Shh-dependent differentiation of intestinal tissue from embryonic pancreas by activin A

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Summary

The pancreas develops from the endoderm to give rise to ducts, acini and islets of Langerhans. This process involves extracellular signals of the Transforming Growth Factor β (TGFβ) family. The aim of this work was to study the effects of activin A, a member of this family, whose potential role in pancreas differentiation is controversial. To this end, we used pancreatic explants from E12.5 mouse embryos. In culture these explants exhibited spontaneous growth, epithelial morphogenesis and endocrine and exocrine differentiation. Exposure to activin A did not affect exocrine or endocrine differentiation. Surprisingly, activin A induced in the explants the appearance of a large contractile structure surrounded by a cylindrical epithelium, a thick basal lamina and a smooth muscle layer. This structure, the formation of which was prevented by follistatin, was typical of an intestinal wall. Consistent with this interpretation, activin A rapidly induced in the explants the mRNAs for fatty acid binding proteins (FABPs), which are markers of the intestine, but not of the pancreas. We also found that induction of the FABPs was preceded by induction of Sonic hedgehog (Shh), a known inducer of intestinal differentiation in the endoderm. Activin B induced neither Shh nor intestinal differentiation. The activin A-mediated intestinal differentiation was blocked by cyclopamine, an inhibitor of Hedgehog signaling, and it was mimicked by Shh. We conclude that activin A does not appear to affect the exocrine or endocrine components of the pancreas, but that it can promote differentiation of pancreatic tissue into intestine via a Shh-dependent mechanism. These findings illustrate the plasticity of differentiation programs in response to extracellular signals in the pancreas and they shed new light on the regulation of pancreas and intestinal development.

Movie available online

Key words: Activin A, Differentiation, Intestine, Mouse embryo, Pancreas, Sonic hedgehog

Introduction

Mammalian pancreas development is being intensively studied as a model of differentiation of pluripotent precursors into several cell types. In the mouse, the pancreas develops at embryonic day (E) 8.5 from a specified dorsal and ventral territory of the posterior foregut endoderm. At E9-9.5, the prepancreatic epithelium buds out of the endoderm and begins to differentiate into ductal precursors, exocrine precursors, or endocrine precursors which will give rise to cells producing insulin, glucagon, somatostatin or pancreatic polypeptide. At E12.5, morphogenetic events take place, with proliferation and branching of the epithelium within the surrounding mesenchyme, segregation of clusters of hormone-producing cells that delaminate from the epithelium, and organization of ductal cells into canaliculi and of exocrine cells into acini. The endocrine cells become organized into islets of Langerhans at E17-18, just before birth, and the acinar cells start secreting their digestive enzymes shortly after birth (Pictet and Rutter, 1972; Kim and MacDonald, 2002).

All these developmental events depend on extracellular signals that mediate precisely timed cross-talks between epithelial and mesenchymal cells (Kim and Hebrok, 2001; Kim and MacDonald, 2002). Among these signals, TGFβs and activins, which belong to the same superfamily, play a prominent role as they are involved at several stages of pancreas development. The ligands of this superfamily bind to heterodimeric receptors composed of a type II and a type I subunit. The type II subunit is required for ligand binding and it phosphorylates and activates the type I subunit. The latter is responsible for downstream signaling events. The TGFβs act via receptors that are distinct from those of the activin ligands. The three activins are dimers of distinct subunits (βA and βB). Activin A (βA/βA), activin B (βB/βB) and activin AB (βA/βB) share the four activin receptors ActRI (also called ALK2), ActRIB (also called ALK4), ActRII and ActRIIB. The actions of activins, but not of TGFβs, are specifically blocked by follistatin, a physiological inhibitor that binds to them (Massagué and Chen, 2000). Because of the differential expression, in time and space, of the members of this complex signaling network, it is possible that the different TGFβ and activin ligands control different developmental events.

Work in chick embryos showed that pancreas specification requires notochord-mediated repression of Sonic hedgehog (Shh) in the prepancreatic endoderm. This repressive activity can be mimicked by activin B which, unlike activin A, is present in the notochord (Hebrok et al., 1998). In the mouse

embryo, TGFβ1 appears to control later events, as it stimulates endocrine differentiation and promotes apoptosis of the acinar, but not canalar, tissue (Sanvito et al., 1994) and it plays a key role in islet morphogenesis (Miralles et al., 1998a).

As to activin A, which is expressed with its receptors in developing pancreas (Furukawa et al., 1995; Verschueren et al., 1995), its role is not clear. Expression of a dominant-negative ActRII in transgenic mice perturbs both endocrine and exocrine differentiation (Shiozaki et al., 1999). In contrast, $ActRII^{-/-}$ and $ActRII^{+/-}ActRIIB^{+/-}$ mice have impaired endocrine development (Kim et al., 2000). Since these receptors bind not only the three activins, but also several other ligands (Vg1, nodal, BMP4, BMP7, Gdf11 and Lefty2), a role of activin A is not conclusively established by such experiments. In pancreatic explants obtained from E12.5 rat embryos and cultured in the presence of mesenchyme, the exocrine compartment develops normally, but endocrine differentiation is inhibited. This phenomenon can be mimicked by follistatin, suggesting that activin receptor ligands promote endocrine differentiation (Miralles et al., 1998b). However, as follistatin inhibits all the activins, it is not possible to be conclusive about the involvement of activin A per se. In a more direct approach, Ritvos et al. (Ritvos et al., 1995) tested the effect of activin A on cultured mouse pancreatic rudiments from E11 embryos. They concluded that activin A inhibits epithelial morphogenesis and that this effect is prevented by follistatin, but they did not study the effect of activin A on exocrine or endocrine differentiation.

The aim of the present work was to reassess the possible influence of activin A on pancreas differentiation. To this end, we have studied the effect of activin A on mouse pancreatic explants obtained from E12.5 mouse embryos. Our data show that activin A does not appear to affect exocrine or endocrine differentiation. However, we find that activin A induces intestinal differentiation of pancreatic epithelium and mesenchyme, and demonstrate that this involves a Shh-dependent mechanism.

Materials and Methods

Dissection and culture of pancreatic explants

The explants were cultured for 7 days on microporous membranes according to the method of Gittes et al. (Gittes et al., 1996). The dorsal pancreatic bud from embryonic day (E)12.5 129/Swiss mice embryos was excised with a dissecting microscope in Hank's balanced salts solution (HBSS) containing 50 Units/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL Life Technologies). Care was taken to avoid pancreatic tissue proximal to the gut so that the explants were derived exclusively from tissue not contaminated by prospective intestinal tissue. Mice were treated according to the principles of laboratory animal care of the University animal welfare committee. The explants were rinsed once in Medium 199 with Earle's salts (Gibco-BRL Life Technologies) supplied with 10% fetal bovine serum (BioWhittaker), 50 Units/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone (Gibco-BRL Life Technologies). The pancreatic explants were then laid on Millicell® culture plate inserts (Millipore) in standard 24-well plates containing 350 μl of the same medium. No medium was added on top of the filter, so that the explants grew at the air/medium interface. Medium was changed every other day. Activin A (15 ng/ml), activin B (15 ng/ml), follistatin (250 ng/ml) and Shh (200 ng/ml) (all from R&D systems) were dissolved in PBS/0.15% bovine serum albumin (BSA) and were used at the final concentration indicated. Unless mentioned otherwise, all these agents were added with each change of medium, namely on day 1 (after one night in culture), on day 3 and on day 5. Cycloheximide (Sigma) was dissolved in dimethylsulfoxide and added at a final concentration of 30 μg/ml 30 minutes prior to addition of activin A. Cyclopamine (Toronto Research Chemicals Inc.) was dissolved in 95% ethanol to 10 mM (stock solution) and was used at 5 μM in the culture medium. Control explants were exposed to the same quantity of vehicle.

Histological and immunofluorescence analysis

Pancreatic explants were fixed for 30 minutes at room temperature in 4% paraformaldehyde/PBS, and stained overnight at 4°C in 2% Certistain® Lightgreen SF yellowish (Merck) in PBS. The stained explants were included in PBS/4% Seaplaque® GTG® agarose (BioWhittaker Molecular Applications). These agarose-embedded explants were embedded in paraffin and cut into 5 μm sections.

Tissue sections were rehydrated and agarose was dissolved in a boiling 0.01 M citrate solution, pH 6.0 for 10 minutes. Tissue sections were permeabilized for 15 minutes with 0.3% Triton X-100-PBS, and blocked in 3% milk/10% BSA/0.3% Triton X-100 in PBS for 45 minutes at room temperature. Primary antibodies and dilutions were: monoclonal mouse anti-human Ki67 at 1:250 (Pharmingen), rabbit anti-Pdx-1 at 1:1000 (a kind gift from C. V. Wright), monoclonal mouse anti-E-cadherin at 1:50 (BD Transduction Laboratories), rabbit anti-carboxypeptidase A at 1:1000 (Biogenesis Ltd.), monoclonal mouse anti-insulin at 1:500 (Sigma), rabbit anti-glucagon at 1:200 (Dako), rabbit anti-laminin at 1:50 (Sigma), monoclonal mouse anti-smooth muscle actin at 1:40 (Dako). Incubation of primary antibodies was performed in 3% milk/10% BSA/0.3% Triton X-100 in PBS for 1 hour at 37°C. Washes were done with 0.1% Triton X-100 in PBS three times for 5 minutes each. Secondary antibodies and dilutions were: fluorescein isothiocyanate- or Texas-Red® dye-conjugated goat anti-mouse or anti-rabbit IgG at 1:100 (Jackson ImmunoResearch Laboratories, Inc.), or biotin-conjugated affinity purified anti-mouse or anti-rabbit IgG at 1:500 (Chemicon) in 10% BSA/0.3% Triton X-100 in PBS for 1 hour at 37°C. In the latter case streptavidin-Alexa Fluor® 488 conjugate at 1:1000 (Molecular Probes) was added to reveal the biotin. Washes were repeated with 0.1% Triton X-100-PBS three times for 5 minutes each. Slides were stained with bis-benzimide (Sigma), 1 μg/ml in PBS (Hoechst staining) for 10 minutes, rinsed in distillated water and mounted in Dako® fluorescent mounting medium (Dako). Follistatin immunofluorescence was detected with a rabbit anti-follistatin antibody at 1:100 (a kind gift from S. Werner) according to the protocol of Wankell et al. (Wankell et al., 2001). Fluorescence was immediately observed with a Zeiss Axiovert 200 inverted fluorescence microscope. All the pictures were taken using a Coolpix 995 digital camera (Nikon).

RNA preparation and RT-PCR analysis

Total RNA was isolated from individual pancreatic explants with the TriPure RNA Isolation kit (Roche). Total RNA was diluted in 10 μl water, denatured at 85°C for 3 minutes, and quickly chilled on ice. Total RNA was incubated in a final volume of 25 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 40 units of RNAsin (Promega), 3 μg of random hexamers (Invitrogen) and 1 mM dNTPs (Amersham Pharmacia Biotech), and was left for 10 minutes at room temperature and subsequently for 1 hour at 37°C. After cDNA synthesis, the sample was diluted with 25 μl of H_2O. For the PCR, 3 μl of the diluted cDNA sample were incubated in a volume of 30 μl of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (vol/vol) Triton X-100, 1.5 mM $MgCl_2$, 40 μM dATP, dTTP, dCTP and dGTP, 10 pmol of each primer and 2.5 units of Taq polymerase (Promega). For the semi-quantitative PCR, 3 μl of the

diluted cDNA sample were incubated in 50 μl of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (vol/vol) Triton X-100, 1.5 mM $MgCl_2$, 40 μM dATP, dTTP and dGTP, 20 μM dCTP, 10 pmol of each primer, 2.5 units of Taq polymerase (Promega) and 2.5 μCi of 3000 Ci/mmol [^{32}P]dCTP (Amersham Pharmacia Biotech). The number of cycles was determined for each amplicon as required to reach mid-logarithmic phase of amplification. Primer sequences were 5′-ACCCTTCACCAATGACTCCTATG-3′ and 5′-ATGATGACTGCAGCAAATCGC-3′ (TBP, 190 bp), 5′-GATCAGAAAGCTTCATGTGG-3′ and 5′-TCAGGAAGAGCCACACTTCTG-3′ (activin A, 220 bp), 5′-CCGAGGAGGATGTGAACGAC-3′ and 5′-CACCACACAAGTGGAGCTGC-3′ (follistatin, 360 bp), 5′- ACCACCAATGTGGGAGACAGC-3′ and 5′-GGGAGGAGAAGATCTTCACAG-3′ (ALK2, 204 bp), 5′-TGCACGAAGATGCAATTCTGG-3′ and 5′-ATGCGCAGAGCTGTCAGACGG-3′ (ALK4, 230 bp), 5′-TTGATGACTCTCAGGACAATGC-3′ and 5′-CTTGTGAGGCAGGTCCAATCTTC-3′ (carbonic anhydrase II, 244 bp), 5′-GTACCAATTGCAGAGCCAGG-3′ and 5′-CCATTTTATTGTCACCTTCC-3′ (l-FABP, 252 bp), 5′-CGGCACGTGGAAAGTAGACC-3′ and 5′-TCCGTCTGCTAGACTGTAGG-3′ (i-FABP, 197 bp), 5′-GAACGATTTAAGGAACTCACC-3′ and 5′-CCCTCATAGTGTAGAGACTCC-3′ (Shh, 217 bp).

Results

Expression of activin A and its receptors during pancreas development

We started our study by assaying the expression of *activin A*, its two specific type I receptors *ALK2* and *ALK4*, and its inhibitor *follistatin*, during the course of mouse pancreas development. RT-PCR experiments performed on pancreatic RNA from E12.5 embryos showed that these genes were all expressed (Fig. 1A). Their expression remained constant until birth, except that of *ALK2*, which decreased after E12.5 (Fig. 1A). While our work was in progress, results compatible with those shown in Fig. 1A were published by Dichmann et al., (Dichmann et al., 2003). These data indicated that all the components to initiate or modulate activin A signaling are present during pancreas development, which prompted us to test the role of activin A on pancreatic explants in culture.

Differentiation of pancreatic explants in culture

The explants were studied over the 7-day culture period to validate them as an ex vivo model system of pancreas differentiation. Just after dissection at E12.5 (day 0 of culture), the dorsal pancreatic buds in culture showed epithelial cells surrounded by mesenchyme (Fig. 2A). At this stage, the epithelial cells visualized by Hematoxylin and Eosin staining did not exhibit an organized structure (Fig. 2B). After 24 hours in culture (on day 1), the epithelium was beginning to branch and was invading the surrounding mesenchyme, with rounding of the explant (Fig. 2A). Growth and branching morphogenesis of the epithelium continued during the following days and on day 7 the explant consisted of small dark clusters of epithelial cells surrounded by mesenchymal tissue (Fig. 2A). At that stage, histological examination showed an impressive reshaping of the epithelium (Fig. 2B). Numerous acini with a pink coloration of the apical pole and a basal localization of nuclei could be observed. This organization and polarization of epithelial cells is typical of differentiated pancreatic exocrine tissue. Other cells were arranged in duct-like structures with a cuboidal epithelium. Endocrine cells could not be identified with this staining (Fig. 2B).

To determine whether cells in the explants were dividing during the culture, we performed double immunofluorescence experiments with anti-Ki67 and anti-Pdx-1 antibodies. Ki67 is a marker of proliferation. Pdx-1 is a transcription factor expressed in the epithelial pancreatic cells during development and it is used as a marker of pancreatic precursor cells (Hogan and Wright, 1996). As shown in Fig. 2C, on day 1 all the epithelium was strongly stained for Pdx-1 and on day 7 many epithelial cells were still Pdx-1 positive. At these two stages, some Pdx-1-expressing cells were also labeled for Ki67, showing that the epithelium kept proliferating. Mitotic figures were indeed observed in Pdx-1-expressing cells (data not shown). Cell proliferation was also observed in the mesenchyme on day 1 and on day 7, as some cells were stained with the Ki67 antibody, but not with the Pdx-1 antibody (Fig. 2C).

To better characterize epithelial differentiation during the culture, we first visualized the exocrine marker carboxypeptidase A (CPA). Epithelial cells were identified with an anti-E-cadherin (E-cad) antibody (Fig. 3A,C). On day 0, some of these cells, usually located at the periphery of the epithelium in contact with the mesenchyme, expressed CPA. At this stage, CPA-positive cells were not polarized and were not organized into acini (Fig. 3A). On day 7, most of the cells that were stained for E-cad were exocrine cells, as they also expressed CPA (Fig. 3C). As was seen with the Hematoxylin and Eosin staining (Fig. 2B), these exocrine cells were organized in acini, with apical CPA staining and basal localization of nuclei (inset of Fig. 3C). Thus, a typical exocrine differentiation process took place during the culture. Some epithelial cells that were E-cad positive, but CPA negative, were most probably ductal cells (arrowheads in Fig. 3C). Consistent with this interpretation, carbonic anhydrase II mRNA was detected by RT-PCR in the explants at that stage (data not shown).

To assess endocrine differentiation during the culture, the explants were double-stained for insulin and glucagon. On day 0, there were a few clusters of

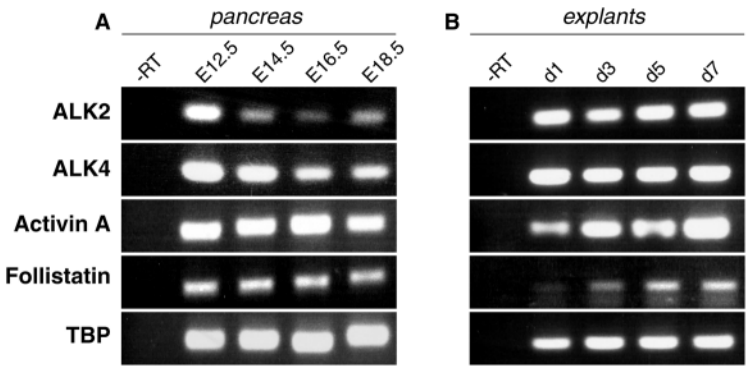

Fig. 1. Expression of activin A signaling components in embryonic mouse pancreas and pancreatic explants. Expression of *activin A*, *follistatin* and type I activin receptors *ALK2* and *ALK4* was assessed by RT-PCR, with TBP mRNA as a reference, on RNA extracted from the pancreas of embryos at the stages indicated (A) or from pancreatic explants cultured for the times (days) indicated (B).

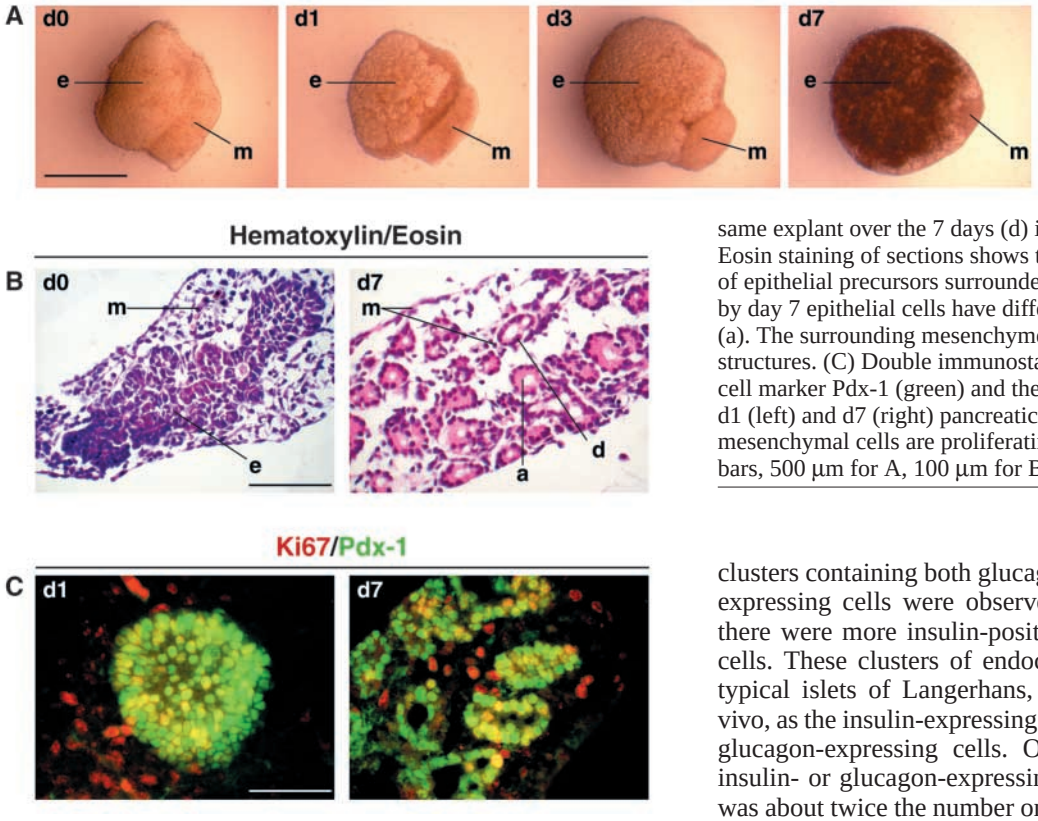

Fig. 2. Differentiation of pancreatic explants in culture. (A) Low magnification microscopy shows rounding of the explant, expansion and branching morphogenesis of the epithelium (e) and redistribution of the mesenchyme (m), for the same explant over the 7 days (d) in culture. (B) Hematoxylin and Eosin staining of sections shows that on day 0 the pancreas consists of epithelial precursors surrounded by mesenchymal cells, and that by day 7 epithelial cells have differentiated into ducts (d) and acini (a). The surrounding mesenchyme has invaded the epithelial structures. (C) Double immunostaining for the pancreatic epithelial cell marker Pdx-1 (green) and the proliferation marker Ki67 (red) in d1 (left) and d7 (right) pancreatic explants. Both epithelial cells and mesenchymal cells are proliferating on day 1 and on day 7. Scale bars, 500 μm for A, 100 μm for B and 50 μm for C.

glucagon-expressing cells and very scarce insulin-expressing cells, scattered in the pancreatic tissue (Fig. 3B). The total numbers of insulin- and glucagon-expressing cells at this stage were 723 and 854 for two explants, as determined by scoring labeled cells on serial sections (150 per explant). On day 7, clusters containing both glucagon-expressing cells and insulin-expressing cells were observed (Fig. 3D). Unlike on day 0, there were more insulin-positive cells than glucagon-positive cells. These clusters of endocrine cells did not yet resemble typical islets of Langerhans, which appear around E18.5 in vivo, as the insulin-expressing cells were not surrounded by the glucagon-expressing cells. On day 7, the total number of insulin- or glucagon-expressing cells (1670±360 s.e.m., n=4) was about twice the number on day 0 (see above). The increase in number of insulin-expressing cells during the culture period could be explained by proliferation of pre-existing insulin-positive cells or, as is the case in vivo, by differentiation of endocrine precursors. As we never detected endocrine cells expressing the proliferation marker Ki67 (data not shown), we assume that these cells differentiate from epithelial precursors,

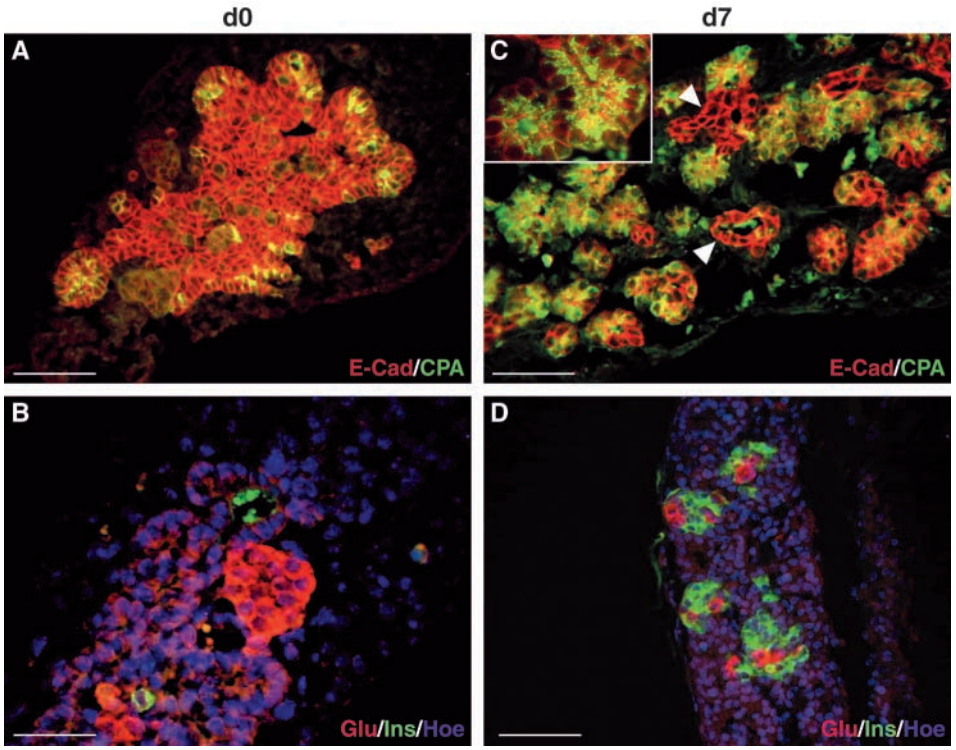

Fig. 3. Ex vivo differentiation of pancreatic precursors. Double immunostaining for CPA (green) and E-cad (red) (A,C) or for insulin (green) and glucagon (red) (B,D) in pancreatic explants on day 0 (A,B) and on day 7 (C,D) in culture. Nuclei are stained blue with Hoechst in B and D. (A) On day 0, only a few of the epithelial cells stained for E-cad express CPA and most of these are located at the periphery (yellow). These CPA positive cells are not polarized and are not organized into acini. (B) On day 0, there are small clusters of glucagon-expressing cells and very few insulin-expressing cells. (C) On day 7, epithelial cells strongly expressing CPA form acini with a characteristic polarization of the cells and a central lumen (inset). Ductal structures that are negative for CPA (arrowheads in C) are also observed. (D) On day 7, insulin-expressing cells, which have appeared during the culture, and glucagon-expressing cells are organized in large endocrine clusters. Scale bars, 100 μm (A-C); 50 μm (B).

thereby reproducing the physiological process of endocrine differentiation.

We concluded from this analysis that the explants of embryonic pancreas cultured under our conditions are a convenient model system to study the differentiation processes that occur in vivo during pancreas development.

Activin A treatment does not affect exocrine or endocrine pancreatic explants

Before testing the effect of activin A on the explants, we verified that they still express the components of this signaling pathway under our culture conditions. RT-PCR experiments showed that *activin A* and its type I receptors *ALK2* and *ALK4* were expressed in the explants as in developing pancreas in vivo (Fig. 1B). Expression of *follistatin* was reduced on day 1, but it returned later to levels similar to those in developing pancreas (Fig. 1).

Activin A was added to the explants on days 1, 3 and 5 of culture, which were observed until day 7. Macroscopic observation of activin A-treated explants (n=89) revealed the appearance of a small cystic structure in 87% of them (Fig. 4). The cyst, which became visible within 24 hours (arrow in Fig. 4), expanded during the following days and, on day 5, it exhibited peristaltic contractions which persisted until the end of the culture period (see Movie 1, http://jcs.biologists.org/supplemental/) (Fig. 4). This phenomenon was also observed after a single addition of activin A on day 1. Occasionally, two such cysts appeared simultaneously in the same activin A-treated explant. Cystic structures were never observed in control cultures (n=96**)** or before activin treatment. Histological analysis of activin A-treated explants revealed that the cystic structure is surrounded by a single-layered epithelium (Fig. 4). This epithelium was metaplasia-like, as both cuboidal cells and cylindrical cells were found on the same sections. In the regions where the epithelium was cylindrical, it sent out villosities into the cavity and the underlying mesenchyme showed a pink coloration, indicating the presence of smooth muscle cells. These features, which were never observed in untreated explants, are typical of the intestinal wall.

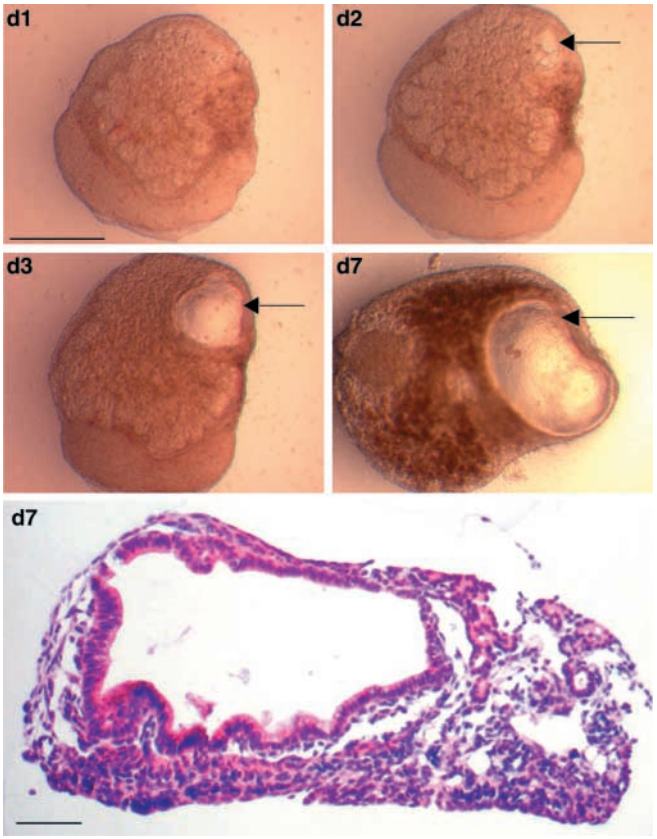

Fig. 4. Morphological and histological changes in pancreatic explants treated with activin A. Activin A, added on day 1 of culture, induces the appearance of a cystic structure within 24 hours (arrow, d2). This structure expands during the following days and peristaltic-like activity of surrounding cells appears on day 5 (see Movie 1, http://jcs.biologists.org/supplemental/). On day 7, Hematoxylin and Eosin staining of sections (bottom panel) shows that the cyst lumen is surrounded by a cylindrical epithelium with villi. Pancreatic tissue is still present, but it is aside of the cyst. Scale bars, 500 μm for upper panels and 100 μm for lower panel.

To confirm the intestinal character of the differentiation process induced by activin A, immunofluorescence experiments were performed with antibodies directed against laminin or smooth muscle actin (SMA) (Fig. 5A). In untreated explants on day 7, all the epithelial structures were surrounded by thin basement membranes, as revealed by laminin staining. Laminin was also found at the periphery of the explant. In contrast, in activin A-treated explants, there was a thick basement membrane underlying the cylindrical epithelium that surrounds the lumen of the cystic structure. Under this basal lamina, mesenchymal cells had differentiated into smooth muscle cells, as they expressed SMA and exhibited spontaneous contractile activity. SMA was never detected in untreated explants (Fig. 5A). As the intestinal structure appeared within 24 hours of activin A treatment, we investigated whether this treatment induced expression of intestinal markers. Fatty acid binding proteins (FABPs) are markers of intestinal differentiation (Gordon et al., 1985), which occur naturally as a liver isoform (l-FABP) and an intestinal isoform (i-FABP). They are coded by different genes and are both present in the intestine. The expression of these genes in our explants was determined by RT-PCR as a function of time. The expression of *i-Fabp* and of *l-Fabp* was induced after 6 and 12 hours of treatment with activin A, respectively (Fig. 5B). These mRNAs were never detected in untreated explants, even after 7 days of culture (Fig. 5B). This ruled out that the culture procedure per se would allow spontaneous differentiation of intestinal precursors present in the explants.

Additional experiments were performed to assess the specificity of the effect of activin A. First, this effect was dose dependent. In explants incubated with 5, 10, 15 or 25 ng/ml the intestinal structure appeared with the latter two concentrations (data not shown). Second, addition of follistatin together with activin A (n=13**)** prevented the appearance of the intestinal structure and neither *i-Fabp* nor *l-Fabp* was expressed in the explants treated in this way (Fig. 5B). Third, unlike activin A, activin B (n=6**)** did not induce an intestinal structure or the FABPs in the explants (Fig. 6). The activin B tested was biologically active since, in transient transfection

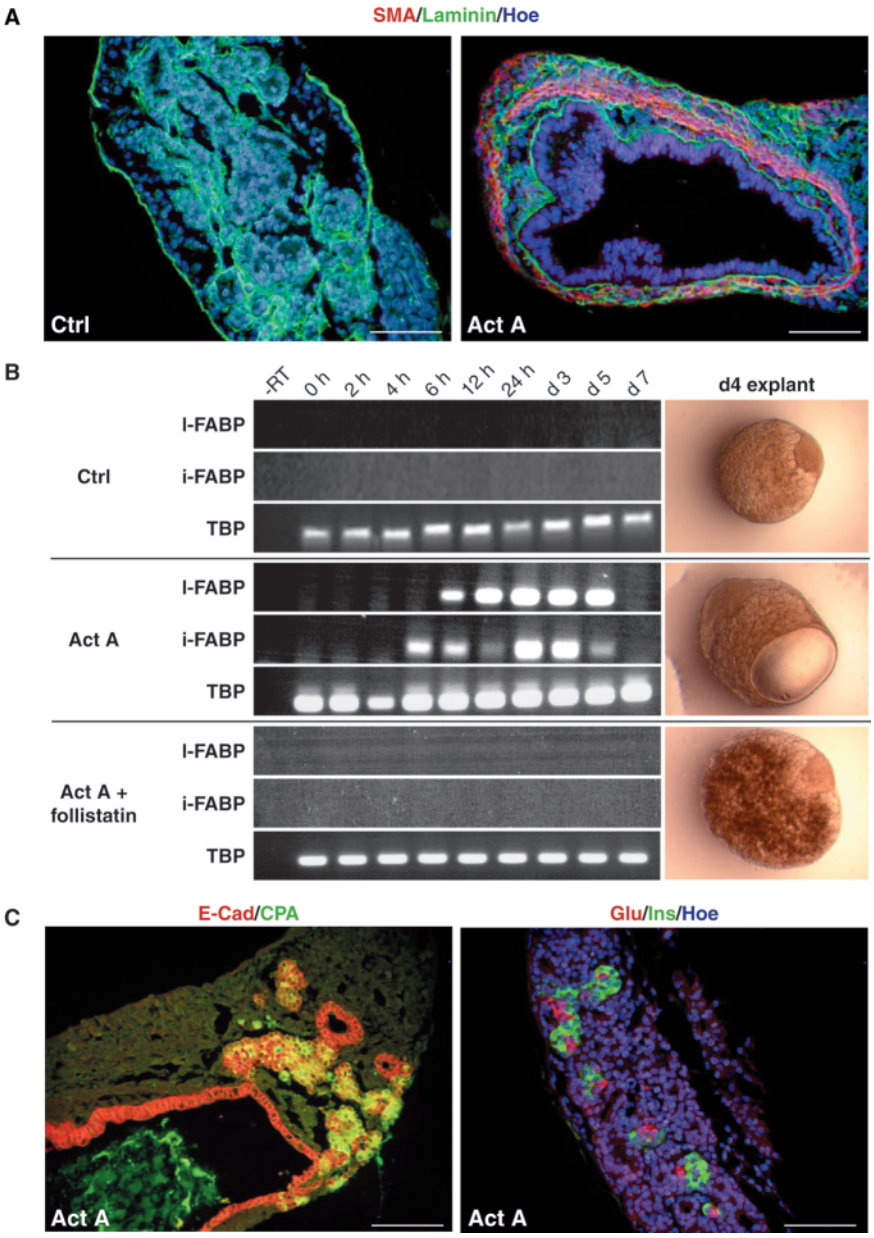

Fig. 5. Pancreatic and intestinal differentiation in explants treated with activin A. (A) Immunostaining for SMA (red), laminin (green) and nuclei (blue) on day 7 in culture in control explants (left panel) or in explants treated with activin A (right panel). In control explants, thin basement membranes, revealed by laminin staining, surround all the epithelial structures. SMA is not detected. In activin A-treated explants, a thick basal lamina separates the cylindrical epithelial cells layer from the smooth muscle layer, which is stained for SMA. This organization is typical of the intestinal wall structure. Scale bars, 100 μm. (B) Expression of FABP mRNAs, with TBP mRNA as a reference, as detected by RT-PCR in untreated explants (upper panel) and in explants treated with activin A (middle panel) or with a mixture of activin A and follistatin (lower panel) for the times indicated starting on day 1 (0 hours). Activin A induces *i-Fabp* and *l-Fabp*, two intestinal markers, and triggers the appearance of an intestinal structure (seen here on day 4 of culture). These effects, which do not occur spontaneously with time in culture, are blocked by follistatin. (C) Immunostaining for E-cad (red) and CPA (green) (left panel) or for glucagon (red), insulin (green) and nuclei (blue) (right panel) on day 7 of culture in activin A-treated explants. Exocrine (E-cad and CPA positive), ductal (E-cad positive and CPA negative) and endocrine (glucagon or insulin positive) cells are found, organized as in control explants (see Fig. 3). Note that the epithelium surrounding the lumen is positive for E-cad, but not for CPA. Scale bars, 100 μm.

assays, it stimulated a CAGA-luciferase reporter gene (which is sensitive to activin signaling) as strongly as did activin A (data not shown). We concluded that induction of an intestinal phenotype in developing pancreas is specific to activin A.

Pancreatic tissue persisted alongside this intestinal structure in the activin A-treated explants (Fig. 4). To determine the possible effect of activin A on exocrine or endocrine differentiation in the pancreatic region of the explant, we performed the same immunofluorescence experiments as described above (Fig. 3) for the untreated explants on activin A-treated explants. As in the latter, E-cad-positive cells expressing CPA were organized into acini (Fig. 5C). Duct-like structures, positive for E-cad but negative for CPA, were also present in the activin A-treated explants. The epithelium surrounding the cystic structure was positive for E-cad, but it did not express CPA. We also analyzed, by insulin and glucagon staining, the consequences of activin A treatment on endocrine differentiation. The pancreatic region of activin A-treated explants showed clusters of cells containing insulin-expressing cells and glucagon-expressing cells (Fig. 5C), as observed in untreated explants (Fig. 3B,D). The total number of endocrine cells, assessed by counting them on 150 serial sections of the pancreatic tissue in day-7 explants, was not increased by the activin A treatment (1402 and 1014 cells for two explants, as compared to a mean of 1670 cells for four untreated explants, see above). Similar data were obtained on explants treated with 5, 10 or 25 ng/ml of activin A.

We concluded from these experiments that activin A does not affect exocrine or endocrine differentiation in our model. However, under the influence of activin A, the pancreatic epithelium and mesenchyme undergo a rapid intestinal differentiation, as shown by molecular markers, by histological and immunohistological criteria and by a functional parameter i.e. spontaneous contractions.

Intestinal differentiation of embryonic pancreas by activin A involves Shh

We next investigated the mechanism by which activin A induces intestinal differentiation of pancreatic tissue. Shh plays

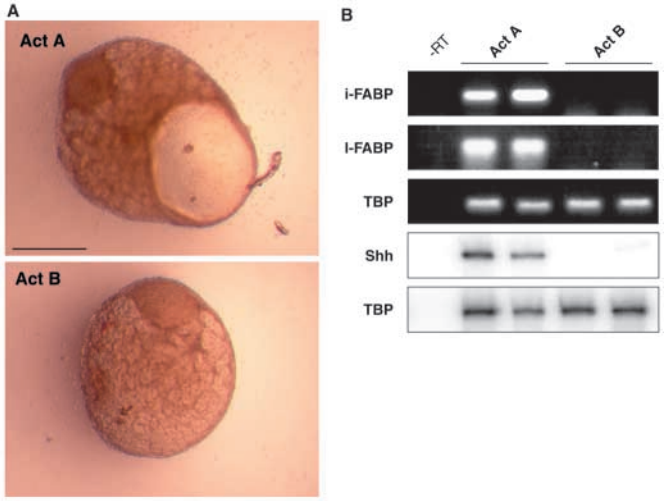

Fig. 6. Intestinal differentiation in pancreatic explants is induced by activin A, but not by activin B. Treatment of explants with activin A leads to the appearance of an intestinal structure (A) seen here on day 4 in culture (scale bar, 500 μm) and induces the intestinal markers *i-Fabp* and *l-Fabp*, detected by RT-PCR on day 7, as well as *Shh*, detected by semi-quantitative RT-PCR on day 7 (B). None of these effects are seen when activin B is used instead of activin A.

a key role in differentiation of the intestine in the chick embryo (Roberts et al., 1998; Sukegawa et al., 2000). We therefore determined by semi-quantitative RT-PCR whether *Shh* expression was induced in activin A-treated explants. Shh was barely detectable in untreated explants over the culture period (Fig. 7A), consistent with previous observations (Apelqvist et al., 1997; Hebrok, 2003). Following a single addition of activin A on day 1, *Shh* expression increased by about 3.5-fold within the two first hours of treatment and showed a further 50-fold increase after 6 hours of treatment. *Shh* expression decreased after 24 hours, but was maintained until day 7 (Fig. 7A). The rapid (less than 2 hours) induction of *Shh* by activin A was consistent with a direct effect of activin A signaling on *Shh* gene expression. To test this, we incubated explants with activin A in the presence of the protein synthesis inhibitor cycloheximide. This prevented induction of *Shh*, as well as of the *Fabps* (Fig. 7B). Thus, it appears that a protein intermediate is required for the induction of *Shh* by activin A. The induction of *Shh* expression by activin A was specific. This effect was not observed with activin B (Fig. 6), which, as mentioned above, did not induce intestinal differentiation. Moreover, when intestinal differentiation was prevented by adding follistatin together with activin A, *Shh* was not induced (Fig. 7A).

After addition of activin A, Shh mRNA increased before the FABPs mRNA. This suggested that Shh is involved in the intestinal differentiation process. To confirm this, we treated three explants with activin A and cyclopamine, a specific inhibitor of Hh signaling that binds to the Smoothened transmembrane protein (King, 2002). As shown in Fig. 7C, no intestinal structure was observed in explants treated with activin A and cyclopamine. If Shh mediates the effects of activin A reported here, these effects should be mimicked by Shh. This was the case. Explants treated with Shh (n=6) exhibited the same intestinal phenotype as activin A-treated explants (Fig. 7C). Moreover, treatment with Shh induced *i-Fabp* and *l-Fabp* (Fig. 7D) as did treatment with activin A. These data showed that induction of Shh by activin A is not an epiphenomenon, but that Shh mediates the effect of activin A in inducing intestinal differentiation of pancreatic tissue.

We conclude from all these data that activin A can induce Shh expression in embryonic pancreas, that this effect occurs prior to induction of the intestinal markers FABPs, and that functional Shh signaling is required for activin A to induce intestinal differentiation from the pancreatic epithelium.

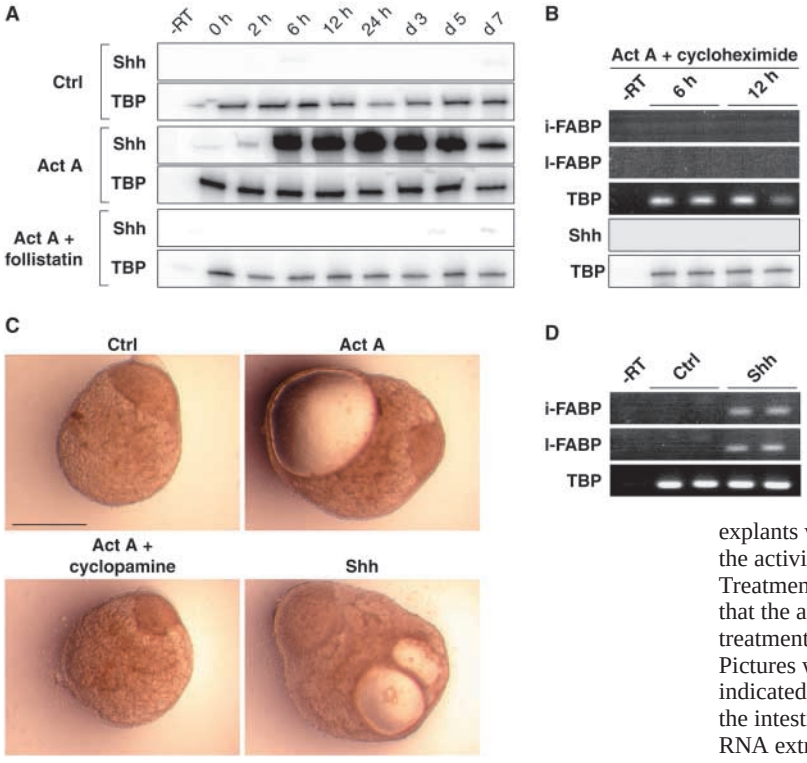

Fig. 7. Intestinal differentiation in pancreatic explants by activin A depends upon an indirect induction of Shh. (A) *Shh* expression in pancreatic explants, measured by semi-quantitative RT-PCR as a function of time for 7 days, is induced rapidly after a single addition of activin A (middle panels). This induction of *Shh*, which does not occur spontaneously with time in culture (upper panels), is inhibited by follistatin (lower panels). (B) Cycloheximide prevents induction of *Shh* and of the *Fabps* (measured as described in Fig. 6) by activin A in pancreatic explants. (C) Treatment of pancreatic explants with cyclopamine, a Hedgehog signaling inhibitor, prevents the activin A-induced appearance of the intestinal structure. Treatment of explants with Shh mimics the effect of activin A. Note that the appearance of two cystic structures is not specific of Shh treatment, as it can also be observed after activin A treatment. Pictures were taken on day 4, after a single addition of the agents indicated. Scale bar, 500 μm. (D). Shh induces in pancreatic explants the intestinal markers *i-Fabp* and *l-Fabp*. RT-PCR were performed on RNA extracted on day 7 of culture.

Discussion

The aim of this work was to investigate the effect of activin A on pancreas differentiation. We resorted to an organ culture which, as we show here, mimicks pancreas development in vivo. Our model differs from those of Ritvos et al. (Ritvos et al., 1995) and of Gittes et al. (Gittes et al., 1996) who use rudiments from E11 embryos. The explants studied here, obtained from E12.5 embryos, contain exclusively pancreatic tissue with its surrounding mesenchyme. Under our culture conditions, these explants exhibited spontaneous developmental changes similar to those observed in the intact embryo. Both the epithelial cells and the mesenchymal cells proliferated, the mesenchyme became reorganized, and the epithelium underwent branching and budding with formation of acini and ducts. Exocrine and endocrine differentiation took place, with segregation of hormone-expressing cells into clusters. Moreover, activin A and its receptors, as well as its physiological inhibitor follistatin, were expressed in the explants. Thus, such explants were appropriate to test the effect of activin A on pancreatic cytodifferentiation.

The data reported here do not support the idea that activin A controls exocrine or endocrine differentiation. Nevertheless, the expression of both activin A and follistatin in developing pancreas suggests a role for the activin-follistatin system. From work on pancreatic rudiments of E11 mouse embryos, Ritvos et al. (Ritvos et al., 1995) concluded that activin A inhibits epithelial morphogenesis. We could not determine conclusively whether this effect of activin A also occurs at a later stage, i.e. in our E12.5 explants. We observed a reduction in the amount of pancreatic epithelium in activin A-treated explants, but this could result from reduced epithelial morphogenesis or from differentiation of part of the epithelium into intestinal epithelium. However, it is likely that the activin-follistatin system still controls epithelial morphogenesis at E12.5 in the pancreas. Indeed, follistatin-treated explants showed more growth and branching, over time in culture, than control explants (Fig. 5B).

Unexpectedly, activin A induced intestinal differentiation in the pancreatic explants. This was confirmed by molecular, histochemical, morphological and functional criteria. The cylindrical epithelium of this intestinal structure strongly expressed Pdx-1 (data not shown), which is not only a pancreatic marker, but also a duodenal marker (Jonsson et al., 1994). However, we did not detect the intestinal marker cdx-2 in this epithelium. In the intact embryo at E15.5, immunofluorescence experiments with an anti-cdx-2 antibody revealed strong labeling of the small intestine, but not of the duodenum (data not shown). We conclude that the intestinal structure induced in the explants by activin A more closely resembles the duodenum than the jejunum or the ileum.

As to the mechanism by which activin A induces conversion of pancreatic tissue into intestinal tissue, we found that this effect involves Shh signaling. Our data extend those of Apelqvist et al. (Apelqvist et al., 1997) who showed that incubating pancreatic rudiments, taken from E10 mouse embryos, with Shh for 7 days induces the conversion of pancreatic mesenchyme into intestinal mesenchyme. We show here that the intestinal phenotype induced in our explants by activin A involves not only the mesenchyme, which differentiates into smooth muscle, but also the pancreatic epithelium, which becomes villous and in which cells are cylindrical and no longer cuboidal. We also demonstrate that activin A can act upstream of Shh in this differentiation program. An increased expression of Shh has been reported when activin A induces dorsal mesoderm in animal caps of *Cynops* embryos (Takabatake et al., 1996). The finding that activin A can induce Shh in differentiating pancreas should be taken into account to interpret the phenotype of knockout mouse embryos lacking activin receptors (Kim et al., 2000). One conclusion of the analysis of such mutants was that activin signaling in the foregut endoderm inhibits Shh expression. It is noteworthy that these receptors bind not only activin A, but also activin B. It has been assumed that activin A and activin B are functionally interchangeable (Dichmann et al., 2003). We show here that this is not the case. This fits with the observation that activin A and activin B transcripts have quite distinct distribution patterns in E12.5 mouse embryos (Feijen et al., 1994). Thus, there is no contradiction in the facts that activin B would repress Shh expression in the prepancreatic territory of the endoderm (Hebrok et al., 1998) and that activin A can induce Shh in committed pancreatic tissue, as demonstrated here. Therefore, the activin pathway can elicit opposite effects on Shh expression, depending on the ligand, target tissue and developmental stage. Similar opposing effects of bone morphogenetic proteins (BMP) at different stages of pancreatic development have been reported (Kumar and Melton, 2003).

Work on transgenic mice has led to a model in which it is Shh expression in a limited lateral territory of the endoderm that promotes intestinal differentiation from that territory, whereas the flanking, Shh-negative, dorsal and ventral regions become pancreatic tissue (Apelqvist et al., 1997). Activin A is expressed with its receptors in developing pancreas in vivo. Since activin A can induce Shh, why does this tissue not differentiate into intestinal tissue? One explanation was that Patched, the Shh receptor, is not expressed in embryonic pancreas, as it was not detectable by in situ hybridization (Apelqvist et al., 1997). However, Patched expression was detected more recently in this tissue by RT-PCR and Patched mutants exhibit developmental defects in the pancreas (Hebrok et al., 2000). Thus, the observations on the activin A-treated pancreatic explants reported here demonstrate that the signaling pathway downstream of Shh is functional in embryonic pancreas and that the latter is not refractory to Shh. We are left with the hypothesis that, in the intact embryo, the pancreas must be refractory to endogenous activin A. Since we were able to trigger a response to activin A, i.e. intestinal differentiation, this refractoriness must be due to a factor upstream of the activin A receptors. An obvious candidate is follistatin, which is expressed in the pancreas at E12.5 (Miralles et al., 1998b) (this paper). This strongly suggests that it is the balance between activin A and follistatin that determines tissue sensitivity, and that this balance would be in favor of activin A in the intestine, but not in the pancreas. Consistent with this hypothesis, immunoreactive follistatin was detected at E12.5 in the pancreas, but not in the intestine (data not shown). In our pancreatic explants, the drop in follistatin expression (Fig. 1B) would allow intestinal differentiation to proceed upon addition of sufficient exogenous activin A.

One condition for pancreas development from the endoderm is repression of Shh by signals from the mesenchyme (Hebrok

et al., 1998). The data reported here suggest that, once endodermal precursors are committed to a pancreatic fate, Shh repression must be maintained in the developing pancreas. Indeed, when Shh was added, or was induced by activin A in pancreatic tissue already specified, this tissue could still assume an intestinal fate. This observation refines our knowledge on the hierarchy of the molecular determinants of developmental processes. Chronologically, these processes involve competence, specification and differentiation. In endoderm-derived organs, such as the pancreas and liver, progress through these three stages is determined by extracellular signals that instruct the tissue to express distinct patterns of transcription factors. These in turn direct gene expression programs that specify the tissue cell type (reviewed by Edlund, 2002; Zaret, 2002). Thus, ectopically expressed transcription factors can drive pancreatic organogenesis and differentiation (Grapin-Botton et al., 2001), or convert pancreatic exocrine cells into hepatocytes (Shen et al., 2000). We show here that, even after specification, a tissue-specific program can still be reshuffled, not only by transcription factors, but also by an extracellular signal such as activin A. This, together with earlier work by others (reviewed by Scharfmann, 2000; Kim and Hebrok, 2001), demonstrates that apparently well-established gene expression programs, i.e. the corresponding transcription networks, remain subordinate to continuous control by extracellular signals. The remarkable developmental plasticity of committed epithelia has interesting implications for the generation of differentiated cells for therapeutic purposes. The present findings raise the important issue of the need to also control the cellular environment of the targeted tissue. Finally, Thayer et al. (Thayer et al., 2003) have recently shown that inappropriate expression of Shh in the pancreas can lead to metaplasia of the epithelium and to adenocarcinoma. This raises the question of the control of the Shh pathway in the pancreas. In this context, our discovery that Shh signaling can be triggered by activin A and inhibited by follistatin is of obvious interest.

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References

Apelqvist, A., Ahlgren, U. and Edlund, H. (1997). Sonic hedgehog directs specialized mesoderm differentiation in the intestine and pancreas. *Curr. Biol.* **7**, 801-804.

Dichmann, D. S., Miller, C. P., Jensen, J., Heller, R. S. and Serup, P. (2003). Expression and misexpression of members of the FGF and TGFβ families of growth factors in the developing mouse pancreas. *Dev. Dyn.* **226**, 663-674.

Edlund, H. (2002). Pancreatic organogenesis – developmental mechanisms and implications for therapy. *Nat. Rev. Genet.* **3**, 524-532.

Feijen, A., Goumans, M. J. and van den Eijnden-van Raaij, A. J. M. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* **120**, 3621-3637.

Furukawa, M., Eto, Y. and Kojima, I. (1995). Expression of immunoreactive activin A in fetal rat pancreas. *Endocr. J.* **42**, 63-68.

Gittes, G. K., Galante, P. E., Hanahan, D., Rutter, W. J. and Debas, H. T. (1996). Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. *Development* **122**, 439-447.

Gordon, J. I., Elshourbagy, N., Lowe, J. B., Liao, W. S., Alpers, D. H. and Taylor, J. M. (1985). Tissue specific expression and developmental regulation of two genes coding for rat fatty acid binding proteins. *J. Biol. Chem.* **260**, 1995-1998.

Grapin-Botton, A., Majithia, A. R. and Melton, D. A. (2001). Key events in pancreatic formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. *Genes Dev.* **15**, 444-454.

Hebrok, M. (2003). Hedgehog signaling in pancreas development. *Mech. Dev.* **120**, 45-57.

Hebrok, M., Kim, S. K. and Melton, D. A. (1998). Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev.* **12**, 1705-1713.

Hebrok, M., Kim, S. K., St-Jacques, B., McMahon, A. P. and Melton, D. A. (2000). Regulation of pancreas development by hedgehog signaling. *Development* **127**, 4905-4913.

Hogan, B. L. M. and Wright, V. E. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* **122**, 983-995.

Jonsson, J., Carlsson, L., Edlund, T. and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **371**, 606-609.

Kim, S. K. and Hebrok, M. (2001). Intercellular signals regulating pancreas development and function. *Genes Dev.* **15**, 111-127.

Kim, S. K. and MacDonald, R. J. (2002). Signaling and transcriptional control of pancreatic organogenesis. *Curr. Opin. Genet. Dev.* **12**, 540-547.

Kim, S. K., Hebrok, M., Li, E., Oh, S. P., Schrewe, H., Harmon, E. B., Lee, J. S. and Melton, D. A. (2000). Activin receptor patterning of foregut organogenesis. *Genes Dev.* **14**, 1866-1871.

King, R. W. (2002). Roughing up Smoothened: chemical modulators of Hedgehog signaling. *J. Biol.* **1**, 8.

Kumar, M. and Melton, D. (2003). Pancreas specification: a budding question. *Curr. Opin. Genet. Dev.* **13**, 401-407.

Massagué, J. and Chen, Y. (2000). Controling TGF-β signaling. *Genes Dev.* **14**, 627-644.

Miralles, F., Battelino, T., Czernichow, P. and Scharfmann, R. (1998a). TGF-β plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. *J. Cell Biol.* **143**, 827-836.

Miralles, F., Czernichow, P. and Scharfmann, R. (1998b). Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development* **125**, 1017-1024.

Pictet, R. L. and Rutter, W. J. (1972). Development of the embryonic endocrine pancreas. In *Handbook of Physiology: The Endocrine Pancreas* (ed. D. Steiner and N. Freinkel), pp. 25-66. Baltimore, Md, USA: Williams and Wilkins.

Ritvos, O., Tuuri, T., Eramaa, M., Saino, K., Hilden, K., Saxen, L. and Gilbert, S. F. (1995). Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mech. Dev.* **50**, 229-245.

Roberts, D. J., Smith, D. M., Goff, D. J. and Tabin, C. J. (1998). Epithelial-mesenchymal signaling during the regionalization of the chick gut. *Development* **125**, 2791-2801.

Sanvito, F., Herrera, P. L., Huarte, J., Nichols, A., Montesano, R., Orci, L. and Vassalli, J. D. (1994). TGF-β1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* **120**, 3451-3462.

Scharfmann, R. (2000). Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. *Diabetologia* **43**, 1083-1092.

Shen, C.-N., Slack, J. M. W. and Tosh, D. (2000). Molecular basis of transdifferentiation of pancreas to liver. *Nat. Cell Biol.* **2**, 879-887.

Shiozaki, S., Tajima, T., Zhang, Y., Furukawa, M., Nakazato, Y. and Kojima, I. (1999). Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mice expressing the truncated type II activin receptor. *Biochem. Biophys. Acta* **1450**, 1-11.

Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S. and Fukuda, K. (2000). The concentric structure of the developing gut is regulated by Sonic Hedgehog derived from endodermal epithelium. *Development* **127**, 1971-1980.

Takabatake, T., Takahashi, T. C., Inoue, K., Ogawa, M. and

Takeshima, K. (1996). Activation of two *Cynops* genes, fork head and sonic hedgehog, in animal cap explants. *Biochem. Biophys. Res. Commun.* **218**, 395-401.

Thayer, S. P., Pasca di Magliano, M., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V. et al. (2003). Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **425**, 851-856.

Verschueren, K., Dewulf, N., Goumans, M. J., Lonnoy, O., Feijen, A., Grimsby, S., Vandi Spiegle, K., ten Dijke, P., Moren, A., Vanscheeuwijck, P. et al. (1995). Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. *Mech. Dev.* **52**, 109-123.

Wankell, M., Kaesler, S., Zang, Y-Q., Florence, C., Werner, S. and Duan, R. (2001). The activin binding proteins follistatin and follistatin-related protein are differentially regulated in vitro and during cutaneous wound repair. *J. Endocrinol.* **171**, 385-395.

Zaret, K. S. (2002). Regulatory phases of early liver development: paradigms of organogenesis. *Nat. Rev. Genet.* **3**, 499-511.