Notochord repression of endodermal Sonic hedgehog permits pancreas development

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Notochord signals to the endoderm are required for development of the chick dorsal pancreas. Sonic hedgehog (SHH) is normally absent from pancreatic endoderm, and we provide evidence that notochord, in contrast to its effects on adjacent neuroectoderm where SHH expression is induced, represses SHH expression in adjacent nascent pancreatic endoderm. We identify activin-βB and FGF2 as notochord factors that can repress endodermal SHH and thereby permit expression of pancreas genes including *Pdx1* and insulin. Endoderm treatment with antibodies that block hedgehog activity also results in pancreatic gene expression. Prevention of SHH expression in prepancreatic dorsal endoderm by intercellular signals, like activin and FGF, may be critical for permitting early steps of chick pancreatic development.

[*Key Words:* Pancreas; endoderm; chicken; fibroblast growth factor; activins; hedgehog]

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Signals between endoderm and mesoderm govern the identity and patterning of vertebrate respiratory and digestive organs including the trachea, lungs, stomach, intestines, and pancreas (Golosow and Grobstein 1962; Wessels and Cohen 1967; Haffen et al. 1989; Bellusci et al. 1997). At least two distinct endoderm–mesoderm interactions are necessary for development of the pancreas. Early signals from the notochord to the endoderm permit dorsal pancreatic morphogenesis, and initiate and maintain expression of genes required for pancreas development (Kim et al. 1997a,b), including the homeodomain transcription factors PDX1 and ISL1 (Jonsson et al. 1994; Ahlgren et al. 1996, 1997; Offield et al. 1996). Later signals from the mesenchyme to the endoderm permit subsequent development of both dorsal and ventral pancreatic buds, which later fuse (Golosow and Grobstein 1962; Le Douarin and Bussonnet 1966; Wessels and Cohen 1967; Dieterlen-Lièvre 1970). Despite various efforts, the signaling molecules involved in pancreatic cell interactions have not been identified (for review, see Slack 1995).

Expression of Sonic hedgehog (*Shh*), a potent intercellular patterning signal, is strikingly absent from pancreatic endoderm, in contrast to uniform endodermal *Shh* expression in the anlagen of organs rostral or caudal to the pancreas (Ahlgren et al. 1997; Apelqvist et al. 1997; Kim et al. 1997b). Ectopic SHH expression results in abnormal morphogenesis and gene expression in lungs (Bellusci et al. 1997), neural tube (Echelard et al. 1993; Pourquié et al. 1993; Ericson et al. 1995), and limb buds (Masuya et al. 1995; Riddle et al. 1995) demonstrating the importance of limiting *Shh* expression in these organs. Ectopic SHH expression in the mouse pancreas during the later epithelial–mesenchymal signaling does not affect endocrine or exocrine cytodifferentiation, but does prevent proper morphogenesis (Apelqvist et al. 1997). This suggests that late maintenance of *Shh* repression is crucial for normal pancreas morphogenesis. Here we ask whether early cell interactions necessary to initiate pancreatic development depend on repression of *Shh* expression in endoderm.

In this study we investigate the early role of notochord signals in patterning chick pancreatic endoderm. The results suggest a simple model in which notochord signals prevent *Shh* expression in foregut endoderm, thereby permitting pancreatic development. Grafting experiments demonstrate that notochord can repress endodermal *Shh*. Notochord removal before completion of notochord–endoderm signaling results in ectopic *Shh* expression in the pancreatic anlage, abnormal morphogenesis, and prevents gene expression required for endocrine and exocrine differentiation. We further show that two signaling factors expressed in notochord during pancreas specification, activin-βB and fibroblast growth factor 2 (FGF2), repress endodermal *Shh* and induce expression of pancreatic genes including *Pdx1* and insulin. Inhibition of SHH in isolated prepancreatic endoderm with an antibody that neutralizes Hedgehog activity is also sufficient to induce pancreatic gene expression. Thus, in con-

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trast to neural and somitic induction that require SHH from notochord, pancreatic endodermal differentiation is inhibited by SHH, and requires notochord signals to prevent SHH activity in endoderm.

Results

Shh *expression is absent in pancreatic endoderm*

In situ hybridization of stage-11 (13 somites) chicks reveals uniform *Shh* expression in columnar epithelium of the prechordal foregut, the extreme rostral limit of definitive endoderm (Fig. 1A). Floor plate expression of *Shh* is evident, and notochord is absent at this rostral level. Ventral foregut cells in the stomach/duodenal anlage at stage 11 have a columnar shape and express *Shh* (Fig. 1B). Dorsal foregut epithelial cells adjacent to notochord, however, have a thinner squamous cuboidal shape and do not express detectable *Shh*. In contrast, expression of *Shh* in endoderm caudal to the anterior intestinal portal, including the pancreatic, midgut, and hindgut anlagen (Fig. 1C), appears as two ventrolateral stripes separated by midline endoderm, which does not express *Shh*. Endoderm in this region has a squamous morphology until later stages when the notochord and dorsal endoderm separate and the endoderm, covered by mesenchyme, assumes a columnar shape (Fig. 1D–F). Thus, patterns of gut epithelial cell shape and *Shh* expression correlate with proximity of the notochord to the epithelial cell layer (Fig. 1; upper schematic). Early endodermal *Shh* expression in mice (Echelard et al. 1993; Bitgood and McMahon 1995; Apelqvist et al. 1997) was known to vary along the rostrocaudal axis, but previous studies did

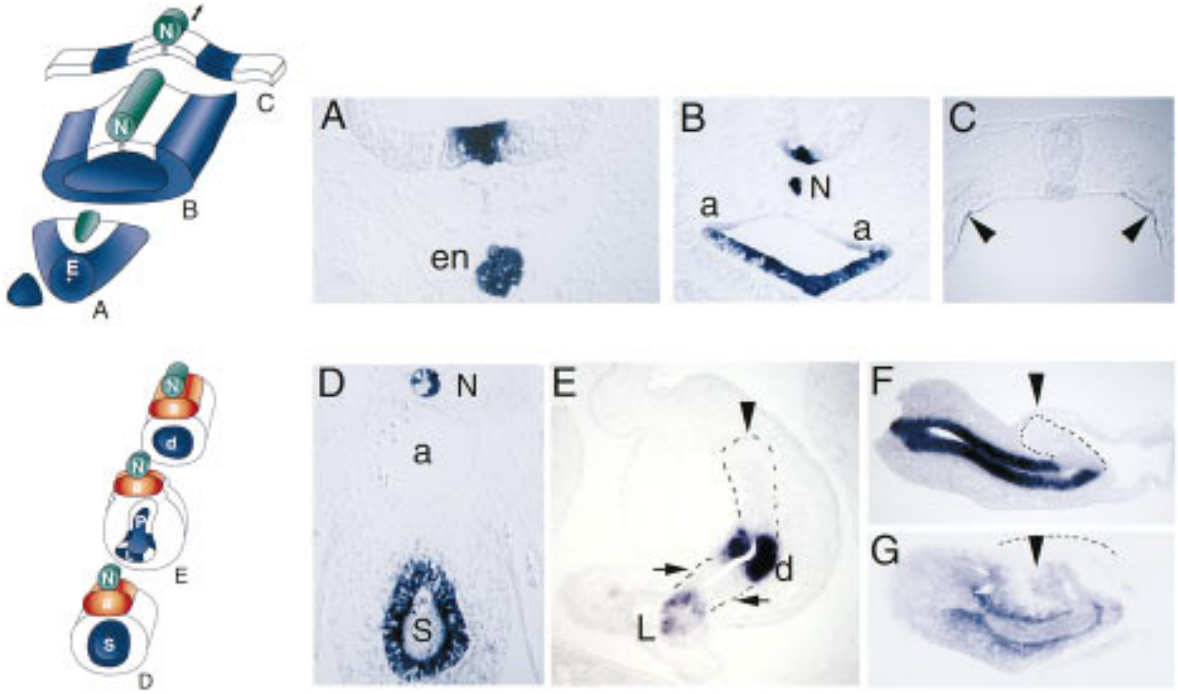

Figure 1. Patterns of *Shh* and *Ptc* expression in endoderm and pancreas. Upper schematic shows transverse sections through gut endoderm (E) and adjacent tissues including notochord (N) at stage 11 (*A–C*). Endoderm without *Shh* expression is white, endoderm with *Shh* expression is blue. Lower schematic shows transverse sections through notochord (N), aorta (a), stomach (S), pancreas (P), and duodenal (d) endoderm and adjacent mesenchyme at stage 18–19 (*D–G*). (*A*) In situ hybridization with *Shh* probe of transverse section through prechordal endoderm at the extreme rostral end of the digestive tract. *Shh* expression in the floor plate of the neural tube is detected dorsal to endodermal (en) cells also expressing *Shh*. Note absence of notochord at this rostrocaudal level. (*B*) *Shh* expression in foregut endoderm rostral to the anterior intestinal portal. *Shh* expression is evident in floor plate cells and notochord (N), dorsal to endoderm expression. Note absence of dorsal endodermal *Shh* expression and the transition from columnar shape of ventral endodermal cells to squamous shape of dorsal midline endoderm. Paired dorsal aortas are marked ''a''. (*C*) Midgut/hindgut *Shh* expression. Arrowheads point to two stripes of endodermal *Shh* expression. Endoderm medial and lateral to these *Shh*-expressing cells does not express detectable *Shh*. Floor plate and regressing Hensen's node cells at this caudal level do not express detectable *Shh* at this stage. (*D*) *Shh* expression in stomach (S) endoderm and notochord (N) after aortas (a) fuse. Expression of *Shh* is evident in dorsal and ventral columnar epithelial cells. (*E*) Endodermal *Shh* expression in the pancreatic anlage. Endoderm in the nascent dorsal (arrowhead) and two ventral pancreatic buds (arrows) does not express *Shh* in contrast to strong expression in duodenal (d) endoderm between the buds and ventral common bile duct endoderm in the liver (L). (*F*) Sagittal section of dissected foregut revealing *Shh* expression anterior to the dorsal pancreas bud (arrowhead and dotted outline) in stomach and duodenal endoderm. Space adjacent to the dorsal pancreas bud is the lumen of the right omphalomesenteric vein. (*G*) Sagittal section revealing foregut expression of *Ptc* in mesenchyme adjacent to stomach and duodenal endoderm and in dorsal pancreas endoderm (arrowhead), but little detectable *Ptc* in dorsal pancreas mesenchyme (outlined by dashed line). (*A–G*) Dorsal is at the top. (*F,G*) Anterior is at *left*.

not correlate notochord–endoderm proximity with endodermal patterns of cell shape and *Shh* expression.

By stage 15 (25 somites), midline fusion of the paired dorsal aortas separates the notochord from the foregut (Fig. 1, bottom schematic). The chick pancreas derives from a dorsal and paired ventral endodermal evaginations that express *Pdx1* and *Isl1* (Kim et al. 1997b), and later fuse. At stage 15 and in later stages, *Shh* is expressed uniformly in enteric endoderm rostral (Fig. 1D) and caudal to the pancreas but is absent from the dorsal and ventral pancreatic endoderm in chicks (Fig. 1E). In mice, which also have dorsal and ventral pancreatic buds, pancreatic *Shh* expression is similar to that in chicks at later stages (Ahlgren et al. 1997; Kim et al. 1997b).

Expression of *patched* (*Ptc*), a receptor for *Shh* (Goodrich et al. 1996; Marigo et al. 1996), is also similar in chicks and mice. *Ptc* transcription is induced by SHH; thus, high levels of *Ptc* expression indicate abundant SHH. Endodermal SHH in the gut, including stomach and duodenum (Fig. 1F), is flanked by high levels of *Ptc* expression in adjacent mesenchyme (Fig. 1G). We detect very little *Ptc* expression in mesenchyme adjacent to pancreatic endoderm, however, consistent with absence of *Shh* in pancreatic endoderm (Fig. 1F). Endodermal *Ptc* expression is also evident in the pancreatic anlage (Fig. 1G, arrow; compare staining of darker dorsal bud endoderm to lighter surrounding dorsal mesenchyme outlined by dashes).

Notochord signals repress endodermal Shh *expression*

Our observations on *Shh* and *Ptc* gene expression in endoderm suggest that signals from the notochord down-regulate adjacent endodermal *Shh* expression. A series of notochord deletion and grafting experiments provide strong evidence for such a notochord–endoderm interaction in chicks (Fig. 2). Eighteen hours after deletion of notochord adjacent to foregut endoderm, ectopic *Shh* expression is observed in squamous dorsal epithelial cells (Fig. 2A); fate mapping studies (Matsushita 1996) indicate that this region of foregut includes the pancreatic anlage. We do not observe a squamous to columnar shape transition in dorsal endodermal cells until later stages when they contact mesenchyme. Grafting an ectopic notochord adjacent to ventral foregut results in down-regulation of *Shh* in adjacent epithelium, and a reproducible change in cell morphology from columnar to cuboidal (Fig. 2B), reminiscent of notochord-induced changes in neuroectodermal cell shape (Schoenwolf and Smith 1990). In contrast, control grafts of somites adjacent to ventral foregut epithelium do not affect *Shh* expression or cell shape (Fig. 2C). Notochord deletion (Kim et al. 1997a) or ectopic notochord grafts do not affect adjacent endodermal expression of *HNF3β* (Fig. 2D), demonstrating that notochord signals may regulate expression of a specific subset of endodermal genes, including *Shh*.

Two days after notochord deletion, we observe ectopic expression of *Shh* and *Ptc* in a rudimentary dorsal pancreatic evagination (Fig. 2E,F), which subsequently fails to develop (Kim et al. 1997a). These results demonstrate that notochord signals prevent initial *Shh* and *Ptc* expression in the dorsal pancreatic anlage.

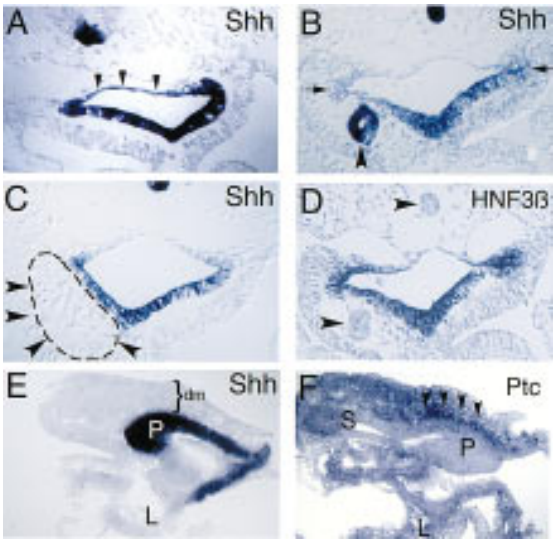

Figure 2. Notochord signals repress *Shh* expression in adjacent endoderm. Transverse sections through foregut adjacent to the anterior intestinal portal at stage 16 (*A–D*). (*A*) Ectopic *Shh* expression in dorsal foregut endoderm after notochord deletion ($n = 10$) at stage 10–11. Arrowheads outline blue dorsal endoderm. Contrast to Fig. 1B. (*B*) Ectopic notochord graft decreases adjacent endodermal *Shh* expression; compare to contralateral control side (arrows). Ectopic notochord also expresses *Shh* (arrowhead). The endoderm adjacent to notochord grafts is reproducibly thinner than corresponding control endoderm on the contralateral side ($n = 18$) The apparent lumen in the transplanted notochord is an artifact of histologic preparation. (*C*) Endodermal *Shh* expression after insertion of the ninth somite (dashed line and arrowheads), from a stage 10 donor, adjacent to endoderm. *Shh* expression in endoderm is unaffected compared to the contralateral side ($n = 4$). (*D*) *HNF3β* expression in foregut endoderm after notochord grafting. Endogenous or grafted notochord (arrowheads) does not express *HNF3β* at this stage. Ventral endodermal *HNF3β* expression is unaffected by ectopic notochord. (*E,F*) Sagittal sections through dissected pancreatic anlage and adjacent anterior foregut at stage 19. (*E*) Ectopic endodermal *Shh* expression in the dorsal pancreas bud (P) after notochord deletion at stage 11. Some liver (L) is seen in this section but liver and stomach endoderm are out of the section plane. Mesenchyme (dm) overlying the dorsal pancreas bud is indicated. (*F*) shows stomach (S), liver (L), and ectopic *Ptc* expression in mesenchyme (arrowheads) adjacent to the dorsal pancreas bud (P) after notochord deletion. Dorsal is toward the top; anterior is toward the left in *E* and *F*.

Notochord factors activin and FGF initiate pancreatic differentiation

An in vitro endoderm culture method (Kim et al. 1997a) provides an assay for factors that can initiate expression of pancreas genes, including *Pdx1* and insulin, in isolated pancreatic endoderm. As shown in Figure 3, midline endoderm and notochord in the pancreatic anlage can be

dissected free from adjacent tissues including somites, aortic endothelium, and splanchnic mesoderm. When first isolated, this endoderm does not express detectable *Shh*, insulin, or *Pdx1* (Fig. 3D, column E0). Previously (Kim et al. 1997a), we have also shown that notochord can be isolated without adherent endoderm and that notochord cultured alone does not express insulin or *Pdx1*. After 3 days of growth in vitro, isolated endoderm does not express *Pdx1* or insulin, but now does express *Shh* (Fig. 3D, column E) and *Ptc* (see below). This shows that separation of pancreatic endoderm from notochord allows endodermal *Shh* expression, which in turn correlates with a lack of *Pdx1* and insulin expression. Growth of isolated notochord with endoderm results in expression of both *Pdx1* and insulin (Fig. 3D, column E+N). Thus, even in the presence of notochord-derived SHH, notochord signals can stimulate endodermal pancreas gene expression (see Discussion).

Activin-βB, a member of the transforming growth factor-β (TGF-β) family, has been shown to repress *Shh* expression in Hensen's node during establishment of avian left–right axial asymmetry (Levin et al. 1995, 1997). In stage-12$^-$ chicks, activin-βB expression is detectable in notochord tissue but not in more lateral mesoderm (Fig. 4A), confirming previous work (Connolly et al. 1995). Activin-βA expression is not detected at this stage (Connolly et al. 1995; data not shown). We tested activin-βB for activity in our pancreatic endoderm assay (Fig. 4B) and found that activin-βB induced *Pdx1* and low levels of insulin expression. At higher concentrations, activin stimulated increased levels of *Pdx1* and insulin expression. No activity was detected in similar experiments with TGF-β1, BMP-4, chordin, or activin at 0.1 U/ml (data not shown).

Activin-loaded beads inserted adjacent to Hensen's node have been shown to repress *Shh* expression (Levin et al. 1995). As shown in Figure 5, beads loaded with activin-βB and inserted adjacent to the endoderm cell layer decrease endodermal *Shh* expression. This suggests that activin-like signals may represent part of the notochord signal that regulates endodermal *Shh*.

This model of activin function predicts that endodermal transcription of *Pdx1* and insulin resulting from activin-βB signaling would be inhibited by addition of SHH protein. Indeed, we find that addition of increasing concentrations of purified bioactive SHH amino-terminal peptide (Martí et al. 1995) with activin-βB to endoderm suppresses expression of endodermal insulin and *Pdx1* (see Fig. 4C). Inhibition by SHH is concentration dependent between 1 to 40 μg/ml, an in vitro activity range similar to that previously shown for SHH activity on motor neuron induction in neural tissue (Martí et al. 1995).

Concentrations of FGF2 >50 ng/ml repress expression of *Xlhbox8* in *Xenopus* endoderm (Gamer and Wright 1995). FGF2 is expressed in notochord and lateral mesoderm of stage-12$^-$ chicks (see Fig. 4A) as well as in endoderm and adjacent tissues (Borja et al. 1993, 1996). Concentrations of FGF2 <0.1 ng/ml have no detectable activity (data not shown). When added at a concentration of 1 ng/ml to pancreatic endoderm, FGF2 induces expression of *Pdx1* and insulin (see Fig. 4B), while suppress-

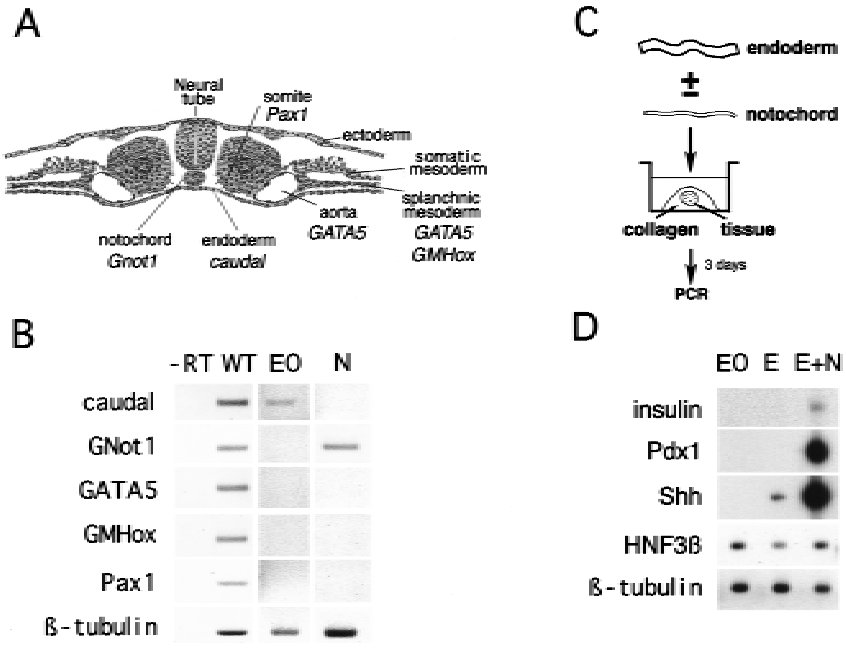

Figure 3. Separation of pancreatic endoderm and notochord promotes *Shh* expression and prevents *Pdx1* and insulin expression. (*A*) Schematic transverse section through stage 12 pancreatic anlage summarizing tissue-restricted expression patterns of *Gnot1* (notochord), *caudal* (endoderm), *GATA5* (endothelial and splanchnic mesoderm), *GMHox* (splanchnic mesoderm), and *Pax1* (ventral somitic mesoderm), adapted from Patten and Carlson (1974). (*B*) Gene expression detected by RT–PCR in dorsal pancreatic endoderm isolated at stage 12. Total RNA was harvested and analyzed by RT–PCR for *caudal, GNot1, GATA5, GMHox, Pax1, HNF3β*, and β-tubulin RNAs in whole torso (WT), which includes ectoderm, mesoderm, and endoderm germ layers, freshly dissected midline endoderm (E0) or notochord (N). β-Tubulin was used as a loading control. No signal was detected in control samples of RNA from whole embryo trunk samples untreated with reverse transcriptase (−RT). (*C*) Schematic of in vitro pancreatic endoderm growth and RT–PCR analysis revealing pancreas marker gene induction by notochord. Endoderm and notochord were removed from stage-12 embryos as previously described (Kim et al. 1997a) and grown in contact in a collagen matrix. (*D*) RT–PCR analysis of RNA from freshly dissected endoderm (EO) shows no insulin, *Pdx1*, or *Shh* expression. Endoderm isolated from stage-12 embryos and grown for 3 days in vitro (E) without notochord expresses *Shh* but not insulin or *Pdx1*. Recombination and growth of endoderm with notochord (E+N) results in insulin and *Pdx1* expression. High levels of *Shh* expression by notochord in the E+N sample are also detected.

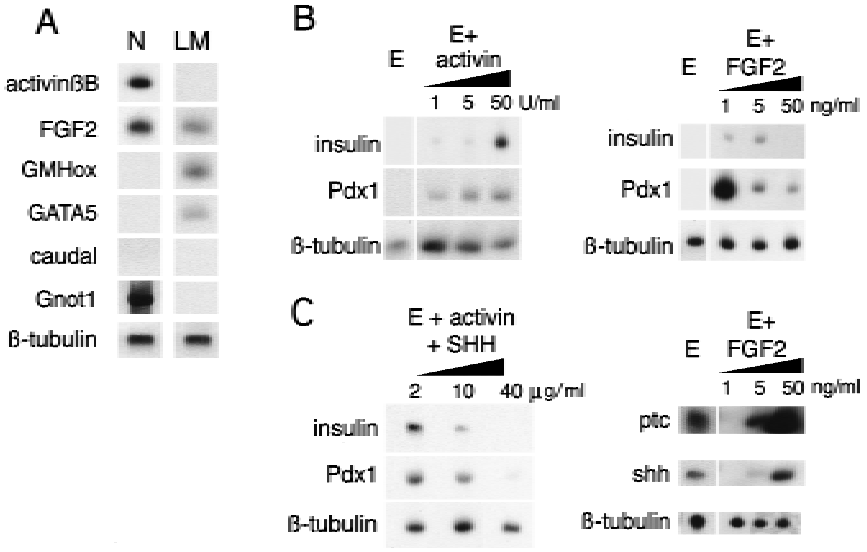

Figure 4. Activin-βB and FGF2 mimic notochord activity in inducing pancreatic genes. (*A*) Notochord (N) isolated at stage 12 expresses FGF2, activin-βB, and *Gnot1* assayed by RT–PCR. Absent *GMHox* or *GATA5* signals indicate lack of adherent lateral mesoderm. Absence of adherent midline endoderm is demonstrated by lack of detectable *caudal* expression. Isolated lateral mesoderm (LM) expresses FGF2, *GMHox*, and *GATA5*, but not activin-βB, *caudal* or *Gnot1*. β-Tubulin was used as a loading control. (*B*) Endoderm isolated at stage 12 was grown in the absence (E) or presence of increasing amounts of activin-βB (E+activin) or recombinant FGF2 (E+FGF2), then analyzed by RT–PCR for expression of insulin, *Pdx1*, or β-tubulin. (*C*) Isolated stage 12 endoderm grown in the presence of 5 U/ml human activin-βB and recombinant *Shh* at increasing doses,or grown in the presence of FGF2 at increasing doses. Endoderm grown in activin and Shh was assayed for expression of insulin, *Pdx1*, and β-tubulin; explants grown in FGF2 were tested for expression of patched (ptc), Sonic hedgehog (shh) and β-tubulin. PCR products of β-tubulin transcripts were used as loading controls.

ing endodermal expression of *Shh* and *Ptc* (see Fig. 4C). In contrast, FGF2 at higher concentrations (10–50 ng/ml) reduces *Pdx1* and insulin expression, while increasing transcription of *Shh* and *Ptc* (see Fig. 4B,C). High concentrations of FGF2 also inhibit the stimulatory effects of activin-βB on endodermal *Pdx1* and insulin expression (data not shown).

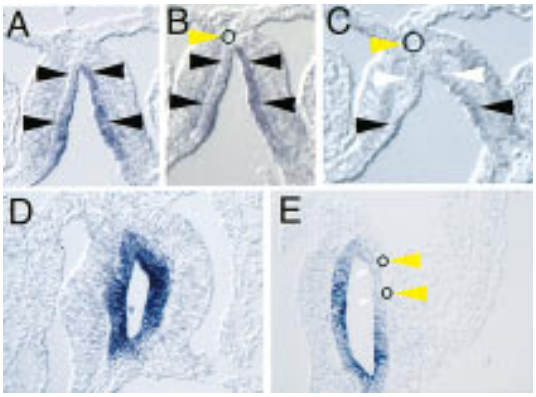

Figure 5. Ectopic activin down-regulates endodermal *Shh*. Black arrowheads indicate wild-type endogenous expression of endodermal *Shh*. White arrowheads indicate where expression is not present in embryos adjacent to ectopic activin-coated beads; yellow arrowheads and black circles indicate beads. (*A–E*) In situ hybridization with *Shh* probe at stage 17–18, (*A–C*) at the level of midgut and (*D,E*) in the stomach anlage. Control bead (*B*) soaked in phosphate buffered saline or activin-soaked beads (*C,E*) were implanted adjacent to midline foregut endoderm in stage 10 embryos. *Shh* expression in unmanipulated endoderm (*A,D*) is the same as in the embryos with control bead implants. In *E*, the beads became detached during processing for in situ hybridization.

Inhibition of endodermal Shh *signaling initiates pancreas gene expression*

To test whether prevention of SHH signaling in pancreatic endoderm is sufficient to initiate pancreatic differentiation, we performed antibody blocking experiments with an affinity-purified antibody (Martí et al. 1995) previously shown to prevent notochord-derived SHH induction of motor neurons in neuroectoderm. Isolated pancreatic endoderm incubated with SHH antibody expresses *Pdx1* and insulin at levels similar to those induced in endoderm by notochord (Fig. 6). Exposure of pancreatic endoderm to an antibody against carboxypeptidase A or preimmune serum does not result in *Pdx1* or insulin expression. These results indicate that one mechanism for initiating pancreatic differentiation in endoderm is to suppress endodermal SHH activity.

Discussion

Dorsal pancreas development in the chick requires interactions (Kim et al. 1997a) between the pancreatic endoderm and the notochord, an established source of signaling molecules including SHH. SHH from notochord induces expression of *Shh* in overlying neuroectodermal floor plate cells (Echelard et al. 1993); thus, we were surprised to find that notochord signals repress *Shh* expression in endoderm, thereby permitting pancreas development. Results in this paper provide an explanation for this apparent paradox; besides SHH, notochord expresses activin-βB and FGF2, two factors that can repress endodermal *Shh* expression. These findings may reconcile the seemingly opposite responses of endoderm and ectoderm to notochord signals and further emphasize that pancreatic endoderm and neuroectoderm (Sherman et al. 1993;

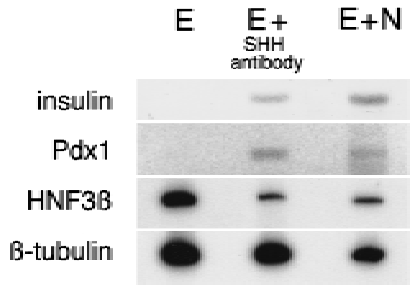

Figure 6. Antibody-mediated induction of pancreatic gene expression by endoderm. Explanted stage 12 endoderm was grown alone (E), in contact with nickel–agarose beads preincubated in an affinity-purified antiserum specific to the 19-kD amino-terminal Shh peptide (E+SHH antibody) or in contact with stage 12 notochord (E+N). Samples were collected after 3 days and analyzed by RT–PCR for expression of insulin, *Pdx1*, *HNF3β*, and β-tubulin.

Dono and Zeller 1994; Liem et al. 1995, 1997) are similarly patterned by integration of activin, SHH, and FGF signaling pathways.

Ventral pancreatic endoderm is not contacted by notochord and previously we have shown that notochord removal does not affect gene expression in the ventral pancreatic anlage (Kim et al. 1997a). The dorsal pancreas in chicks develops larger, insulin-secreting islets than the ventral pancreas, which develops mainly into exocrine acinar tissue mixed with smaller, glucagon-secreting islets (Beaupain and Dieterlen-Lièvre 1974). Although our conclusions are limited to development of the dorsal pancreas in chicks, two results described in this work suggest that mechanisms that initiate dorsal and ventral pancreas development are similar. First, ventral pancreatic endoderm, like dorsal endoderm, does not express *Shh*, suggesting that there must be mechanisms that initiate and maintain *Shh* repression in ventral, as well as dorsal pancreatic endoderm. Second, ectopic notochord grafts adjacent to ventral endoderm decrease endodermal *Shh* expression, demonstrating that the ventral endoderm is competent to transduce intercellular signals that lead to *Shh* repression. Tissues adjacent to ventral pancreatic endoderm include ventrolateral splanchnic mesoderm and vascular endothelium and currently we are studying their effects on initial repression of ventral endodermal *Shh*.

The possible role of activin and FGF in specification of pancreatic endoderm

Several lines of evidence from previous work suggested activin-βB as a candidate for a notochord factor that permits pancreas differentiation. Activin-βB was shown to be expressed in notochord when prepancreatic endoderm requires notochord signals (Connolly et al. 1995). Studies on endoderm differentiation in *Xenopus* suggested that activin and mature Vg1, a related (TGF-β) factor, could induce endodermal expression of *XlHbox8* (Gamer and Wright 1995; Henry et al. 1996). Activin has also been shown to induce insulin expression by islet cells (Yasuda et al. 1993). Lastly, activin-βB had been shown to repress *Shh* expression in Hensen's node during establishment of avian left–right axial asymmetry (Levin et al. 1995, 1997). Our results show that activin-βB can decrease *Shh* expression, while inducing expression of *Pdx1* and insulin by chick endoderm.

BMP-4, TGF-β1, and chordin lack activity in our assays, and activin-βA expression between stages 2 and 20 of chick embryogenesis has not been detected (Thomsen et al. 1990; Connolly et al. 1995; S. Kim and M. Hebrok, unpubl.). Expression of cVg1, the chick homolog of Vg1, has been demonstrated in somitic mesoderm but is absent in notochord until stage 14 (Seleiro et al. 1996; Shah et al. 1997), and we have not yet detected an effect of somites on endoderm (Fig. 2C). Thus, our studies identify activin-βB as a possible candidate signal from notochord that induces pancreatic endoderm differentiation and make it unlikely that activin-βA, cVg1, BMP-4, TGF-β1, or chordin account for this notochord activity. Our studies, however, cannot yet establish activin-βB as an endogenous initiating signal of pancreatic endoderm development.

In contrast to activin-βB expression, which is restricted to the axial midline, at least four isoforms of FGF2 generated by alternate splicing have been detected in mesodermal structures including notochord, as well as in neural tube, endoderm, and endothelium (Borja et al. 1993, 1996). FGF2 has been shown to repress *XlHbox8* expression in *Xenopus* vegetal endodermal explants (Gamer and Wright 1995) at concentrations >50 ng/ml. Separate studies of limb bud development have established a role for FGF2 or FGF4 in the apical ectodermal ridge, where FGF functions both to maintain *Shh* expression and to promote competence to SHH activity in underlying limb bud mesenchyme (Riley et al. 1993; Fallon et al. 1994; Laufer et al. 1994; Niswander et al. 1994). We have shown that FGF2 regulates endodermal gene expression in a concentration-dependent manner. Thus, similar to activin-βB, FGF2 may govern pancreas differentiation by regulating levels of endodermal SHH. In combination, these activin and FGF-like activities may be sufficient to offset potentially inhibitory input from notochord SHH (Figs. 3D, column E+N, and 4C). Further studies are required to determine whether FGF2 is an endogenous notochord signal that initiates pancreatic endoderm differentiation.

Shh *and pancreas development*

We have correlated effects of FGF2 on *Pdx1* and insulin expression in chick endoderm with FGF2 effects on *Shh* and *Ptc*. Similarly, we have shown that addition of purified SHH inhibits induction of endodermal *Pdx1* and *Shh* expression by activin-βB. In contrast, antibody inhibition of endodermal SHH activity is sufficient to allow pancreatic gene expression. Thus, mechanisms that restrict *Shh* expression in endoderm appear to be critical for initial pancreatic development. We have shown that misexpression of *Shh* in pancreatic endoderm after no-

tochord deletion results in absence of further pancreatic differentiation (Kim et al. 1997a) and correlates with increased mesenchymal *Ptc* expression and a thickened dorsal pancreatic mesenchymal layer. Misexpression of SHH in embryonic lung endoderm (Bellusci et al. 1997) also results in hypercellular mesenchyme, increased mesenchymal *Ptc* expression, and decreased endodermal branching.

Recently Apelqvist et al. (1997) showed that pancreatic misexpression of *Shh* from a *Pdx1* promoter in otherwise wild-type mice resulted in abnormal pancreas morphogenesis, ectopic intestinal smooth muscle, and an absent spleen. However, endocrine and exocrine cell differentiation was detectable in these mice. We observe a more severe, essentially apancreatic phenotype from ectopic dorsal pancreatic *Shh* expression after notochord deletion (Kim et al. 1997a). These differences may arise because notochord deletion results in both early ectopic endodermal *Shh* expression and lack of endodermal *Pdx1* expression. Thus, both initial repression of endodermal *Shh* expression (results presented here) and maintenance of *Shh* repression after notochord–endoderm separation (Apelqvist et al. 1997) are necessary for pancreatic cytodifferentiation and morphogenesis. Absence of *Shh* in dorsal endoderm along most of the rostral–caudal axis in early vertebrate development, however, indicates that *Shh* repression is not *sufficient* for pancreatic development outside the pancreatic anlage.

A cell interaction model of pancreas development

Our results suggest that cell interactions between notochord and endoderm govern chick pancreas development. We have shown that members of the TGF-β and FGF families, which are expressed in notochord, can stimulate responses in dorsal endoderm that prevent *Shh* expression. Lack of endodermal *Shh* permits mesenchymal (and possibly endodermal) *Ptc* function, resulting in expression of genes encoding transcription factors including *Pdx1, Isl1,* and *Pax6;* thus, notochord–endoderm interactions can affect subsequent mesenchymal–epithelial interactions. These transcription factors are critical for later cell differentiation (Ahlgren et al. 1996, 1997; Sander et al. 1997; St.-Onge et al. 1997) and expression of the genes necessary for normal pancreas function, including glucagon, insulin, and digestive enzymes like carboxypeptidase A and amylase. Furthermore, our results suggest that cell interactions can also repress *Shh* in ventral pancreatic endoderm.

The results presented here and previously (Kim et al. 1997a) suggest that the endoderm is prepatterned and notochord factors act as *permissive* signals during initiation of pancreas development. The activity of cell signals that permit dorsal pancreas development may be refined by local stimulatory and inhibitory activities. These include inhibitory (high) levels of FGF expressed in surrounding mesoderm, as well as SHH from adjacent notochord, floor plate, and lateral endoderm. Other factors including follistatin (Connolly et al. 1995; Miralles et al. 1998) may modulate the response to activin, and extracellular matrix components like heparan (Handler et al. 1997) may augment the activity of FGF. Localized expression of receptors or other signal transduction components of activin, FGF, and SHH may account for the observation that rostral, but not caudal, endoderm responds to notochord signals by expressing pancreas genes (Kim et al. 1997a). After the notochord separates from the endoderm, mechanisms must maintain *Shh* repression in the pancreas (Apelqvist et al. 1997). It will be interesting to study whether epithelial–mesenchymal interactions participate in the maintenance of pancreatic *Shh* repression. Mutations affecting *Shh*/*Ptc*, *activin*, and *FGF* signaling in mice exist and will allow us to test whether regulatory cell interactions in chicks described here are conserved during mammalian pancreas development.

Materials and methods

Dissection, grafting, and in vitro endoderm growth methods

Methods for chick embryo growth, pancreas dissection and notochord removal, and in vitro growth of pancreatic endoderm are described in Kim et al. (1997a). Embryos were staged according to Hamburger and Hamilton (1951). Ectopic tissue, including notochord or the penultimate somite from stage 10–11 donor embryos, or protein-loaded nickel–agarose beads (Quiagen, Inc.) were inserted under an endoderm flap created by a rostrocaudal incision made lateral to the dorsal aorta in the recipient embryo (Sundin and Eichele 1992). After recovery at room temperature for 45–60 min, manipulated embryos were grown in vitro at 38°C for ~18 hr to stage 16. By this stage, manipulated lateral endoderm has folded over and forms the ventral floor of the closing gut tube (Rosenquist 1971; Matsushita et al. 1996).

In situ hybridization, histochemistry, and RT–PCR

In situ hybridizations using *Shh* (Riddle et al. 1995) and *Ptc* (Marigo et al. 1996) sense and antisense probes were performed as described by Henry et al. (1996). Microscopy and photography were performed as previously described (Kim et al. 1997a). RT–PCR and electrophoretic analysis of PCR products was performed as described (Wilson and Melton 1994; Kim et al. 1997a). Primer sequences used for chick β-tubulin, *HNF-3β*, *Pdx1,* and insulin have been described (Kim et al. 1997a). Other primer sequences are listed forward then reverse, 5′ to 3′: *Gnot1* (Knezevic et al. 1995), GCAAGAGTTCCTCAAGCAGCAGTAC and AAAGCTCAACCTCCACTGTGTCC; *caudal* (Frumkin et al. 1994), TGGACCATCCTGAGGAGGTTTTG and CCAGCTATCCATCATCTTGTGCC; *GATA5* (Laverriere et al. 1994), CTAACTGCCACACAACCAACACC and TCGTAATGGAAGAAGGGGAGTTC; *GMHox* (Kuratani et al. 1994), CAAATCCTACTCAGGGGATGTGAC and TGACTGTGGGCACTTGATTCCTC; *Shh* (Riddle et al. 1995), TGGAGGATATGAAGGGAAGA and CTGAGTTTTCTGCTTTGACG; *Ptc* (Marigo et al. 1996), TACATTGGGCTTCGTCATTGGCTCC and CAATCAGGATAACCACAGGCACTG; *Pax1* (Ebensperger et al. 1995), ATTCGACCGTGCGACATCAG and ATGTGCTTGACCACGTTGGG; FGF2 (Borja et al. 1993), GGCACTTCAAGGACCCCAAG and AAAGGATAGCTTTCTGTCCAGGTC; activin-βB (GenBank accesssion no. Z71594; Connolly et al. 1995), GAACTTGGATGTTCAATGTGAGGG and GCAGTCTGTGCTTTTGCCTGAG.

Endoderm assays with growth factors

Recombinant human FGF1, FGF2, FGF7, and porcine TGF-β1 were from R&D Systems Inc. Activin A and B were provided by K. Symes (Boston University, MA) and A. Schneyer (Massachusetts General Hospital, Boston), and murine activin A was obtained as described (Sokol et al. 1990). Mouse BMP4 was obtained by transfection of COS cells (Basler et al. 1993) provided by K. Liem, Jr. and T. Jessell (both of Columbia University, New York, NY). Purified 19-kD amino-terminal SHH and affinity-purified SHH antiserum AB 80 were provided by E. Martí (Instituto Cajal de Neurobiologia, Madrid, Spain) and A. McMahon (Harvard University, Cambridge, MA). *Xenopus* chordin was provided by E. De Robertis (UCLA). Pancreatic endoderm was grown in vitro, in collagen, and in serum-free medium (Kim et al. 1997a) supplemented with the appropriate growth factor. Nickel–agarose beads were soaked in SHH antiserum as described (Martí et al. 1995) for 1 hr at room temperature, then wrapped with endoderm (notochordside contacting the bead) before embedding in collagen matrix. Control beads were soaked in PBS, preimmune serum, or undiluted polyclonal antiserum against carboxypeptidase A (Biogenesis).

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Notochord repression of endodermal Sonic hedgehog permits pancreas development

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