity (red) is also observed within the E14.5 stomach epithelium expressing Pdx1 (green). (H, I) In situ hybridization of FGF10 mRNA in WT (H) and TG pancreas (I). Heterogeneous expression of FGF10^{FLAG} is observed only within TG epithelium.

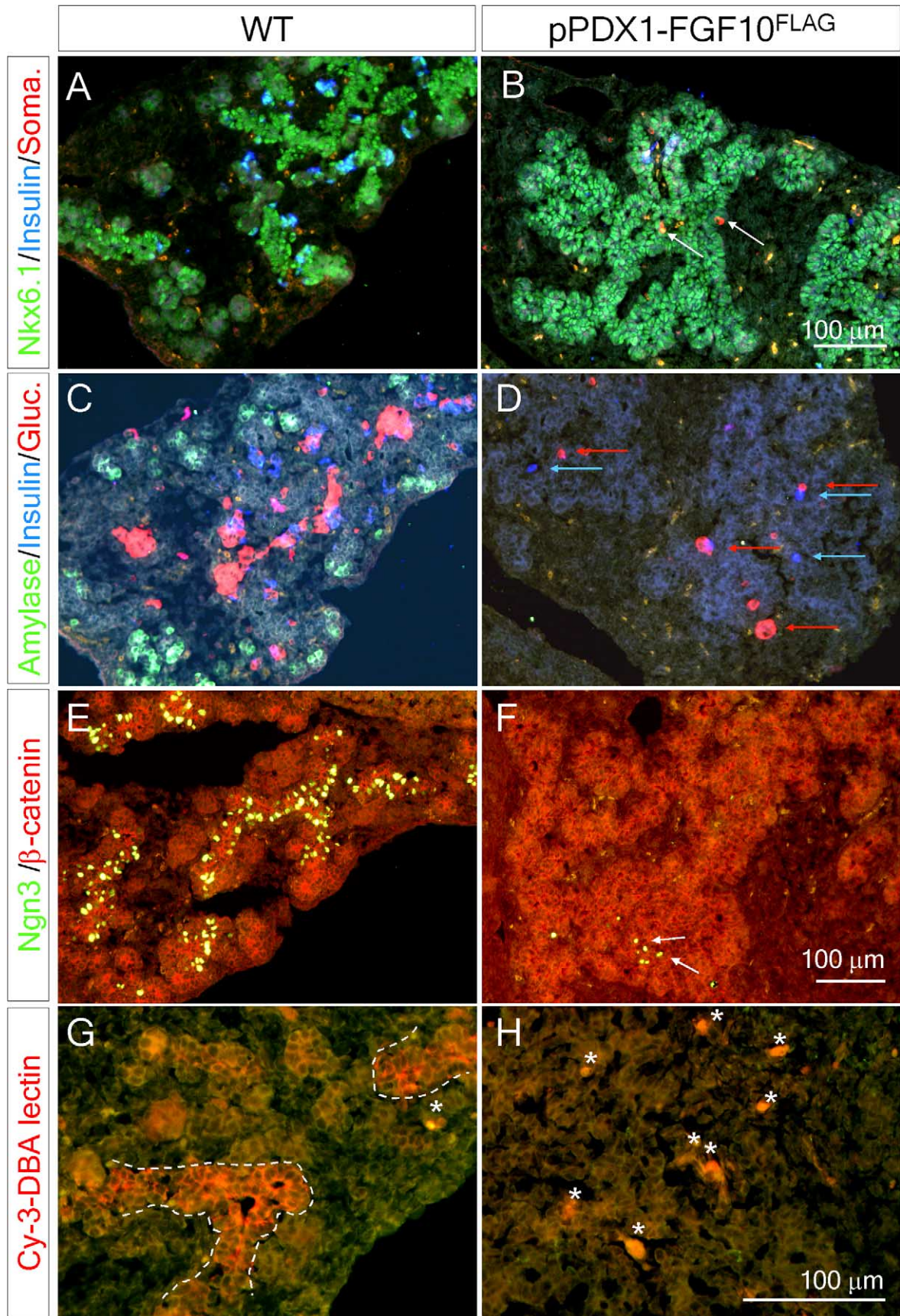


Fig. 2. Expression of pancreatic markers. (A, B) Triple staining of Nkx6.1, insulin, and somatostatin of WT (A) and TG (B) E14.5 pancreas. Insulin cell number is strongly reduced, but few somatostatin cells (arrows) are present only in TG pancreas. Two types of Nkx6.1⁺ cells are observed: the mature β -cell type, which is Insulin/Nkx6.1 double-positive (white nuclear appearance, abundant in WT), and the remaining precursor cells expressing Nkx6.1 only (abundant in TG). (C). Triple staining of the exocrine marker amylase (green), with insulin (blue) and glucagon (red) in the WT. Exocrine cells differentiate in the periphery. (D) Same, TG littermate. No amylase-positive cells are observed. Insulin (blue arrows) and glucagon (red arrows) cells are few. (E) Double-staining of β -catenin (red), with ngn3 (green). Large numbers of ngn3-positive cells are observed. (F) Same, TG littermate. Ngn3⁺ cells are scarce. (G) Ductal differentiation in the WT E14.5 pancreas is signified by DBA-lectin binding (outlined). (H) TG littermate shows no binding of DBA lectin. Asterisks (*) denote erythrocytes.

of endocrine versus exocrine cell differentiation (Gittes et al., 1996; Miralles et al., 1998) Also, under such conditions, the branching process of the epithelium was completely inhibited.

Little is known of why experimental abolition of the mesenchymal–epithelial interaction has such a profound impact upon differentiation and morphology. Most of the genes known to control pancreatic cell fates do not seem to be rigidly patterned prior to development of the individual cell types. This is true for *Pdx1* (Jonsson et al., 1994; Øster et al., 1998; Guz et al., 1995), *Nkx2.2* (Sussel et al., 1998), and *Nkx6.1* (Øster et al., 1998). Also, many seem to play a role farther down in the differentiation pathway instead of initial lineage establishment [*Pax6* (Sander et al., 1997; St-Onge et al., 1997), *Isl1* (Ahlgren et al., 1997), *NeuroD* (Naya et al., 1997)]. Therefore, except for the proendocrine gene *Atoh5* (encoding *ngn3*), which is expressed only in the central domains of the pancreas as β -cells form (Gradwohl et al., 2000; Gu et al., 2002; Jensen et al., 2000a), information remains lacking as to how patterning within the pancreatic organ is conferred prior to differentiation of pancreatic cellular subtypes.

Recently, FGF10 was found to be critically involved in pancreagenesis. FGF10 is normally expressed by the distal-most pancreatic mesenchyme at early stages, and *Fgf10*-null mouse embryos display a hypoplastic pancreas, attributed to loss of the proliferative phase of the pancreas epithelial progenitor cells (Bhushan et al., 2001). The progenitor cells normally express the homeodomain factor *Pdx1*, which itself is required for pancreatic progenitor cell expansion. Thus, the effects of FGF10 were explained by a loss of *Pdx1* expression. The detrimental effect of the mutant, however, significantly hindered interpretation of the role of FGF10 in pancreatic development at later stages.

For this reason, we generated a transient transgenic (TG) mouse model (pPDX1-FGF10^{FLAG} mice) with the objective of revealing later effects of FGF10 in pancreatic development. Furthermore, we aimed to test the hypothesis that mesenchymal FGF10 signaling acts not only to maintain epithelial growth through induction of epithelial cell proliferation (Bhushan et al., 2001), but in addition may serve to maintain Notch-signaling active in pancreatic epithelial cells, thereby simultaneously suppressing cell fate determination. Our data suggest that FGF-signaling has such a dual effect upon pancreatic development, providing a molecular framework that may help in understanding the link between pancreatic cytodifferentiation and morphogenesis.

Materials and methods

Cloning of pPDX1-FGF10^{FLAG}

The open reading frame for mouse FGF10 was amplified from lung tissue and fused C-terminally in-frame with the FLAG sequence, incorporated in the downstream primer.

The flank of the upstream primer codon was changed to match the consensus Kozak sequence (CCACC). Primers included *EcoRI* sites for cloning. The fragment was inserted into the pPDX1-*EcoRI*-vector (Jacob Hald), which includes the *Pdx1* regulatory region and a β -globin splice cassette. The pPDX1-FGF10^{FLAG} fragment for oocyte injection was released by *Bss*HIII digest (Fig. 1A).

Transgenic mice derivation and embryo isolation

Viable oocytes (FVB \times FVB, 100–150/day) were injected (DNA conc. 20 ng/ μ l) and transferred to pseudopregnant recipients. A total of eight independent injection rounds were produced for this study. Genotyping was performed on either yolk sac or paw tissue using gene-specific primers. Date of transfer was set as gestational day E0.5. Embryo/tissue isolation was performed in ice-cold PBS under a stereomicroscope (Olympus SZX12) allowing visual inspection of the entire gut. BrdU (20 mg/kg) was injected 2 h prior to isolation of embryos.

Histology

Histochemistry was performed on frozen sections (7 μ m). At day of staining, slides were dried at 37°C for 30 min, transferred to PBS, and microwaved at boiling for 2 \times 5 min in 10 mM citric acid buffer, pH 6.0. Slides were allowed to cool (30') prior to 3 \times washes in 1 \times PBS. Blocking of unspecific reactivity was performed at RT/1 h using a proprietary TSA-block, supplied by Perkin-Elmer. A mix of diluted 1°-antibodies was added following blocking with no washing, and left O/N. The next morning, excess 1°-antibodies were removed by 3 \times washes by 1 \times PBS. A matching mix of fluorophore-conjugated secondary antibodies was added (1:100, e.g., Cy2-Donkey-anti-rabbit, Texas Red-donkey-anti-mouse, AMCA-donkey-anti-guinea pig) for 1 h. Secondary antibodies were obtained from Jackson ImmunoResearch. Excess secondary antibody was washed off by 3 \times washes in 1 \times PBS. In certain cases, TSA-stimulated amplification was performed to increase signal strength. TSA-amplification of a select color channel (green) was performed by using FITC-TSA reagent (Perkin Elmer, cat. #: NEN-701). Primary antibodies were here detected by using "Histostain kit" reagents (Zymed). HRP activity was revealed by using the HRP-catalyzed tyramide reaction of FITC-TSA. Controls without primary antibodies were included. Antibody identity, source, and dilution are as follows: rabbit α -IDX1 (PDX1), Joel Habener, Boston, MA, USA, 1:2000 (DIF); rabbit α -HES-1, Nadean Brown, Ann Arbor, MI, USA, 1:5000 (TSA); rabbit α -Nkx6.1, Ole D. Madsen, Gentofte, Denmark, 1:500 (DIF); mouse α -Somatostatin, DAKO, 1:500 (DIF); rabbit α -Amylase, A8273, 1:1000 (DIF) Sigma, St. Louis, MO, USA; mouse α -BrdU, Bu20A, 1:100 (DIF) DAKO A/S, Denmark; G. pig α -insulin, GP31, 1:1000 (DIF) Novo-Nordisk, Denmark; mouse α -glucagon, K79BB10, 1:100 (DIF) Sigma, St. Louis, MO,

USA; rabbit α -chromogranin A, 18-0094, 1:100 (DIF) Zymed, CA, USA; α -PP, 18-0043 rabbit 1:500 (DIF) DAKO, Denmark; rabbit α -Ngn3, 1:500 (TSA) Dr. H. Edlund, Umeå, Sweden; rabbit α -IAPP, G017-11, 1:100 (DIF), Phoenix Pharmaceuticals, AZ, USA; rabbit α -pHistoneH3, 06-570, 1:500 (DIF), Upstate, Charlottesville, VA, USA; rabbit α -PCNA, FL-261, 1:50 (DIF) Santa Cruz Biotech., CA, USA; rabbit α - β -catenin Ab-1, 1:100 (DIF) Neomarkers/labvision, CA, USA; rat α -E-cadherin, ECCD2, 1:200 (DIF), Zymed, CA, USA; mouse α -FLAG-Cy2, M2, 1:100 (DIF), Sigma, St. Louis, MO, USA; Rhodamine-DBA lectin RL-1032, 25 μ g/ml Vector Labs, CA, USA.

Three E12.5, 6 E14.5, and 3 E18.5 TG embryos were histologically characterized. These groups were qualitatively similar (based on Pdx1, Nkx6.1, and FLAG staining patterns). $N = 3$ WT littermate embryos were included per time point in pairwise comparisons.

ISH was performed as follows. First, 1 μ g of linearized template DNA was in vitro transcribed and labeled with digoxigenin-UTP (DIG). The RNA was subsequently precipitated adding 1 μ l glycogen (20 μ g/ml; Roche), 7 μ l 7.5 M ammonium acetate, 75 μ l cold 100% EtOH, resuspended in H₂O, reprecipitated using ammonium acetate/cold EtOH, and resuspended in DEPC water. Slides were dried for 30' at 37°C. Hydrophobic barriers around individual sections were generated by using a PAP-pen (Zymed). Sections were refixed by using 4% PFA for 10' followed by 3 \times 5' washes in 1 \times PBS. Proteinase K digestion was not performed. Sections were acetylated followed by washing in 3 \times 5' in 1 \times PBS. Then, 500 μ l preheated (55°C) hybridization solution was added to each slide for prehybridization [5 \times SSC, 50% formamide, 50 μ g/ml yeast tRNA (Sigma), 1% SDS, 50 μ g/ml heparin (Sigma)]. Hybridization mix was prepared by adding 1 μ l probe to 100 μ l hybridization solution. Slides were coverslipped during hybridization O/N in a humidified chamber at 70°C. The next day, coverslips were removed by immersion in preheated 5 \times SSC followed by posthybridization washes: 0.2 \times SSC, 3 h, 70°C; 0.2 \times SSC, 5', RT, and 1 \times MAB, 5', RT. Blocking was performed for 1 h by submersion into blocking solution (2% blocking reagent (Roche), 10% heat-inactivated sheep serum, 0.1% Tween 20, 1 \times MAB). Slides were incubated with anti-DIG-AP or anti-FLU-AP (1:5000) conjugated secondary antibodies in blocking solution O/N at 4°C. The next day, slides were washed 3 \times /20' at RT in 1 \times MAB/0.1% Tween 20. Finally, slides were transferred to NTMT (0.1 M Tris, 400 mM NaCl, 50 mM MgCl₂, 0.1% Tween 20). Signal was visualized by using the AP-substrate BM-purple (Roche). Probe information is as follows (Gene name, vector, source): *Fgf10*, pCR4, Own; *Notch1* and *Notch2*, pBK-CMV, Dr. Jacob Hald, Denmark; *Jagged1*, pCMV-SPORT6, IMAGE: 3500602, ResGen; *Jagged2*, pSK, Dr. Gerry Weinmaster, UCLA; *Hes1*, pBS-SKII+, Dr. R. Kageyama, U. Kyoto, Japan; *Ptfla*, pCR-bluntll, Dr. Lori Sussel, UCHSC, USA.

Morphometry

All morphometric analyses were done on digitized images by using ImagePro software v4.5 (Media Cybernetics). Defined areas of interest were quantified by using area integration. Cell numbers were manually counted for each field-of-vision (FOV). At least 10 independent FOVs were quantified per time point on different slides ($n = 3$). Total cell number analyzed was (WT): 466 BrdU⁺/1907 β -Catenin⁺, (TG): 2518 BrdU⁺/6928 β -Catenin⁺. Cell density was calculated as cells/mm² \pm s.d. A similar method was used for quantifying phosphohistone H3 (pHH3) frequency/area.

Results

Generation of pPDX1-FGF10^{FLAG} transgenic mice

We generated a construct containing a full-length, C-terminally FLAG-tagged FGF10 coding region under control of the *Pdx1* promoter (Fig. 1A). This vector contains 4.5 kb of the upstream regulatory elements of the mouse *pdx1* promoter, which has been shown to faithfully mimic endogenous *Pdx1* expression (Stoffers et al., 1999; Apelqvist et al., 1997; Li and Edlund, 2001). Thus, this promoter allows for abundant expression in the developing pancreas and lower levels of expression in the posterior stomach and duodenal epithelium. The pPDX1-FGF10^{FLAG} construct was injected into fertilized oocytes, and TG embryos were isolated at various ages through gestation; no line establishment was attempted. Gross anatomical evaluation combined with histological analysis showed that all carriers but one (94%, 15/16), at all ages studied, displayed a pronounced endodermal phenotype affecting lung, stomach, pancreas, and intestinal development. We describe here the effects on pancreatic development.

We obtained a total of $n = 16$ independent carriers at three different time points: E12.5 ($n = 3$), E14.5 ($n = 9$), E18.5 ($n = 4$). At E14.5, visual inspection of microdissected foregut–midgut regions revealed an alteration on overall morphology of the TG carriers compared with wild type (WT) littermates. Pancreatic development was disturbed. The regular triangular shape of the dorsal pancreatic rudiment did not develop; instead, an enlarged bulk of tissue was positioned on the side of the stomach. Farther down on the presumptive duodenum, a large mass of ventral pancreatic tissue was observed. At E18.5, the morphological characteristics described above of the E14.5 guts were exacerbated.

We first determined expression of the FGF10^{FLAG} transgene. At both E12.5 (not shown) and E14.5, homogeneous expression of FGF10^{FLAG} protein was found in Pdx1-expressing cells throughout a hyperplastic pancreatic epithelium (Fig. 1C). No FLAG-staining was observed in WT Pdx1⁺ cells (Fig. 1B). Expression of FGF10 FLAG protein

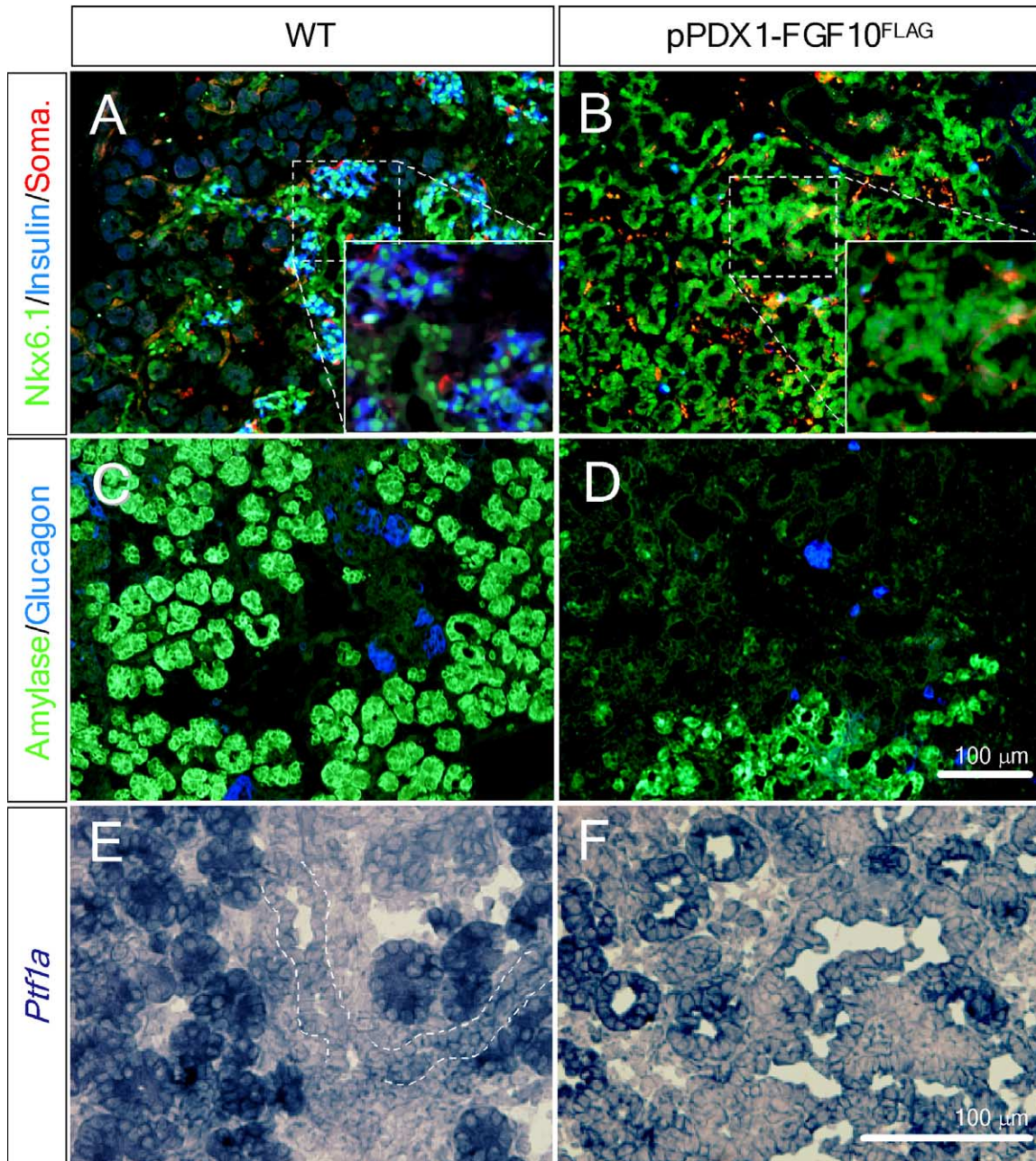


Fig. 3. Continued suppression of cytodifferentiation by FGF10 in the late-stage pancreas. (A) Triple staining of Nkx6.1 (green), insulin (blue), and somatostatin (red), WT pancreas. At E18.5, only few remaining precursor cells are remaining (single- positive Nkx6.1⁺ cells). These extend to the stem of the exocrine Nkx6.1⁻ acini. Most ductal cells do not express Nkx6.1. All insulin-positive cells express Nkx6.1. Somatostatin-positive cells are scarce. (B) Same, TG littermate. Few scattered insulin/Nkx6.1 coexpressing cells are observed. Very few somatostatin⁺ cells are detectable. The majority of cells in the expanded pancreatic epithelium express Nkx6.1. (C) Exocrine differentiation is pronounced in the WT pancreas, signified by formation of acinar structures (amylase, green). Glucagon cells cluster in a similar manner as the insulin-producing cells. (D) Same, TG littermate. A limited number of cells have taken an exocrine fate (amylase, green). The glucagon cell number is strongly reduced. (E, F) *Ptf1a* in situ hybridization. *Ptf1a* is expressed both in the WT and TG pancreatic cells. Low-level expression of *Ptf1a* remains in certain ductal cells in the WT (outlined).

persisted in E18.5 pancreas epithelium (Fig. 1E). Surprisingly, at all time points, the strongest staining was observed in the plasma membrane rather than in the cytoplasm (Fig. 1F). FGF10^{FLAG} protein was also observed in Pdx1-expressing stomach epithelial cells, albeit at lower levels (Fig. 1G). The staining for the FLAG-tagged protein was always homogenous within the Pdx1-expressing cells, an unex-

pected finding as the embryos correspond to independent integration events and some of which may be mosaic. As FGF10 is a secreted protein, it seems possible that by visualizing the FLAG-tagged protein we might detect FGF10^{FLAG} postsecretion, likely binding to cognate receptors and possibly internalized. To clearly define the cellular subset expressing the exogenous FGF10 gene, we per-

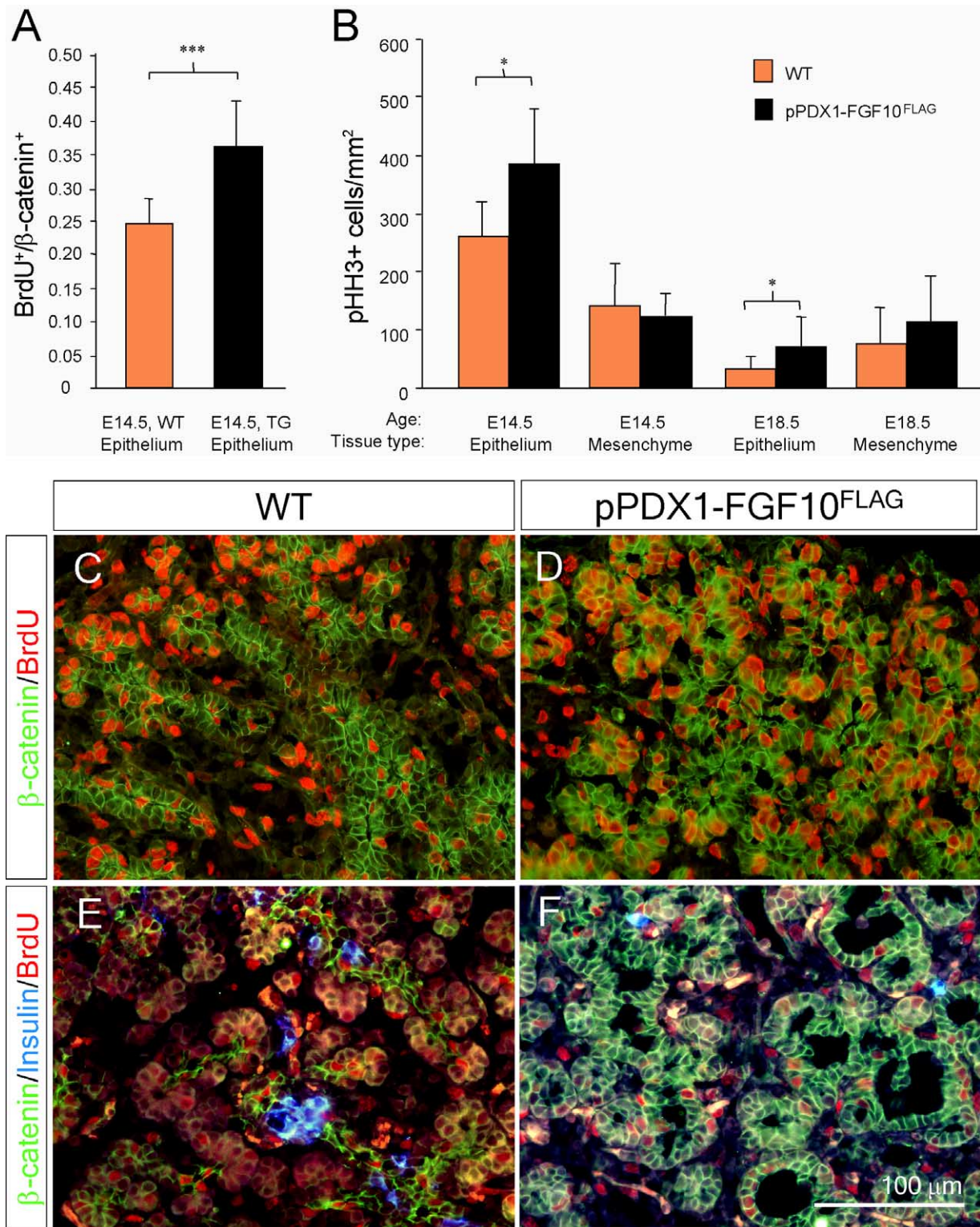


Fig. 4. Cell proliferation. (A) Morphometric analysis of BrdU incorporation. Data are based on β -catenin/BrdU double staining of E14.5 pancreas. BrdU⁺/ β -catenin⁺ cells were counted relative to total β -catenin⁺ cells. (B) Morphometric analysis of phosphorylated Histone H3 frequency. Data based on colabeling with E-cadherin and pHH3. Error bars, s.d.; *, $P < 0.05$; ***, $P < 0.001$. (C, D) Double staining of BrdU (red) and β -catenin (green), E14.5. (E, F) Triple staining of BrdU (red), insulin (blue), and β -catenin (green), E18.5. Fully differentiated exocrine cells, as well as β -catenin⁺ ductal-type cells are BrdU⁺ in WT (E). In the transgenic pancreas (F), β -catenin⁺ cells within the extended ductal network are labeling with a low frequency compared with E14.5 (D).

formed in situ hybridization for FGF10. High levels of exogenous FGF10 mRNA were detected in the developing E14.5 pancreas epithelium of TG carriers (Fig. 1I), and none in WT littermates (Fig. 1H). No mesenchymal expression of FGF10 was noted. Lack of mesenchymal FGF10 expression was expected as the endogenous FGF10 expression is normally restricted between E9.5 and E12.5 (Bhushan et al., 2001). TG Pancreatic FGF10 mRNA expression was highly heterogeneous. Thus, local dispersion of the FGF10 protein may explain why all pancreatic cells display a membranous staining pattern of the protein. Due to the secreted nature of FGF10, we speculated that the unexpected phenotype in the lung might be due to diffusion of FGF10 produced by the pancreas and stomach, thereby conditioning the lumen of the gut. Western blot analysis of transgenic amniotic fluid failed to reveal the presence of FGF10^{FLAG} protein (data not shown). However, RT-PCR revealed the presence of low amounts of exogenous FGF10 mRNA in transgenic lung (data not shown).

Pancreatic cell differentiation in pPDX1-FGF10^{FLAG} mice

In order to characterize FGF10-mediated effects on pancreatic cell differentiation, we first wished to clarify which cells of the midgut region were truly in a pancreatic state. As Pdx1 is not restricted to the pancreas, we took advantage of the pancreas-restricted endodermal marker Nkx6.1. In E14.5 WT embryos, Nkx6.1 is restricted to few centrally located remaining progenitor cells and mature β -cells (Fig. 2A). In TG littermates, almost all cells of the pancreatic-type epithelium expressed Nkx6.1 (Fig. 2B). Costaining for Pdx1 and Nkx6.1 revealed that most cells coexpressed these markers (data not shown). By analyzing the expression of terminal markers of differentiated pancreatic cells, we found that insulin expression was strongly reduced (Fig. 2B and D), while in the normal pancreas, large numbers of insulin-expressing cells have developed (Fig. 2A and C) at an approximate 1:1 ratio with glucagon-expressing cells (Fig. 2C). Glucagon-expressing cells were found in TG pancreas, but in reduced numbers (Fig. 2D). A few Somatostatin-expressing cells were observed in the E14.5 TG pancreas (Fig. 2B, and data not shown), whereas these cells were not present in the WT littermates at this age (Fig. 2A). This could signify an altered fate allocation for cells in the β/δ -cell lineage. The exocrine-specific marker amylase was absent in TG pancreas at E14.5 (Fig. 2D), but readily detectable in the WT littermates (Fig. 2C).

*FGF10-mediated suppression of endocrine differentiation occurs upstream of the proendocrine gene *Atoh5/ngn3**

The almost complete absence of endocrine cell formation could be explained by a repressive effect of FGF10 signaling at various levels of endocrinogenesis. Multiple cell intrinsic regulators play roles at different levels of endocrine cell formation. For instance, both *Isl1* and *Pax6* are required

for proper endocrine differentiation but act after an endocrine cell type has made a fate commitment and has become postmitotic. Extremely few *Pax6*-positive cells were observed in E14.5 TG pancreas compared with WT, and the majority of these expressed either insulin or glucagon (data not shown). The reduced number of *Pax6* cells suggested that the repressive effect of FGF10 might have targeted the initiation of endocrine development. To clarify this, we analyzed expression of the proendocrine factor *Ngn3* (Gradwohl et al., 2000). *Ngn3* was expressed in the TG pancreas at a greatly reduced cell number compared with WT (Fig. 2E and F). Therefore, FGF10 seem to have inhibited the propensity of any given pancreatic progenitor cell to initiate an endocrine program by blocking *Ngn3* activation.

FGF10 arrests pancreatic epithelial cells in an undifferentiated state

The absence of terminal differentiation and the homogeneous *Nkx6.1* and *Pdx1* expression at E14.5 suggested that the FGF10^{FLAG}-expressing cells were trapped in a phenotypic state resembling that of the normal expanding pancreatic epithelium during its major growth phase between the primary and secondary transitions. We next asked if this state was maintained throughout embryonic development by analyzing expression of pancreatic markers at E18.5. At this point, most WT pancreatic precursors have undergone a fate allocation toward either an endocrine (insulin⁺ or glucagon⁺; Fig. 3A and C) or exocrine (amylase⁺; Fig. 3C) fate, with very few *Pdx1*⁺/*Nkx6.1*⁺/hormone⁻ progenitor cells remaining (Fig. 3A). In the E18.5 TG pancreas, most cells expressed *Pdx1*, where some of these also expressed *Nkx6.1*, albeit at strongly reduced levels compared with E14.5 (data not shown). In E18.5 WT pancreas, *Nkx6.1* and *Pdx1* colocalize in β -cells (data not shown), and *Pdx1* is only expressed at very low levels in mature exocrine cells. Total endocrine cell number in the E18.5 TG pancreas was considerably reduced as evaluated by the panendocrine marker ChromograninA (data not shown) and scattered insulin⁺, glucagon⁺, somatostatin⁺, and PP⁺ cells were found adjacent to the TG epithelium (Fig. 3B and D, and not shown). The overall pancreatic morphology was highly cystic, consisting almost entirely of tubular networks. At this time point a relatively small portion of cells in the peripheral regions had adopted an exocrine fate (Amylase⁺; Fig. 3D). We conclude that cellular differentiation is not irreversibly blocked in TG embryos. To evaluate a possible mechanism to account for the reduction of exocrine cell differentiation, we analyzed expression of the *Ptf1a* gene, which is normally expressed both in pancreatic progenitor cells, and later is involved in the exocrine fate choice (Kawaguchi et al., 2002). *Ptf1a* was expressed in both WT and TG E14.5 pancreas epithelium (not shown), as well as in the cystic epithelium of the E18.5 TG pancreas and mature WT exocrine cells (Fig. 3E and F). Thus, suppression of exocrine differentiation occurs downstream of *Ptf1a*.

Early pancreatic progenitor cells have been shown to express particular cell-adhesion molecules. β -Catenin is a protein with pleiotropic functions, one of which is associated with maintenance of the epithelial morphology through its interaction with cell adhesion molecules of the cadherin family. Both β -catenin and E-cadherin are normally expressed strongly in pancreatic epithelial progenitors (Selander and Edlund, 2002; and data not shown). E-cadherin and β -catenin were expressed homogeneously in E14.5 TG epithelial cells, contrasting the heterogenous pattern of the WT pancreas. Uniform staining was also observed in the epithelial structures at E18.5 (data not shown). Again, such a pattern was not observed in WT littermates, where only ductal cells ($Pdx1^{-}$) and few $Pdx1^{+}/hormone^{-}$ cells display a similar qualitative staining (data not shown). Thus, a possibility was that the FGF10-expressing epithelial cells had taken a ductal fate. Therefore, we performed staining for Dolichos Biflorus Agglutinin (DBA lectin); (Kobayashi et al., 2002) which we find exclusively staining duct cells. No cells labeled with DBA lectin in the TG epithelium [analyzed at E14.5 (Fig. 2G and H) and E18.5 (not shown)], arguing that the TG pancreatic cells had not undergone ductal differentiation.

Triple immunofluorescence of BrdU (5-Bromo-deoxy-Uridine) S-phase incorporation together with pancreatic markers (β -catenin and insulin) at both E14.5 (Fig. 4C and D) and E18.5 (Fig. 4E and F) allowed us to discriminate between the mitotic activities of pancreatic progenitor cells and mesenchymal cells (Fig. 4A). Insulin⁺ cells did not incorporate BrdU in E14.5 or E18.5 TG pancreas, whereas WT insulin cells had a low mitotic activity at E18.5 (data not shown) and none at E14.5, in agreement with previous data (Jensen et al., 2000a). In contrast, the β -catenin-expressing epithelial cells were strongly proliferating in both WT and TG pancreas. Still, a 1.49-fold increase in the BrdU-labeling index was observed between TG vs. WT pancreas (Fig. 4A). To complement the BrdU measurements, we performed double staining of phosphorylated histone H3 (pHH3) in combination with E-cadherin (Fig. 4B). A similar fold increase of the proliferative index (1.47-fold) between TG and WT E14.5 pancreas epithelium was observed. At E18.5, the proliferative capacity had strongly declined in both the TG as well as the WT pancreas (Fig. 4B). However, a relative increase in the proliferation of the TG pancreas epithelium at E18.5 was still observed (Fig. 4B). No significant difference in mesenchymal proliferation was observed at E14.5 or E18.5 (Fig. 4B).

FGF10-expressing pancreatic progenitor cells remain active in Notch signaling

As precursor cell arrest combined with inhibition of differentiation is the expected outcome if Notch signaling is sustained in the developing pancreas (Apelqvist et al., 1999), we analyzed expression of components of this signaling system. *Notch1* and *Notch2* are normally expressed

by the developing mouse pancreas. Normal expression of *Notch1* is restricted to the developing pancreatic buds at E10 (Apelqvist et al., 1999; Lammert et al., 2000) and continue in the pancreatic epithelial progenitors prior to endo- and exocrine differentiation, as well as in the remaining progenitors following the secondary transition (Apelqvist et al., 1999; Lammert et al., 2000; Jensen et al., 2000a). *Notch2* is normally expressed both by pancreatic epithelium and mesenchyme early (Jensen et al., 2000b) but becomes restricted to remaining ductal progenitor cells at E15.5 (Lammert et al., 2000). In E18.5 FGF10^{FLAG} TG embryos, *Notch1* and *Notch2* expression persisted in the arrested epithelium (Fig. 5D and F), while we noted that expression of *Notch1* and *Notch2* was absent in the few exocrine cells that differentiated, similar to the exocrine cells of the WT (Fig. 5C and E). Expression of *Notch1* and *Notch2* suggests competence for Notch signaling but does not reveal whether Notch signaling is active. Therefore, we investigated the expression status of *Hes1* as this gene is a known downstream target for Notch1 and 2 (Jarriault et al., 1998; Beatus et al., 2001) and generally accepted to signify active Notch signaling (Jouve et al., 2000; Kuroda et al., 1999; Solecki et al., 2001; Jarriault et al., 1998). We found that *Hes1* was expressed in most cells of the E18.5 TG epithelium, contrasting its restriction to ductal-type cells in the WT (Fig. 5A and B).

To evaluate the mechanism for the continued Notch activation, we addressed the expression of Notch-ligand genes. *Delta1* (encoded by *Dll1*), which is normally expressed in the earliest differentiating endocrine cells (Lammert et al., 2000), was only expressed in very few cells of both WT and TG pancreas at E12.5 (data not shown) and only a low level expression of *Dll1* in both the WT and TG pancreas at E14.5 (not shown). We did not detect expression of *Dll1* in WT or TG E18.5 pancreas (data not shown). In contrast, we observed a strong and uniform expression of *Jagged2* (Fig. 6D) in the E18.5 TG pancreas. Similarly; *Jagged1* was expressed broadly in the TG epithelium at lower levels (Fig. 6B). These domains overlapped those regions that still expressed FGF10^{FLAG} mRNA (Fig. 6F). *Jagged1* and *Jagged2* were abundantly expressed in the forming vasculature (Fig. 6A and B) within the pancreas interstitial area of both TG and WT. The epithelial expression of *Jagged1* and *Jagged2* could imply that these ligands play a role during the normal epithelial expansion prior to the secondary transition. Epithelial-restricted pancreatic expression of *Jagged1* prior to differentiation has previously been reported (Mitsiadis et al., 1997). We find *Jagged2* expressed in both WT and TG pancreatic epithelium at E12.5 (data not shown), overlapping cells also expressing *Notch2*, *Notch1*, and *Hes1* (data not shown). Thus, *Jagged1*, *Jagged2*, *Notch1*, *Notch2*, and *Hes1* are coexpressed within the normal pancreatic progenitor cells prior to the secondary transition. This pattern is maintained in the TG epithelium.

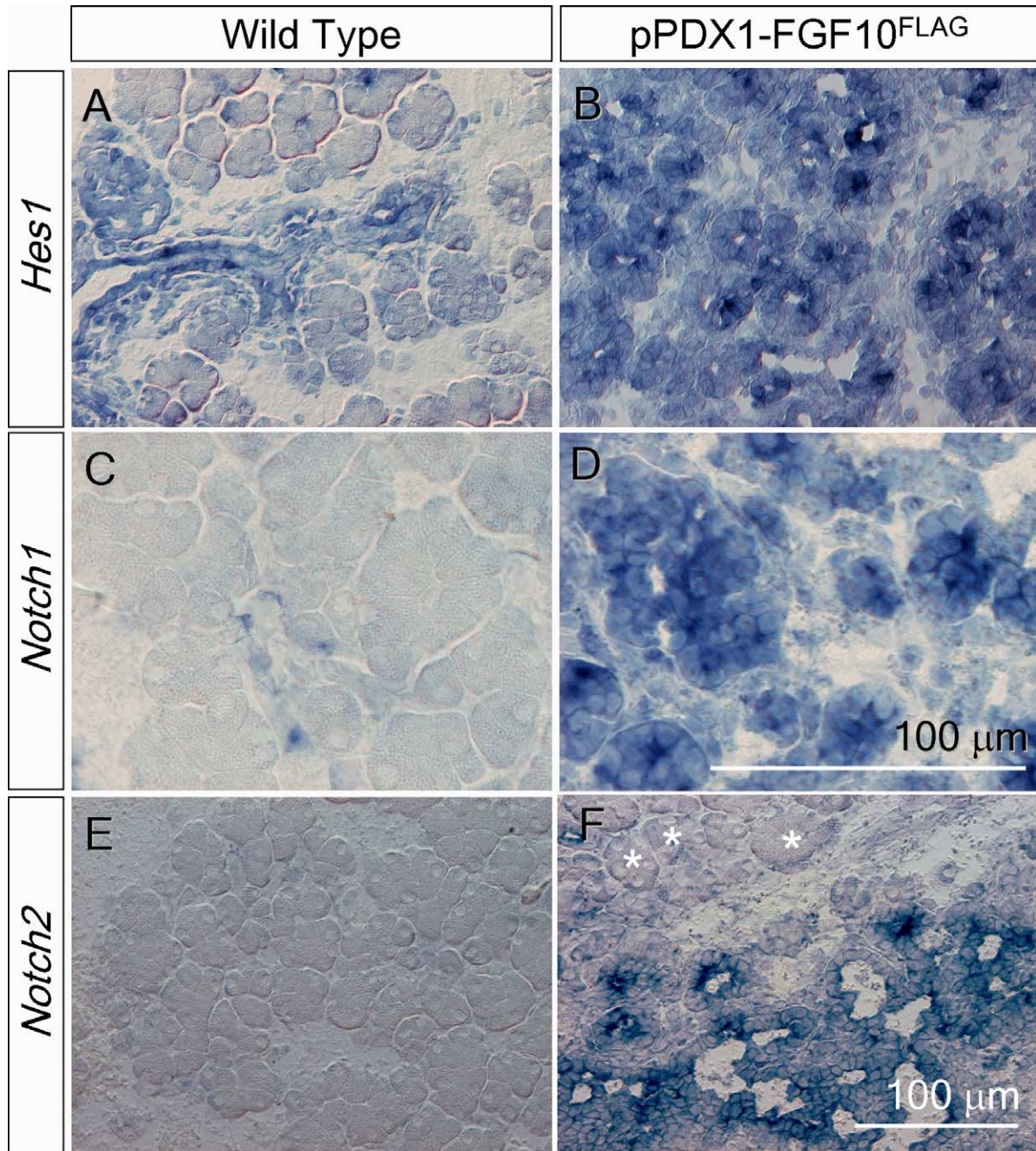


Fig. 5. Sustained activation of the Notch signaling system by FGF10. (A) In situ hybridization of *hes1*, E18.5 WT. *Hes1* is expressed almost exclusively in ductal structures of the normal pancreas. (B) TG littermate, *Hes1* is expressed throughout the epithelium, with varying intensity. (C) *Notch1* is only expressed in pancreatic duct cells, and absent in exocrine pancreatic cells. (D) In contrast, *Notch1* is expressed throughout the epithelial cells of the TG pancreas. (E) *Notch2* is not expressed in the normal pancreas at E18.5. (F) *Notch2* remains expressed at high levels in the precursor epithelium of the TG pancreas. The cells that undergo exocrine differentiation cease to express *Notch2* (asterisks), similar to WT exocrine cells. Mesenchymal cells do not express *Notch2* at this time point.

Discussion

In the current study, FGF10 was overexpressed in the pancreatic epithelial progenitor cells using the Pdx1 promoter. FGF10 is thus targeted to a cell type not normally expressing the ligand, but to one that is responsive to the factor (Bhushan et al., 2001; Miralles et al., 1999). It is

conceivable that epithelial-expressed FGF10 sensed by the mesenchyme subsequently signaling back to the epithelium could explain some of the observed effects. However, this appears less likely than the probability that exogenous FGF10^{FLAG} protein exerts auto- or paracrine effects. We base this argument on the fact that the predominant cognate receptor for FGF10, the III-b-splice form of the FGFR2, is

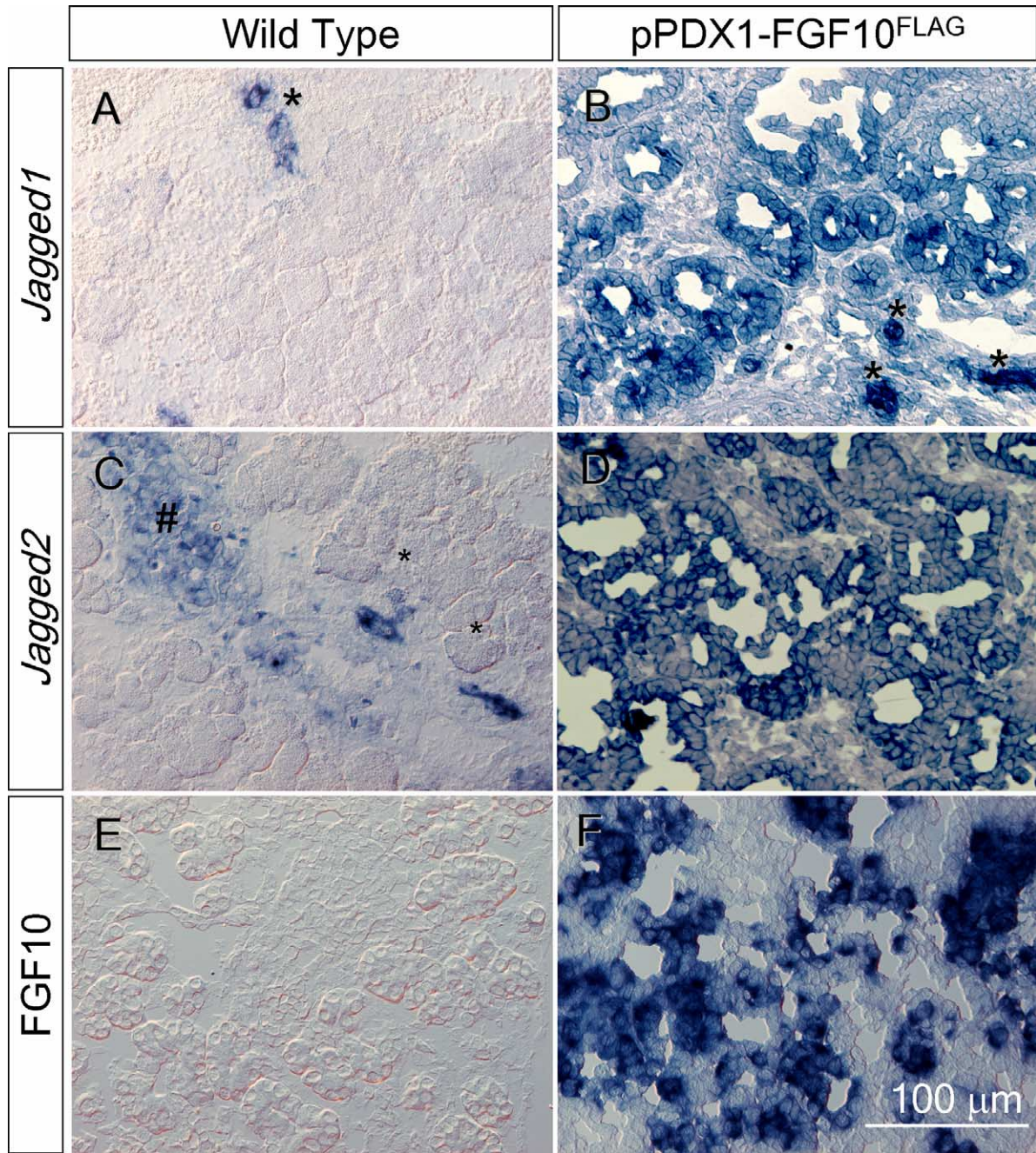


Fig. 6. Expression of *Jagged*-type Notch-ligand genes in pPDX1-FGF10 pancreas. (A) In situ hybridization of *Jagged1*, E18.5 WT. *Jagged1* is expressed exclusively in endothelial cells (*). (B) TG littermate, *Jagged1* is expressed in endothelial cells (*). In addition, most cells of the pancreatic epithelium express *Jagged1*. (C) *Jagged2* is expressed in endothelial cells (*), and absent in exocrine pancreatic cells. Some staining is observed in endocrine regions (#). (D) *Jagged2* is expressed in TG pancreas throughout the pancreatic epithelial cells. (E, F) In situ hybridization for FGF10 mRNA reveals continued expression of the transgene in the E18.5 pancreas (F), whereas the control WT pancreas is negative (E). Expression in the TG pancreas (F) varies greatly between individual epithelial cells. The general domains of transgene expression overlap those expressing the various *Notch* genes.

expressed in the pancreas epithelium and not in the adjacent mesenchyme (Elghazi et al., 2002; Miralles et al., 1999). Furthermore, FGF10 protein was only detected within the epithelial cells of the pancreas. Additionally, mesenchymal cell proliferation is not altered in the TG embryos. In normal lung and pancreas, FGF10 is secreted by the distal-most mesenchyme, causing outgrowth of those epithelial cells in

proximity to the signaling source. The production of FGF10 in lung creates a morphogen gradient that influences spatial gene expression (Bellusci et al., 1997b; Weaver et al., 2000; Park et al., 1998) and we assume the existence of a similar morphogen gradient in the pancreas. In the current model, the autocrine FGF10 production would effectively disrupt such a gradient. Given the high level of expression

achieved, it is likely that the pancreatic epithelium would be exposed to FGF10 concentrations that may correspond to that of a proximal position to the mesenchyme. Cells in such a proximal position in the lung activate *bmp4* expression in response to FGF10 (Bellusci et al., 1996; Weaver et al., 2000). However, as we cannot detect *Bmp4* mRNA in WT nor TG pancreas (data not shown), this genetic network does not seem to operate in the developing pancreas.

Active suppression by FGF10 of pancreatic cell differentiation

Based on the results of the *FGF10*-null mice, we anticipated that ectopic FGF10 secretion would lead to an increase in pancreatic epithelial cell proliferation. The ~1.5-fold upregulation in mitotic index of β -catenin/E-cadherin-positive epithelial progenitors at E14.5 is consistent with this and would account for the hyperplastic pancreas observed. Somewhat unexpectedly we find that cytodifferentiation of the principal cell types in the pancreas (endocrine, exocrine and ductal) is strongly repressed. Bhushan and coworkers found that glucagon cells did not form prematurely, or in excess, in FGF10-deficient embryos thereby suggesting that FGF10 did not play a role in repressing differentiation. Also, Miralles et al. (1999) proposed that FGF-signaling is instructive for the exocrine pancreatic fate, as explanted pancreatic epithelia were found to contain increasing numbers of exocrine cells when cultured in the presence of FGF10 (or FGF7, FGF1). Our data do not support the notion that FGF10 is instructive for exocrine development, as this fate choice is completely repressed at E14.5. The discrepancy between these studies might be explained by the absence of the precursor cell expansion phase in isolated epithelial explant cultures without FGF signaling, as this would lead to reduced numbers of exocrine cells developing in total. Thus, the addition of exogenous FGF would rescue the mesenchymal trophic effect, allowing for normal pancreatic epithelial development and later differentiation of exocrine cells. In such a case, the role of FGF10 should be viewed as a permissive, rather than instructive, signal for exocrine cell development. Our data also contrast the results by Hart et al. (2000) where expression of a presumed dominant negative FGFR2b under control of the *Pdx1* promoter does not lead to developmental defects. Considering the degree of the phenotype of *Pdx1-FGF10^{FLAG}* transgenic embryos observed here, it is surprising that the *pPDX1-DN-FGFR2b* mice do not display a detectable embryonic phenotype manifestation. One possibility explaining this difference is that the FGFR2b receptor may not mediate all signaling from FGF10, and that such an alternative pathway is not blocked by the DN-FGFR2b receptor. In turn, this can also explain the relatively weak pancreatic hypoplasia phenotype manifested by FGFR2b mutant mice (Pulkinen et al., 2003). Predicting the components of such an alternative signaling pathway is not

straightforward, as multiple FGFRs (FGFR1b/c, FGFR2 b/c, FGFR3b/c, FGFR4, and FGFR5) are all expressed during pancreatic development (Dichmann et al., 2003). Of note, Dichmann et al. (2003) recently described the effects of ectopic expression of FGF4 in the pancreas, suggesting some similarity between the *pPDX1-FGF4* and *pPDX1-FGF10^{FLAG}* mice (this study), such as cystic epithelial formation and the attenuation of endocrine development in both models. Yet, important differences are clear as exocrine development was not blocked in the *pPDX1-FGF4* mice, and perhaps more important, the pancreatic mesenchymal mitotic activity was highly elevated; whereas this parameter is unchanged in *pPDX1-FGF10* mice. FGF4 is known to signal through the FGFR1,2,3-c but not the FGFR1,2,3-b splice forms nor FGFR4 (Bellosta et al., 2001). Due to the different signaling pathways of FGF10 and FGF4, it is not straightforward to determine the reason for the similarities between these models. In our opinion, secondary effects caused by the increase in pancreatic mesenchymal tissue mass in the *pPDX1-FGF4* model could well be responsible for the observed effects on endocrine development and cystic network formation. It would be of interest to determine whether FGF10 is expressed in the *pPDX1-FGF4* mesenchymal tissue.

We find that FGF10-expressing pancreatic progenitor cells become arrested in the undifferentiated precursor state, characterized by the expression of *Pdx1*, *Nkx6.1*, cell adhesion molecules, *Notch*, *Hes1*, and *Jagged* genes. Thus, ectopic FGF10 appears to have blocked the normal wave of differentiation events occurring at the secondary transition. In the endocrine differentiation pathway, the block occurs upstream of the proendocrine factor *Ngn3*. The loss of *Ngn3* expression in TG embryos signifies that the earliest known cue for endocrine development is not activated; however, this does not unambiguously demonstrate that the consequences upon cytodifferentiation are due to an active repression mechanism, rather than a default block of maturation as the cells remain in the cycling state due to an otherwise independent trophic effect of FGF10. Cell cycle exit is an integral part of the endocrine differentiation pathway, as endocrine cells become postmitotic upon differentiation (Ahlgren et al., 1997; Jensen et al., 2000a). Importantly, the mitotic arrest in endocrine formation occurs downstream of *Ngn3* gene activation (Jensen et al., 2000a), showing that this event is a consequence, rather than the basis for initiating the endocrine program. Consequently, we deduce that FGF10 actively suppresses initial *Ngn3* promoter activation. Our finding that most pancreatic cells are still blocked in differentiation at E18.5 while showing reduced mitotic activity supports this conclusion.

By what mechanism then is FGF10 actively suppressing pancreatic cell differentiation? Notch-signaling normally acts to repress *Atoh5/Ngn3* expression during normal endocrine pancreatic development. One aspect of this inhibition pivots around a lateral specification mechanism, whereby

cells of proendocrine character signal laterally between neighbors, restricting a primary fate allocation to one and simultaneously repressing the process in the other (Fig. 7B). Such a mechanism is supported by investigations of the effects upon mutation of either the Notch downstream gene *Hes1*, the Su(h)-type transducer *RBP-Jκ*, or the Notch ligand *Dll1* (Apelqvist et al., 1999; Jensen et al., 2000b). However, the almost complete conversion of pancreatic progenitors at E9.0–E10 and the lack of endocrine cell clustering in the *Hes1*^{-/-} intestine, combined with the observation that *Hes1* is widely expressed in epithelial cells of the normal presecondary transition pancreas even in cells not neighboring a laterally signaling cell (Jensen et al., 2000b), suggest that Notch signaling is not required for every aspect of *Hes1* expression, or alternatively, that a second mechanism beyond lateral inhibition activating Notch/*Hes1* ubiquitously in the pancreatic progenitor cells exists. The second possibility has received experimental support, as mice mutant for *Hnf6* express *Notch* and *Hes1* at nonreduced levels despite the almost complete absence of *Atoh5/Ngn3* expression and loss of endocrine cells (Jacquemin et al., 2000).

“Suppressive maintenance” by Notch: a possible role for Notch/Hes1 prior to lateral inhibition

Our findings of the effects of FGF10 signaling further supports such an additional role of Notch-activation, as *Hes1* is clearly activated by a process different from lateral inhibition. This role is linked to precursor cell maintenance, and to discriminate it from the lateral inhibition mechanism, we will refer to this as “suppressive maintenance” defined by Notch-mediated *Hes* gene activation throughout the precursor cell population with the outcome that cell differentiation cues are suppressed and the progenitor state is maintained. The difference between such a suppressive maintenance role of Notch and that of Notch in lateral inhibition is depicted in Fig. 7. A distinguishing feature between these mechanisms is the ubiquitous expression of the ligand and receptor in suppressive maintenance, in contrast to the typical speckled expression of a lateral inhibitory ligand, such as that reported for *Dll1* (Lammert et al., 2000). Both *Jagged1* and *Jagged2* exhibit such a uniform pattern in the normal pancreatic epithelial cells prior to the secondary transition, and are thus overlapping with *Notch1* and *Notch2* (this study and Jensen et al., 2000a; Apelqvist et al., 1999; Lammert et al., 2000; Mitsiadis et al., 1997). This pattern is maintained in the presence of ectopic FGF10. The control of Notch activation during suppressive maintenance would be expected to be distinct from that of lateral inhibition. Due to the established effect of the pancreatic mesenchyme in assisting epithelial growth prior to differentiation, such distinction may be conferred through mesenchymal signaling (Fig. 7A). Supporting this are the results of isolated epithelial culture, where differentiation occurs toward an endocrine fate, rather than exocrine. An additional, although

generally overlooked, conclusion from such experiments is that the isolated epithelial cells do not rest in the precursor state, but in fact undergo an accelerated differentiation. Such an outcome is similar to the effects on pancreatic development in vivo of an absence of *Hes1* (Jensen et al., 2000b), where the endocrine, and not the exocrine, fate is accelerated, and occurs in excess.

FGF signaling and Notch-mediated suppressive maintenance: a conserved molecular cassette?

Is FGF-stimulated progenitor cell maintenance via the Notch system a unique feature of the developing pancreas? Although a link between FGF signaling and Notch activation as a conserved gene regulatory cassette is not commonly recognized several observations suggest such an association in several developmental contexts, such as the developing limb (Crosnier et al., 2000; Vargesson et al., 1998) where FGF8 and 10 signaling regulates expression of *Jagged1* and *Jagged2* during distal outgrowth (Shawber et al., 1996; Valsecchi et al., 1997). Likewise, in the developing tooth (Harada et al., 2002), FGF10 is capable of maintaining the dental epithelial precursor pool via stimulation of *Hes1* (Mustonen et al., 2002). A specific model of FGF/Notch interaction combined with a temporal definition of two modes of Notch signaling is that of tracheal development in *Drosophila* (Ikeya and Hayashi, 1999), where the fly gene *branchless* (FGF-type) leads to FGF-receptor activation (*breathless*) and downstream MAPK signaling causing upregulation of *delta* in the inductive process of tracheal cell invagination. Later, *notch* plays a second role (lateral inhibition) in the selection of fusion versus tracheal fates. A related bimodal system may be also involved in vertebrate somite differentiation. In Zebrafish (Sawada et al., 2001) and chicken (Dubrulle et al., 2001), FGF8 secreted by the caudal presomitic mesoderm (PSM) has been proposed as a wavefront generator in the clock-and-wavefront model of somitogenesis. Dubrulle and colleagues observed that an increase in FGF8 protein levels did not affect the periodicity of the clock mechanism, but resulted in an increase in the Notch-target gene *c-hairy2* in the PSM (Dubrulle et al., 2001). Reciprocally, the lowering of FGF-signaling resulted in reduced *c-hairy2* expression. Therefore, the role of FGF signaling in the PSM could be to set a noncyclical Notch activity threshold, which thus can be viewed as the actual wavefront (Holley and Takeda, 2002) [i.e., suppressive maintenance restricting condensation] on top of which a cyclical oscillating Notch output (Evvard et al., 1998; Zhang and Gridley, 1998) serves to operate, and against which differentiation cues push. If such a system indeed exists one may expect a ligand for Notch to be under control of FGF signaling, expressed in a decreasing gradient rostrally in the PSM. Both *Dll1* and *Dll3* display such a distribution within the PSM (Kusumi et al., 1998).

This study shows that increased FGF10 signaling results in the maintenance of Notch signaling in pancreatic devel-

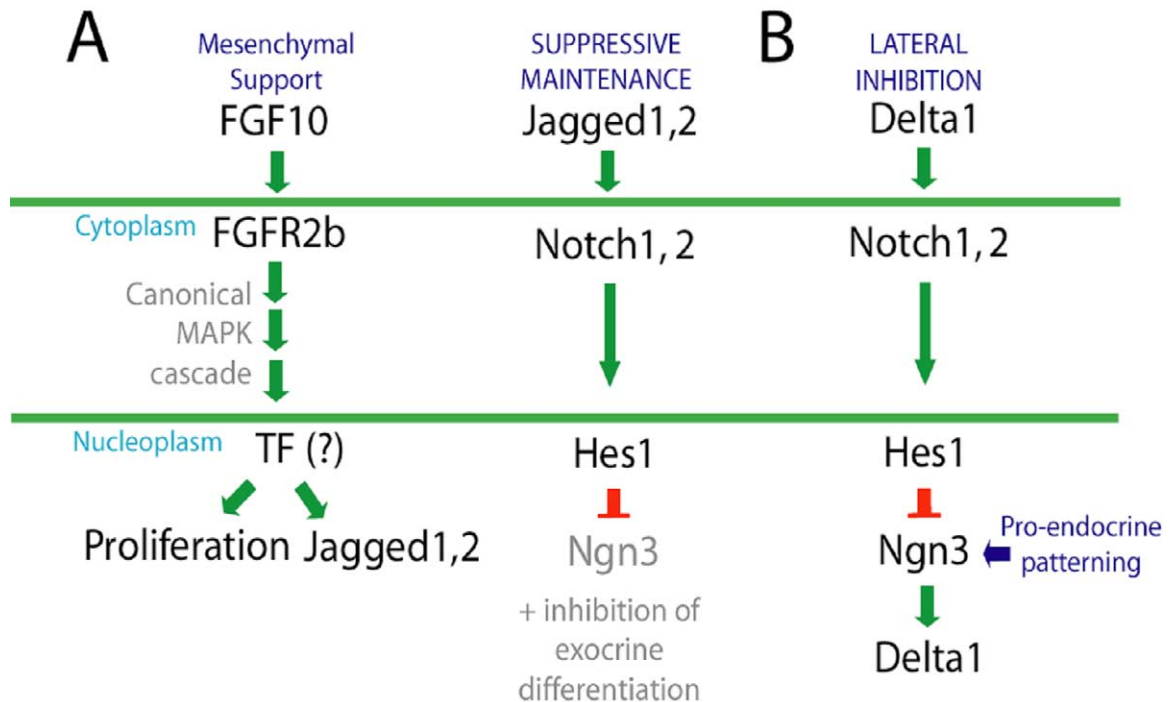


Fig. 7. Bimodal model of Notch signaling. We postulate that Notch activity may be controlled by two independent mechanisms in which either FGF signaling (A, leading to suppressive maintenance), or lateral inhibition (B) may serve to activate Notch receptors. FGF10, normally provided by the mesenchyme, sustains epithelial expression of *Jagged1* and *Jagged2* that may activate *Notch1* and *Notch2*, which are expressed in the same cells. This leads to a general block of differentiation, through *Hes1*. Suppression of differentiation is likewise the outcome of lateral inhibition, although this may be limited to cell–cell contact. Furthermore, this process is dependent on morphogenetic cues (presently unknown) that stimulate *Ngn3* promoter activation in a proper spatial manner (i.e., proendocrine patterning).

opment. Future investigations designed to test for a causal link between these two signaling mechanisms could help clarify to what extent FGF and Notch signaling may interact. Of particular importance is to identify the cell intrinsic regulator(s) that exert the transcriptional effects of FGF10 in the pancreas. Such component(s) could well belong to a family of MAPK-regulated transcription factors. Presently, no good candidates exist among those genes that are known to play roles in the development of the pancreas. Our data suggest that *Jagged1* and *Jagged2* may link FGF10 and Notch signaling. Currently, our data do not allow us to prove that the *Jagged*-genes are immediate targets of the FGF signaling cascade, and further studies are needed to test such a causal link. Of key importance will be to test if *Jagged1* and *Jagged2* promoter activation is a direct or indirect response to FGF10 signaling. Also, it remains a possibility that other signaling pathways may influence the activity state of Notch, some of which may include other ligand/receptor pairs that regulate a MAPK-cascade. Notwithstanding, our data provide evidence that FGF10 is capable of maintaining the pancreatic progenitor cell state, and we believe this knowledge could aid attempts to control pancreatic precursor pool expansion, followed by controlled differentiation in a future β -cell replacement therapy for patients with diabetes.

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