

Notochord to endoderm signaling is required for pancreas development

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SUMMARY

The role of the notochord in inducing and patterning adjacent neural and mesodermal tissues is well established. We provide evidence that the notochord is also required for one of the earliest known steps in the development of the pancreas, an endodermally derived organ. At a developmental stage in chick embryos when the notochord touches the endoderm, removal of notochord eliminates subsequent expression of several markers of dorsal pancreas bud development, including insulin, glucagon and carboxypeptidase A. Pancreatic gene expression can be initiated and main-

tained in prepancreatic chick endoderm grown in vitro with notochord. Non-pancreatic endoderm, however, does not express pancreatic genes when recombined with the same notochord. The results suggest that the notochord provides a permissive signal to endoderm to specify pancreatic fate in a stepwise manner.

Key words: pancreas, organogenesis, endoderm, notochord, chicken, cell signaling

INTRODUCTION

Organ development requires interactions between different tissues to coordinate steps that culminate in morphogenesis and function. Epithelium derived from the endodermal germ layer forms the lining of organs that differentiate from the pharynx and the respiratory and gastrointestinal tracts, including the thyroid, thymus, lungs, esophagus, stomach, intestines, liver and pancreas. Thus, determining how the endoderm is patterned is crucial for understanding the development of many internal organs.

Development of the pancreas, a vital organ with both endocrine and exocrine functions, has been studied in mice and chickens, and a brief comparison of pancreatic morphogenesis follows. The pancreas forms from the fusion of distinct dorsal and ventral buds. Prior to morphogenesis, the endoderm fated to become the dorsal pancreas bud is a single layer of epithelial cells. In chick embryos at the 18 somite stage, radioablation studies show that the pancreatic anlage is at a level adjacent to somites 7-15 along the rostrocaudal axis (Le Douarin, 1964). Laterally, the prepancreatic endoderm is lined by splanchnic mesoderm and along the dorsal axial midline the endoderm contacts the notochord (Wessells and Cohen, 1967; Pictet et al., 1972). From the time it is formed, the notochord remains in contact with the dorsal prepancreatic endoderm until about the 13 somite stage in mice (embryonic day (E) 8) and the 22 somite stage in chickens (embryonic day 2.5) when it is then separated from endoderm by midline fusion of the paired dorsal aortas. The first indication of morphogenesis occurs later, at 22-25 somites in mice (E9.5; Golosow and Grobstein, 1962; Wessells and Cohen, 1967) and at the 26 somite stage in chickens (Romanoff, 1960; Dieterlen-Lièvre, 1965, 1970) when dorsal mesenchyme condenses and underlying endoderm

evaginates, forming the dorsal pancreatic bud. Stimulated by a growing cap of mesenchyme, the dorsal bud endoderm proliferates and branches, and over the next 2 days formation of islets of Langerhans and exocrine acini ensues. By embryonic day 4, the paired ventral buds form in chickens as evaginations from the primitive common bile duct within the liver primordium (Romanoff, 1960; Dieterlen-Lièvre, 1965); thus, the ventral buds derive from endoderm which is not contacted by notochord.

Previous studies have primarily focused on the relatively late interactions between committed pancreatic endoderm and mesenchyme. These studies have shown that permissive signals from mesenchyme to endoderm are necessary for pancreas development (Golosow and Grobstein, 1962; Wessells and Cohen, 1967; Spooner et al., 1970; Dieterlen-Lièvre, 1970; Gittes et al., 1996). The roles of genes crucial to later epithelial-mesenchymal interactions in the pancreas have also been reported. The homeodomain-containing transcription factor PDX1 is expressed in the epithelium where dorsal and ventral pancreatic evaginations appear (Jonsson et al., 1994). Mice with homozygous null mutations in *pdx1* are born apancreatic (Jonsson et al., 1994; Offield et al., 1996) but do initiate the first steps in pancreas bud formation and produce dorsal buds that express glucagon and, in some cases, insulin. These results argue against a role for *pdx1* in initially specifying pancreatic fate. Coculture studies with epithelium and mesenchyme from normal and *pdx1*-mutant animals suggest instead that PDX1 function results in early pancreatic epithelial competence to mesenchymal signals and subsequent epithelial growth and branching (Ahlgren et al., 1996). Recent studies of the LIM-homeodomain protein Islet-1 (ISL1) show that ISL1 function is required both for dorsal pancreatic mes-

enchyme formation and differentiation of all pancreatic islet cells (Ahlgren et al., 1997).

While much has been learned about later developmental interactions between mesenchyme and committed pancreatic endoderm, little is known about the mechanisms that initially pattern endoderm to a pancreatic fate. Studies of the differentiation of endoderm isolated from the dorsal pancreatic anlage suggest that commitment of endoderm to a pancreatic fate may occur as early as the 10-12 somite stage in mice (Spooner et al., 1970; Wessells and Cohen, 1967) and the 13 somite stage in chickens (Dieterlen-Lièvre and Beaupain, 1974; Sumiya and Mizuno, 1987). In vitro culture of endoderm committed to a pancreatic fate with pancreatic or heterologous mesenchyme results in pancreatic endocrine and exocrine differentiation (Le Douarin and Bussonnet, 1966; Golosow and Grobstein, 1962; Wessells and Cohen, 1967; Spooner et al., 1970; Dieterlen-Lièvre, 1970; Ahlgren et al., 1997). This suggests that the competence to form pancreas is intrinsic to the pancreatic epithelium, but it is not understood what tissues, inductive signals or genes are important for committing endoderm to a pancreatic fate.

It is well established that inductive interactions between tissues adjacent to prepancreatic endoderm accomplish the patterning of neuroectoderm (reviewed by Placzek and Furley, 1996) and somitic mesoderm (Watterson et al., 1954; Pourquié et al., 1993). For both neuroectoderm and somitic mesoderm, dorsal-ventral patterning is partly controlled by interactions with the notochord, an axial mesoderm derivative and a known source of many intercellular signals. Could notochord signals also pattern endoderm? The anlage of the dorsal pancreas is in direct contact with the notochord during the period when commitment to a pancreatic fate occurs (Wessells and Cohen, 1967; Pictet et al., 1972), in contrast to other organs formed from endodermal evaginations which derive from lateral or ventral endoderm (Remak, 1854 cited in Dieterlen-Lièvre, 1965). The expression pattern of genes in the neural tube including *Isl1* (Yamada et al., 1991), *pax6* (Goulding et al., 1993), *HNF-3 β* (Ruiz i Altaba et al., 1995) and *nkx2.2* (Barth and Wilson, 1995) can be influenced by signals from notochord, and each of these genes is known to be expressed early in the development of the dorsal pancreas bud (Ahlgren et al., 1997; Turque et al., 1994; Ahlgren et al., 1996; Rudnick et al., 1994). These observations suggest that the notochord may signal adjacent endoderm to acquire a pancreatic fate (Ohlsson et al., 1993; reviewed by Slack, 1995).

We developed methods to analyze morphogenesis and gene expression of the dorsal pancreas anlage in the chicken prior to the time when pancreas fate is specified. We show that removal of the notochord prior to the 13 somite stage prevents subsequent expression of genes, including *Isl1* and *pdx1*, which are crucial for pancreas development. In contrast, expression of these genes during ventral pancreas development appears unaffected by notochord removal. In vitro growth of isolated endoderm from the dorsal pancreatic anlage with adjacent notochord shows that the notochord can initiate and maintain expression of early pancreas genes. This notochord does not initiate pancreas marker gene expression in dorsal endoderm isolated from a posterior region outside the pancreatic anlage. These data demonstrate that the notochord signals competent endoderm to form the pancreas.

MATERIALS AND METHODS

Chick embryos, dissection methods and germ layer growth in vitro

All experiments were performed on white Leghorn chick embryos from SPAFAS (Preston, Connecticut). Eggs were incubated at 38°C and staged according to Hamburger and Hamilton (1951). Stage 10-12 embryos were explanted as described by Sundin and Eichele (1992). For complete notochord removal in stage 11⁻ (12 somites) embryos, tungsten needles were used to cut endoderm lateral to the notochord. This cut extended from the level of somite 8-9 to a caudal level opposite the nonsegmented mesoderm fated to become somite 16-17. The rostral and caudal limits of exposed notochord were severed without creating a hole in ventral neural tube. Forceps were used to grab the freed rostral end of notochord and detach notochord from the ventral neural tube. The endoderm flap was replaced in the midline and the embryo incubated at 38°C. Midline endoderm healed within the next 12-18 hours prior to stage 16. Over 150 embryos were manipulated in this manner. Two control dissections were performed. In the first, the caudal end of notochord was not severed and notochord was detached from the free rostral end and peeled off the neural tube until just rostral to the regressing Hensen's node. Then notochord was replaced in the midline and the endoderm flap maneuvered over it. In the second control, notochord was completely removed and replaced in the midline with notochords dissected from synchronous stage 11⁻ donor embryos. Endoderm was maneuvered over the exogenous replacement notochord. Midline axial endoderm healed over the next 12-18 hours. Over 80 "control" embryos were manipulated in this manner.

Endoderm including the anlage of the dorsal pancreatic bud was isolated from stage 11⁺ or 12⁻ embryos using needles and forceps as described above. The position of the dorsal pancreatic anlage was determined from the endodermal mapping studies of Le Douarin (1964) and Rosenquist (1971) and studies of chick pancreas formation by Dieterlen-Lièvre (1970). A rectangle of endoderm (see Fig. 6A schematic) with rostral and caudal limits at the level of somites 5-6 and future somite 16, respectively, was isolated. To isolate posterior endoderm outside the pancreatic anlage (see Fig. 6B schematic), a rectangle was isolated with rostral and caudal limits at the levels of future somite 16 and the regressing Hensen's node, respectively. Endoderm-notochord recombinants were formed by wrapping notochord with endoderm from the same embryo (notochord-face of endoderm in contact with notochord). Tissue recombinants were grown in collagen-matrix gels as previously described (Dickinson et al., 1995) with the following modifications. 10 \times M199 medium pH 4.0 (Gibco-BRL) was substituted for 10 \times DMEM to form collagen gel. Explants embedded in collagen were covered with 0.7 ml of 1 \times M199 supplemented with a 1:100 dilution of ITS+ Premix culture supplement (Becton Dickinson, 40352) at the start of incubation and this was replaced with fresh medium after 48 hours.

RNA preparation and RT-PCR

Dissected embryonic pancreas rudiments were dissolved in Trizol (Gibco-BRL) and total RNA prepared according to the manufacturer's instructions. RT-PCR was performed as described by Wilson and Melton (1994) with the following conditions: 1 cycle of 94°C for 3 minutes, 60°C for 1.5 minutes, 72°C for 1 minute; then 39 cycles of 94°C for 1 minutes, 60°C for 1.5 minutes, 72°C for 1 minute; and lastly 1 cycle of 72°C for 5 minutes. Primer sequences used are listed forward then reverse, 5' to 3'. One-fifth of the PCR reaction volume was electrophoresed in a 1.5% agarose gel and photographed after ethidium bromide staining. [³²P]dATP labelled PCR products were electrophoresed in 5% polyacrylamide and processed as described by Wilson and Melton (1994).

Primers used for β -tubulin (Valenzuela et al., 1981):

AGATGCTGAACGTGCAGAACAAG and CCTTCTCTTCAA-ATTCACCCTG.

HNF-3 β (Ruiz i Altaba et al., 1995):

TCGCACAAAATGGACCTCAAG and CGGGAATACACAC-CTTGGTAGTAAG.

Isl1 (Tsuchida et al., 1994):

ATGGTGGTTTACAGGCGAACC and GGGCAGAAACAA-CATCAGAACTCTG.

glucagon (Hasegawa et al., 1990):

CCATTTCCATGCTCTGGTGATC and TGGTGATAAGACAGAAAGTCCTG.

insulin (Hasegawa et al., 1991):

TCTTCTGGCTCTCCTGTCTTTC and CGGCTTCTTGGC-TAGTTGCAGTAG.

pax6 (Li et al., 1994):

TTCCATGTTGGGCAGGACAG and AGGGCACAGTTGC-AGTACAGAAG.

Cloning of chick *pdx1* and carboxypeptidase A cDNA

To identify chicken homologues related to human and mouse *pdx1*, rat STF and *Xenopus laevis* *XlHbox8*, degenerate oligonucleotides were used as PCR primers. The sequence of two *pdx1* primers which yielded the appropriately sized DNA product, 5' to 3' are:

CCTTTCCCATGGATGAAATCCACCAAA and TGGAACCA-GATTTTTATGTGCTCTC.

The 232 base-pair fragments obtained from embryonic stage 20 chick pancreas cDNA were subcloned into pCR2.1 (Invitrogen) and DNA sequence obtained from individual clones. NCBI BLAST sequence analysis showed 87% identity between chick *pdx1* and the sequence between nucleotides 366 and 604 of the human *pdx1*, and 82% identity between chick and mouse *pdx1*.

A similar method was used to identify the chick homologue of human, rat and bovine carboxypeptidase A. The sequence of primers which yielded the appropriately sized DNA product, 5' to 3' are:

TGGAGAT(CT)GTCACCAACCCTGATGG and GTGAA(AG)-GAGTACTTGATGCC.

BLAST analysis of DNA sequences from 480 base-pair length PCR products showed 86% identity between chick and human procarboxypeptidase A sequences.

Histologic analysis, microscopy and photography

6 μ m paraffin sections were stained by hematoxylin-eosin (H and E; Sechrist and Marcelle, 1996), by in situ hybridization (Henry et al., 1996) using *shh* sense and anti-sense digoxigenin probes (Riddle et al., 1993), or by immunoperoxidase techniques. Primary antibodies to the following antigens were used at the indicated dilutions. Guinea pig anti-insulin (Incstar) 1:200; rabbit anti-glucagon (Dako) 1:100; rabbit anti-carboxypeptidase A (Biogenesis) 1:500. Secondary antibodies (Jackson ImmunoResearch) were used at 1:500 dilution and included donkey anti-rabbit biotin IgG or donkey anti-guinea pig biotin IgG. After rehydration, slides were incubated in 0.3% peroxide in methanol for 30 minutes at room temperature, treated with primary and secondary antibodies as described (Hogan et al., 1994), and peroxidase activity detected with the ABC immunoperoxidase system (Vector Labs). Slides were photographed on a Zeiss Axiophot microscope, embryos and dissected organs on a Leica WILD M10 microscope. Photos were scanned and formatted using Adobe Photoshop 3.0. Images of ethidium bromide stained DNA fragments were black/white inverted.

RESULTS

Morphology and gene expression during pancreas development in vitro

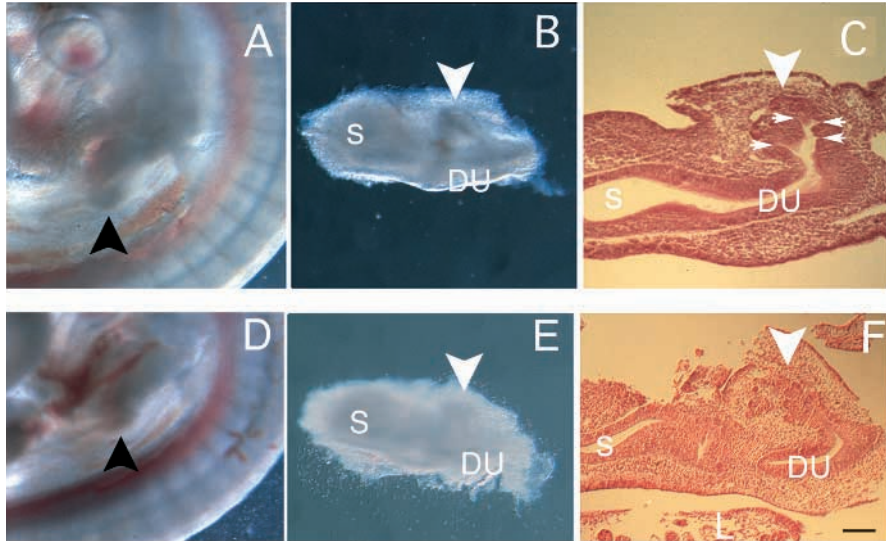
The dorsal pancreas is first seen as a condensation of mesenchyme on the dorsal duodenum at the level of the liver. In chick embryos, this occurs at about stage 16 (26 somites). By

stage 19 (38 somites) the dorsal bud is readily visible as a dark, condensed dorsal projection just caudal to the stomach and ventral to the dorsal aorta (Fig. 1A). At this stage, no ventral pancreas bud is visible (Fig. 1B). Histologic examination of the dorsal pancreas bud (Fig. 1C) reveals that it is composed of an endodermal stalk ending in terminal branches, capped by a layer of loose connective tissue called mesenchyme.

To simplify the manipulation and analysis of pancreas development we adapted a system of in vitro embryonic growth used to study development of external organs (Flamme, 1987; Sundin and Eichele, 1992). Embryos were explanted at stage 10⁺ (11 somites) or 11⁻ (12 somites) and grown with their endoderm-side up. Particularly prominent at this stage are notochord and somites, but there is no morphological sign of pancreatic bud formation. At this stage, the dorsal pancreatic anlage is located in the caudal axial midline endoderm (Rosenquist, 1971), adjacent to the most recently formed somites and the nonsegmented paraxial mesoderm (Le Douarin, 1964). The major morphogenetic events occur during the next 3 days of incubation. By stage 17 (30 somites) head turning and flexion, optic cup formation, gut closure, midline dorsal aorta fusion and extensive vascular development are observed. After 3 days' growth in vitro (stage 22) the embryo has outgrown its vascular supply and further development is limited, but by then extensive development of multiple organ systems, including the gastrointestinal system, has occurred (data not shown). The embryo grown in vitro to stage 19 has formed a visible dorsal pancreatic bud (Fig. 1D,E) which in size, shape and anatomic position is indistinguishable from its in ovo counterpart. Terminal branching of the pancreatic endodermal layer of the embryonic pancreatic duct under a mesenchymal layer is seen (Fig. 1F).

During the period when formation of the dorsal pancreas begins, we characterized the expression patterns of seven pancreatic genes in embryos grown in ovo (Fig. 2A) or grown in vitro (Fig. 2B). Although none of these genes is expressed exclusively in pancreas, coexpression of these genes in embryonic endoderm suggests that we are describing pancreatic differentiation. A similar approach has been used to characterize pancreas gene expression in the mouse (Gittes and Rutter, 1992). HNF-3 β is a transcription factor expressed in both chick endoderm and floor plate cells of the neural tube by stage 11 (Ruiz i Altaba et al., 1995). As expected, HNF-3 β is found to be expressed in the dorsal pancreatic anlage throughout the first four days of pancreas development (Fig. 2A). Islet-1 is a LIM-homeodomain protein required for dorsal pancreatic mesenchyme and islet cell development (Ahlgren et al., 1997). PAX6 is a paired-box containing transcription factor whose early expression in chick endoderm is initially limited to the midgut (Li et al., 1994) and is expressed later in chick pancreatic endocrine cells (Turque et al., 1994). Expression of *Isl1* and *pax6* is initially detected in endoderm isolated from the dorsal pancreatic anlage as early as stage 11 (Fig. 2A) and is detected in the dorsal pancreatic anlage at all subsequent stages tested. At stage 15, just prior to the formation of the dorsal pancreatic bud from dorsal endoderm adjacent and caudal to the anterior intestinal portal, glucagon, *pdx1* and insulin mRNA are first detected. *pdx1* and insulin transcription increase over the next 4 hours and, by stage 16, are detected in tissue along the dorsal gut tube marked by condensed mesenchyme and a dorsal endodermal bulge. Glucagon, *pdx1*, insulin, *Isl1* and

Fig. 1. Morphogenesis of chicken pancreas occurs in vitro. (A-C) Embryos grown in ovo. (A) Stage 19 embryo with left anterior limb bud removed to reveal the dorsal pancreatic bud (arrowhead). Anterior is toward the top. (B-C) Dissected stage 19 embryonic dorsal pancreatic bud. Anterior is toward the left. B shows the relationship of the dorsal pancreas (arrowhead) with the stomach (S) and duodenum (DU). C shows hematoxylin-eosin staining of a sagittal section through the stage 19 pancreas (arrowhead), stomach (S) and duodenum (DU). The liver has been removed. Endodermal branches are marked by small arrows. (D-F) Embryos grown in vitro. (D) View of the developing gut tube and the dorsal pancreas bud (arrowhead) from a stage 19 embryo. (E,F) Dissected embryonic dorsal pancreas bud from stage 19 embryos. E shows the dorsal pancreas (arrowhead), stomach (S) and duodenum (DU). (F) Hematoxylin-eosin stained sagittal section through the pancreas shown in E. Liver tissue (L), ventral to the duodenum, is visible in this section. Stages of development were determined according to Hamburger and Hamilton (1951). Scale bar, 300 μ m in A,B,D,E; 150 μ m in C and F.



pax6 transcripts are not detected in adjacent tissues including liver, proximal stomach or ventral duodenal wall at this stage (data not shown). Carboxypeptidase A is the first exocrine marker expressed in pancreatic development and its onset of expression presages acinar morphogenesis. Transcription of carboxypeptidase A was first clearly detected at stage 19. The levels then reproducibly decreased over the next 12 hours (stage 22, Fig. 2A) before increasing during the postsomite period (stage 23; Fig. 2A).

Fig. 2B summarizes a similar analysis of the expression patterns of these seven pancreas-expressed genes in dorsal pancreas buds from embryos grown in vitro. At stage 14, *Isl1* and low levels of *pax6* are detectable and at stage 19 in vitro, expression of all marker genes is detected. Thus the transcription pattern of this marker gene panel from embryos grown in vitro is quite similar to the pattern obtained from embryos grown in ovo, demonstrating that the initiation of pancreas formation is not markedly affected by in vitro growth of the embryo host.

To further assess pancreas development in embryos grown in vitro and in ovo, we analyzed protein expression by immunohistochemistry. Pancreatic development in chick embryos grown in ovo has been characterized using antibodies (Beaupain and Dieterlen-Lièvre, 1974; Dieterlen-Lièvre and Beaupain, 1974). We analyzed dorsal pancreas buds from different stage embryos to determine the onset of expression of glucagon and insulin (endocrine proteins) and carboxypeptidase A (exocrine protein). As summarized in Table 1, glucagon is detected by stage 16 in embryos grown either in ovo or in vitro. Subsequently, insulin is detected in pancreas rudiments from stage 19 embryos grown either in ovo or in vitro. Lastly, we detect carboxypeptidase A in dorsal pancreas buds

from embryos grown to stage 22 in ovo or in vitro. Thus by numerous criteria the initiation of pancreas development is similar during embryonic growth in vitro or in ovo.

Notochord removal prevents expression of pancreas genes

To study possible influences of notochord on adjacent endoderm, embryos were explanted prior to stage 11 and notochords removed as shown in Fig. 3A. At this stage, prior to the onset of pancreas gene expression, the notochord is less

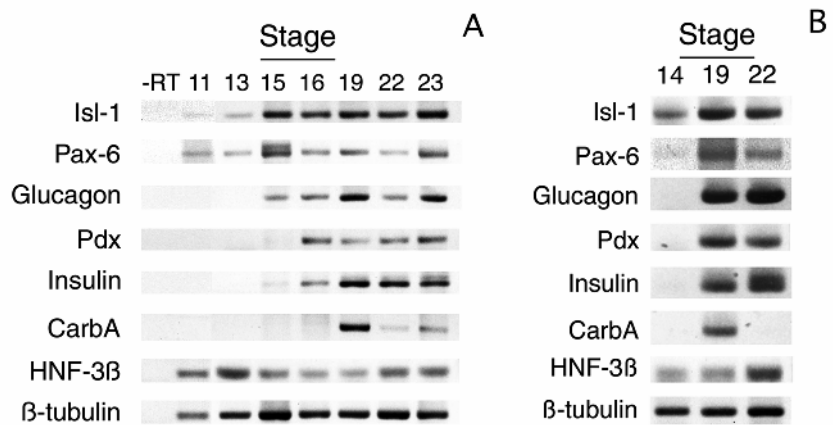
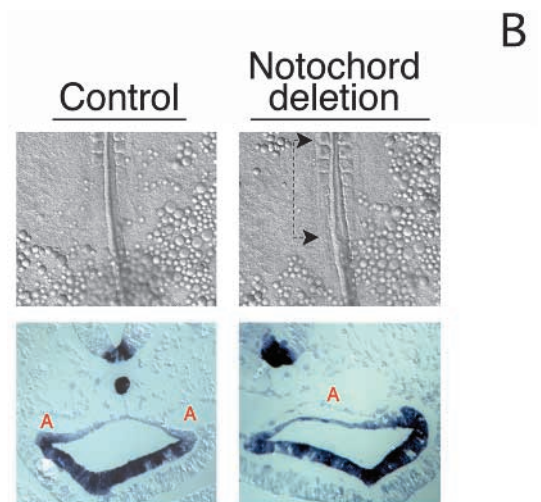
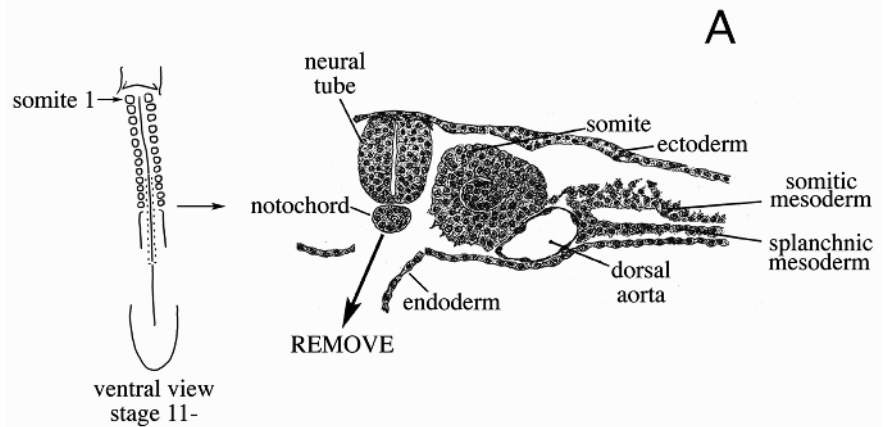


Fig. 2. The pattern of gene expression in dorsal pancreas is similar in embryos grown in ovo and in vitro. (A) Gene expression detected by RT-PCR in the dorsal pancreas removed from embryos at the indicated stage after incubation in ovo. Total RNA was harvested and analyzed by RT-PCR for the presence of *Isl1*, *pax6*, glucagon, *pdx1*, insulin, carboxypeptidase A (CarbA), *HNF-3β* and β -tubulin. No signal was detected in control samples untreated with reverse transcriptase (-RT). PCR products of *HNF-3β* (endodermally expressed) and β -tubulin (ubiquitously expressed) were used as a DNA loading control. (B) The timing of expression of pancreas-expressed genes in chicken embryos grown in vitro. Embryos were removed at stage 11⁻ and grown for up to 3.5 days in vitro to stage 22. As in embryos grown in ovo, the first detectable markers were *Isl1* and *pax6*. Note that carboxypeptidase A expression is reduced after stage 19, similar to the reduction seen in ovo.

Fig. 3. Notochord deletion from chicken embryos grown in vitro. (A) Schematic of notochord removal at stage 11⁻. The notochord in ventral view is represented by the black line between the row of somites, and more posteriorly, between the nonsegmented presomitic mesoderm. A flap of endoderm ventral to the notochord was displaced (dotted line). The notochord was deleted along the rostrocaudal axis from the level of somite 9 to the level adjacent to the presomitic mesodermal anlage of somites 16-17. After notochord removal, the endoderm flap was replaced.

Relationship of notochord and endoderm to adjacent tissues is shown in the schematic of a transverse section through somite 10 (adapted from Patten and Carlson, 1974). (B) Notochord removal does not significantly alter neural tube or initial endoderm morphology. Upper two panels show light micrographs of stage 11⁻ embryos before and after notochord deletion; anterior is toward the page top. Bottom two panels show *shh* in situ hybridization of transverse sections made from embryos incubated until stage 17; dorsal is toward the top. Prior to removal (upper left), the notochord is visible as a dark midline condensation. At stage 17 (bottom left), *shh* is detected in the notochord as well as the ventral floor plate of the neural tube and the columnar cells of the ventral foregut endoderm but not squamous cells of the dorsal endoderm. The medially ingressing paired aortas are marked (A). After microsurgical removal of the notochord (upper right), the ventral floor plate appears as a thin dark furrow between the ventrolateral neural tube. The anterior-posterior extent of the notochord deletion is indicated by the arrowheads and the dotted line. By stage 17 (bottom right), the dorsal endoderm cell layer has healed and the dorsal aorta (A) is fusing in the midline between the endoderm and the floor plate. Note evidence of *shh* expression in both dorsal and ventral endoderm.



adherent to underlying structures and can be removed without immediate visible effect on adjacent ventral floor plate, aorta or somites (Fig. 3B). Midline fusion of the aorta prevents notochord removal by this method after stage 12. In situ hybridization with sonic hedgehog (*shh*), after 1 day in vitro growth, specifically stains floor plate, notochord and ventral endoderm in the unmanipulated control. By this time, the endodermal sheet has formed a tube with squamous epithelium dorsally and columnar epithelium ventrally. The paired dorsal aortas have begun to move medially, separating the notochord and endoderm (Fig. 3B). Absence of staining by the *shh* probe beneath the floor plate shows that notochord removal is complete. A reproducible, but faint expression of *shh* in dorsal endoderm from embryos without notochord, compared to similar stage control embryos, is also noted (Fig. 3B).

After deletion of notochord to the level of the 18th somite, expression of *pax6*, *pdx1*, insulin, glucagon, carboxypeptidase A and, in five of six experiments, *Isl1*, is undetectable (Fig. 4A). In one of six independent experiments we have observed low levels of *Isl1* expression in the dorsal pancreas bud after development to stage 23. Otherwise, only *HNF3 β* transcription in the dorsal bud appears unaffected by notochord deletion. In contrast, mock deletion of notochord did not affect the timing or levels of expression of any of these pancreas genes (Fig. 4A).

Expression of *HNF-3 β* in spite of notochord removal

indicates that this manipulation does not eliminate all endoderm transcription in the region of the dorsal pancreatic anlage. To further evaluate the effects of notochord removal, ventral pancreas buds from the junction between liver and duodenum (Fig. 4B schematic) were isolated from embryos

Table 1. Expression of pancreas endocrine and exocrine markers is similar in chicken embryos grown in ovo and in vitro

Hamburger-Hamilton stage	Glucagon	Insulin	Carboxypeptidase A
In ovo			
Stage 16	+	-	n.d.
Stage 19	+	+	-
Stage 21-22	+	+	+
In vitro			
Stage 16	+	-	n.d.
Stage 19	+	+	-
Stage 22	n.d.	n.d.	+

Dorsal pancreas rudiments were dissected from staged embryos grown in vitro and in ovo and assayed for expression of glucagon, insulin and carboxypeptidase A by whole mount antibody staining. For each antibody, at least six embryos per stage were examined. The plus sign indicates visible DAB staining within the dorsal pancreas bud (above background staining levels). The minus sign indicates no visible DAB staining above the background level of staining (determined by omission of primary antibody). n.d. indicates not determined. Hamburger-Hamilton stages are indicated.

grown for 2 days to stage 20 after notochord removal at stage 11⁻. Morphogenesis of the ventral buds is not detected until about stage 22-23 (Romanoff, 1960; Dieterlen-Lièvre, 1970), about 2 days after the appearance of the dorsal bud. Similar to the dorsal bud, gene expression precedes morphogenesis in the ventral bud; transcription of *Isl1*, *pax6*, *pdx1* and glucagon in the ventral pancreatic anlage is first detected at stage 19-20. As shown in Fig. 4B, gene expression in the ventral pancreatic bud is not affected by notochord deletion. This further suggests that notochord removal specifically affects development of the dorsal pancreas.

To monitor dorsal pancreas bud morphogenesis, embryos grown for 2 days after notochord removal, to stages 20-22, were sectioned and stained. Fig. 5 shows that the initial endoderm evagination that leads to dorsal pancreas bud formation clearly occurs in embryos after notochord deletion. Terminal branches in the distal portions of the dorsal bud endoderm are detected at the end of in vitro development (Fig. 5D) but these are less numerous than in similar stage controls (compare to Fig. 1F). By this stage we also observed that the layer of mesenchyme over the dorsal endodermal bud appears slightly thicker than in control embryo pancreas buds (compare Fig. 1C and Fig. 5D). At this early stage, the ventral pancreatic bud is either not present or too small to assess. The morphology of adjacent liver, stomach (Fig. 5D) aorta, anterior intestinal portal, and more distant structures such as the heart and limb buds appears unaffected.

Immunohistochemical analysis using antibodies specific for endocrine and exocrine markers was also performed. Expression of glucagon, insulin (Fig. 5A-C) and carboxypeptidase A (data not shown) was detected in dorsal pancreas buds from control embryos grown in vitro. No evidence of expression of these proteins was detected in dorsal buds from similar stage notochord-deleted embryos (Fig. 5E,F).

Germ layer culture reveals notochord induction of pancreas genes

Our experiments support the hypothesis that the notochord signals the endoderm to initiate pancreatic development. However, it is also possible that our manipulations somehow expose endoderm to factors that repress pancreas development. To test directly whether notochord induces gene expression in prepancreatic endoderm, we adapted a chick tissue culture system (Dickinson et al., 1995) to grow the embryonic endoderm layer alone or recombined with notochord in vitro. As shown in Fig. 6A, dorsal endoderm, which included the dorsal pancreatic anlage, and adjacent notochord were isolated from stage 12⁻ embryos. By this stage, pancreatic endoderm expresses *pax6*, *Isl1* and *HNF3β*, but not glucagon, *pdx1* or insulin (Fig. 2A). Ideally we would have removed endoderm from stage 11⁻ embryos to match the stage of notochord removal in previous experiments (Fig. 3). However, prior to stage 11⁺/12⁻, dorsal endoderm, which is one cell layer thick, adheres to notochord and even more firmly to somites and medial splanchnic mesoderm, pre-

venting removal of an intact endoderm sheet large enough for tissue culture; protease digestions dispersed endoderm instead of separating tissues (S. K. K., unpublished results). As a further control for these manipulations, notochord adjacent to prepancreatic endoderm was recombined with non-pancreatic posterior endoderm (Fig. 6B).

Endoderm isolated in this manner expresses *caudal* (*cdxA*: Frumkin et al., 1994) a homeobox gene known to be expressed

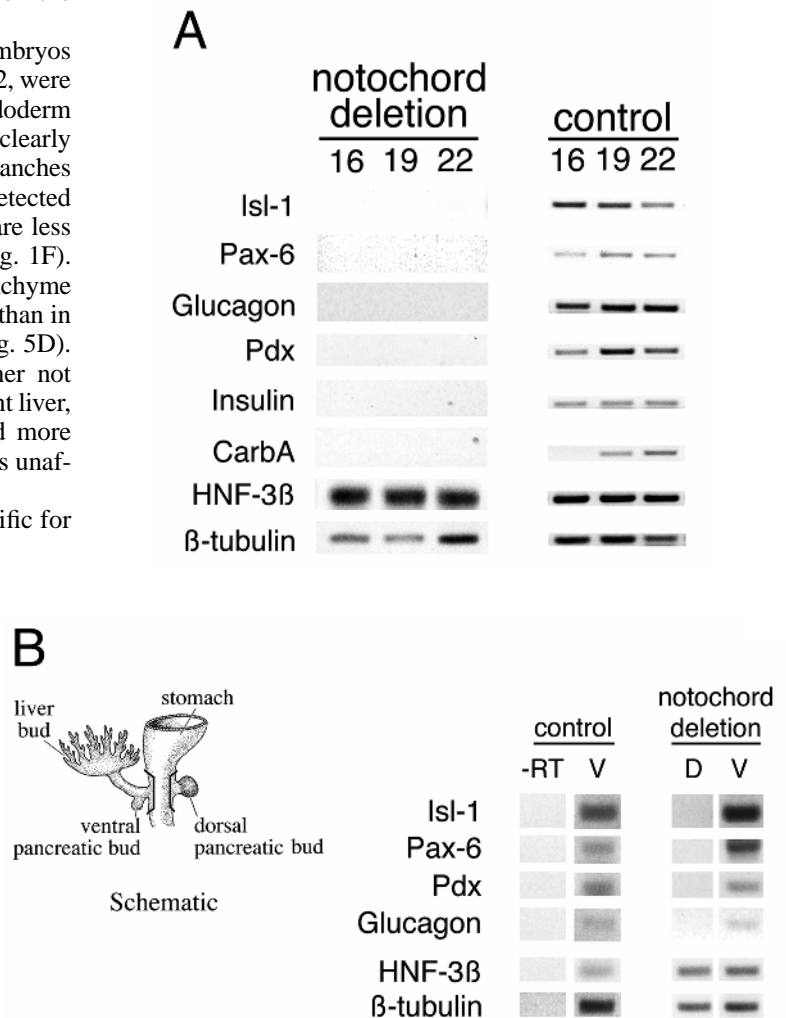


Fig. 4. Effect of notochord removal on pancreas gene expression. (A) Marker gene expression analysis by RT-PCR was performed as described in the Fig. 2 legend. Embryos were explanted at stage 11⁻ and notochord removed as described in the text. In the control, the endoderm flap was cut and displaced and the length of exposed notochord removed from underlying neural tube, then replaced (see Materials and Methods). The endoderm flap was replaced and these embryos grown until the stage indicated (16, 19 or 22). (B) Deletion of notochord does not affect early gene expression of ventral pancreas. Stage 11⁻ control embryos without notochord manipulation and stage 11⁻ embryos with notochords deleted were grown for 2 days in vitro to stage 20. The midgut from these embryos was dissected as shown in the schematic and tissue corresponding to the ventral pancreatic anlage (V) separated from the dorsal pancreas tissue (D). RNA was extracted and analyzed by RT-PCR for expression of *Isl1*, *pax6*, *pdx1*, glucagon, *HNF-3β* and β -tubulin. After growth to stage 20 in vitro, insulin and carboxypeptidase A expression could not be detected in the ventral pancreatic bud. No signal was detected in control samples of ventral pancreatic RNA untreated with reverse transcriptase (-RT).

in endoderm-derived gut epithelium, but showed no detectable expression of *Gnot1*, a homeobox gene expressed in axial mesoderm including the notochord (Knezevic et al., 1995), demonstrating the absence of adherent notochord in dorsal endoderm explants. Isolated notochord, in contrast, was found to express *Gnot1* but not *cdxA*, demonstrating the absence of adherent endoderm in isolated notochords (data not shown). After growth of notochord for 3 days in vitro (Fig. 6C, column N) expression of pancreas marker genes was not detected, further suggesting the absence of pancreatic endoderm cells adhering to isolated notochords.

After growth of prepancreatic endoderm, isolated at stage 12⁻, in collagen matrix for 3 days in vitro, expression of *pax6*, *Isl1*, *HNF-3 β* and glucagon was detected; no *pdx1* or insulin expression was detected (Fig. 6, column E12). This suggests that by as early as stage 12⁻ (15 somites), pancreatic endoderm may be partly, but not fully, committed toward a pancreatic fate. In contrast, when explants of prepancreatic endoderm were recombined with notochord, we detected additional expression of *pdx1* and insulin (Fig. 6C, column N + E12). When this notochord is recombined with endoderm from a region posterior to the pancreatic anlage (Fig. 6C, column N + pE12) only *HNF-3 β* and β -tubulin expression are detected. These results provide direct evidence that the notochord can initiate expression of early pancreatic genes in competent endoderm.

In contrast to endoderm isolated at stage 12⁻, endoderm isolated later, at stage 13⁻ (18 somites), and grown in vitro for 3 days expresses *pax6*, *Isl1*, *HNF-3 β* , glucagon and, additionally, *pdx1* (Fig. 6C, column E13). After 3 days in vitro, growth of endoderm isolated at stage 14⁻ (21 somites), expression of insulin, in addition to the other pancreas marker genes, is detected (Fig. 6C, column E14). These results suggest that endoderm may acquire a pancreatic fate in a series of steps, and that the period when notochord signals endoderm to initiate pancreatic differentiation may be completed by stage 14⁻.

DISCUSSION

The mechanisms that initiate the development of complex endodermally derived organs like the pancreas have remained obscure. A cell interaction model for specifying pancreatic endoderm predicts that removal of the tissue source(s) of intercellular signal(s) prior to commitment might alter expression of the earliest genes required for pancreas development. Results of earlier studies of mouse (Wessells and Cohen, 1967; Spooner et al., 1970) and chicken embryos (Dieterlen-Lièvre and Beaupain, 1974; Sumiya and Mizuno, 1987) suggest that as early as the 10-13 somite stage, the dorsal endodermal bud is specified to become pancreas. However, the mechanisms for specifying endoderm to a pancreatic fate are unknown.

Our studies of chick pancreas development provide evidence for a prepancreatic state when endoderm fate can be influenced by cell interactions, and we identify the notochord as the source of a signal required for pancreatic differentiation. At stage 11⁻ in chicks, when no mesodermal tissue other than notochord contacts the axial-midline endoderm, removal of the notochord prevents the expression of genes required for pancreatic development and function. Our results suggest that some signal(s)

from notochord may directly initiate gene expression in the dorsal endoderm, and that cell interactions crucial for initiating dorsal pancreatic differentiation are not required for the first steps of ventral pancreas development. The competence of dorsal endoderm to respond to notochord signals and initiate pancreatic gene expression appears to be localized; endoderm caudal to the pancreatic anlage does not respond to these notochord signals. These results show that, in addition to patterning neural and somite differentiation, the notochord can pattern endoderm.

Timing of pancreatic endoderm induction

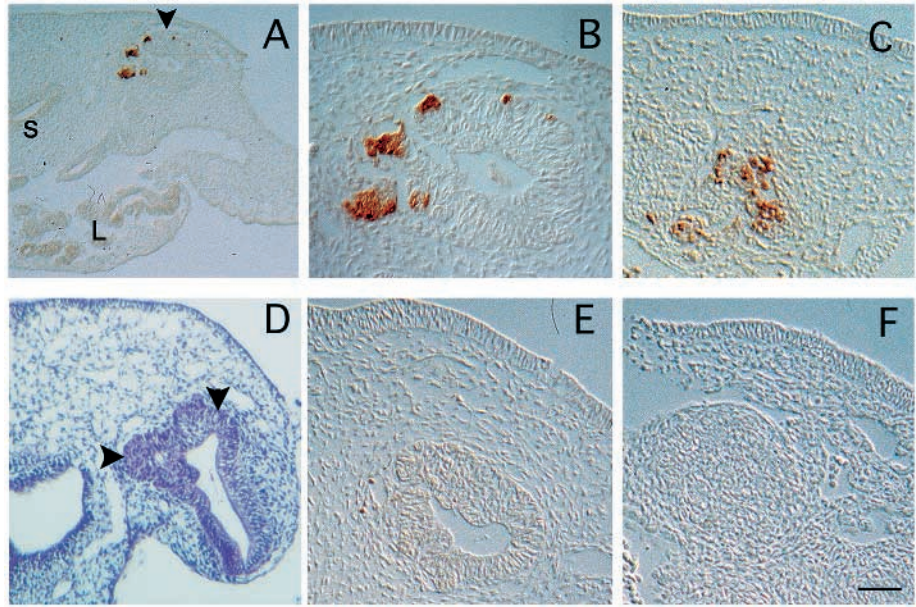
Prior to pancreatic morphogenesis in the chick embryo, we detect expression of *Isl1*, *pax6* then *pdx1*, insulin and glucagon. Expression of these genes prior to morphogenesis has also been observed in the mouse (Gittes and Rutter, 1992; Ahlgren et al., 1996) although in this species *pdx1* precedes *Isl1* expression (Ahlgren et al., 1997). Removal of notochord at stage 11 of chick embryogenesis prevents further dorsal pancreas expression of *Isl1* and *pax6* and subsequent expression of *pdx1*, insulin, glucagon and carboxypeptidase A genes. Analysis of mouse embryos deficient in ISL1 function also reveals markedly reduced PDX1 expression in the dorsal pancreatic anlage and no subsequent expression of endocrine or exocrine genes (Ahlgren et al., 1997). This suggests that the severe effect on dorsal pancreatic gene expression we observe in chick embryos after notochord removal may partly result from loss of ISL1 function.

After notochord removal at stage 11, we observe evagination of a dorsal pancreatic bud consisting of branching endoderm covered by a mesenchymal cap in the absence of *Isl1* expression. In ISL1-deficient mice, however, dorsal pancreatic mesenchyme is virtually absent (Ahlgren et al., 1997). Thus, initial dorsal pancreatic morphogenesis in chicks may occur independently of ISL1 function or of notochord induction. *Isl1* expression in endoderm (Fig. 2) is detected at the stage when the notochord was removed (Fig. 3), raising an alternate possibility that ISL1-dependent functions governing dorsal pancreatic mesenchyme growth may occur before notochord removal prevents further dorsal *Isl1* expression. Our notochord deletion studies further suggest that initial pancreas morphogenesis may not require the continuing presence of notochord. This is reminiscent of studies showing that once ventral neural tube is induced by the notochord, later differentiation of the ventral floor plate does not require the continued presence of notochord (Kitchin, 1949; Watterson et al., 1954).

The main conclusion from results of tissue culture experiments (Fig. 6) is that the notochord can initiate expression of pancreatic genes in isolated pancreatic endoderm. These results also provide evidence that signaling to endoderm by notochord may occur in a stepwise sequence, in agreement with previous work suggesting that endoderm gradually acquires the capacity to differentiate into pancreas (Wessells and Cohen, 1967; Sumiya and Mizuno, 1987). Endoderm commitment to express pancreatic genes is likely incomplete by stage 11 because removal of notochord at stage 11 prevents subsequent expression of *Isl1*, *pax6* and other genes necessary for pancreatic development. Endoderm isolated from notochord at stage 12⁻ and grown for 3 days in vitro expresses some pancreas genes including glucagon, *pax6* and *Isl1*. Later, by stage 14⁻, endoderm isolated from notochord can express all pancreas

Fig. 5. Pancreas morphology and altered expression of endocrine proteins after notochord deletion. (A-F) Dorsal is toward the top and anterior is toward the left. (A-C) Dorsal pancreas from embryos grown in vitro from stage 11 to stage 20.

(A) Detection of glucagon expression by anti-glucagon antibodies in sagittal section of midgut from embryos grown to stage 20. Arrowhead indicates dorsal bud and brown staining, glucagon-expressing cells. The lumen of the stomach (S) and liver rudiment (L) are marked. (B) 2.5× magnification of cells in A. (C) Detection of insulin expression by anti-insulin antibodies in stage 20 embryonic dorsal pancreas. (D-F) Dorsal pancreas from embryos grown to stage 20 in vitro after notochord deletion at stage 11⁻. (D) H and E staining of dorsal pancreas bud from embryo grown for 3.5 days. Some dorsal bud endoderm branching (arrowheads) is observed here and in the majority of embryos grown after notochord removal. (E) Glucagon and (F) insulin antibody staining of sections taken from an embryo grown for 3.5 days. No staining is observed. Scale bar, 80 μm in A and D; 30 μm in B,C,E and F.

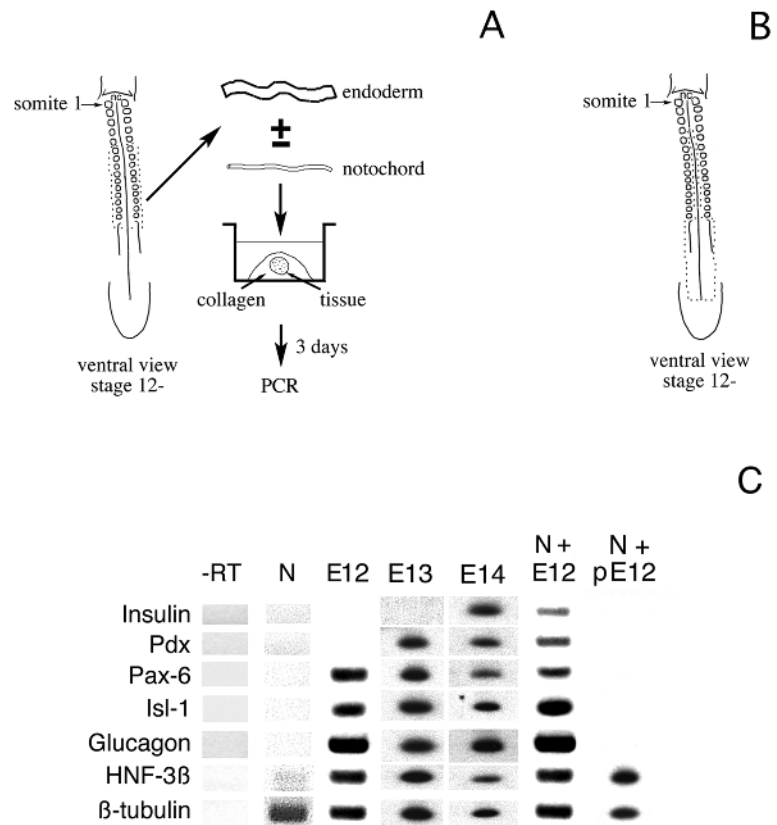


marker genes tested, including *pdx1* and insulin. Cell interactions which require direct contact between the notochord and endoderm must be completed by stage 14 when the notochord is separated from the dorsal pancreatic endoderm by the interposing aorta (see Fig. 3B).

Targets of notochord signaling

Although our studies have identified the notochord as a source of a signal necessary for endoderm differentiation, the actual sequence of cell interactions and the target(s) of the notochord signal are unclear. Expression of two genes by endoderm in

Fig. 6. In vitro endoderm culture reveals that notochord induces pancreas-expressed genes. (A) Schematic of microsurgery for explantation of prepancreatic endoderm. Endoderm from the region marked by the dashed lines was removed from stage 11⁺ and stage 12⁻ embryos as described in Materials and Methods and cultured for 3 days in a collagen matrix covered by M199 medium with or without contact with notochord harvested from the same embryonic region. (B) Schematic of microsurgery for explantation of non-pancreatic endoderm with notochord. Endoderm and notochord, as marked by the dashed lines, were removed from stage 12⁻ embryos and grown in contact, as described in Materials and Methods. (C) Induction of pancreas-expressed marker genes by notochord. RT-PCR analysis of RNA from notochord (N) grown for 3 days in vitro shows no *pdx1*, insulin, *pax6*, *Isl1* or glucagon expression. In this cell culture system, *Isl1*, *pax6* and glucagon expression are detected in endoderm isolated at stage 12⁻ after explanting and growth for 3 days without notochord (E12). Endoderm isolated from stage 13⁻ (E13) or stage 14⁻ (E14) and grown for 3 days without notochord expresses additional marker genes. After recombination of notochord with stage 12⁻ endoderm (N+E12), *pdx1* and insulin expression are induced. *HNF-3β* and β-tubulin are loading markers. Recombination of notochord with stage 12⁻ endoderm isolated from a region posterior to the pancreatic anlage (N+pE12) resulted in expression of only HNF-3β and β-tubulin. No signal was detected in control samples of RNA from notochord-endoderm recombinants untreated with reverse transcriptase (-RT).



tissue culture, the chick homologue of *pdx1*, and insulin, appears to require notochord, although it is unclear whether actual contact between notochord and endoderm is necessary. Our studies suggest that one direct target of notochord signals may be the dorsal endoderm, although we cannot exclude the possibility that signaling of endoderm by notochord may also occur indirectly, through notochord signals to tissues adjacent to endoderm. The competence of dorsal endoderm to respond to notochord signal(s) appears to be restricted along the rostrocaudal axis. It is unclear if notochord activity is also spatially restricted and we are currently testing this possibility.

Ventral pancreatic endoderm is not contacted by notochord and is therefore not expected to be affected by notochord removal. In the chick, the ventral pancreas buds develop mainly into exocrine acinar tissue mixed with smaller islets than those differentiated from the dorsal anlage. Our results demonstrate that the expression of pancreas genes, including *pdx1* and *Isl1*, in the ventral pancreas buds is not markedly altered by notochord removal prior to stage 11. If cell interactions also pattern ventral endoderm to a pancreatic fate, our observations suggest that tissues other than notochord may provide the necessary signals for ventral pancreas development.

Common mechanisms controlling neural and pancreatic differentiation?

The results presented here also raise interesting parallels between the polarizing activity of notochord on neural tube and notochord activity on pancreatic endoderm. In both cases gene expression in a tube of cells recently folded from a single layer of cells is influenced by the same midline axial mesoderm structure. Genes expressed early in the developing neural tube including *pax6* (Goulding et al., 1993), *Isl1* (Ericson et al., 1992), *HNF-3 β* (Ruiz i Altaba et al., 1995) and others (reviewed by Placzek and Furley, 1996) are also expressed in the dorsal pancreatic anlage (Dieterlen-Lièvre and Beaupain, 1974; Le Douarin, 1988; Slack, 1995). Recent demonstration of ISL1 expression in dorsal but not ventral pancreatic mesenchyme (Ahlgren et al., 1997) suggests that the mesenchyme surrounding the early pancreatic epithelium is patterned along a dorsal-ventral axis. Regulatory signals from the notochord are known to influence the dorsal-ventral expression pattern of *Isl1* and *pax6* in the ventrolateral neural tube (Yamada et al., 1991; Goulding et al., 1993) and we have shown that continuing *Isl1* and *pax6* expression in the dorsal pancreas may require permissive signals from the notochord. It seems likely that there may exist common elements of regulation of neural and endodermal development (see Ahlgren et al., 1997).

Two well-known classes of intercellular signals which regulate neural tube differentiation are SHH (Roelink et al., 1994; Tanabe et al., 1995; Martí et al., 1995) and members of the TGF β family (Dickinson et al., 1995; Liem et al., 1995). Could either of these proteins account for the endoderm-signaling activity we have detected in the notochord? Recent studies on endoderm differentiation in *Xenopus* suggest that members of the TGF β family including activin and Vg1 can induce expression of *XlHbox8* (Gamer and Wright, 1995; Henry et al., 1996), the *Xenopus* homologue of *pdx1* (Wright et al., 1988). In addition, caudal notochord adjacent to the pancreatic anlage is known to express SHH (Echelard et al., 1993), BMP7 (Lyons et al., 1995) and activin (Connolly et al., 1995)

during the period when prepancreatic endoderm appears to require notochord-derived signals.

We have tested purified proteins for activity when added to isolated stage 11⁺/12⁻ dorsal endoderm grown in tissue culture. We have not detected pancreas gene-inducing activity in biologically active purified SHH, TGF- β 1 or BMP4 (S.K., M.H. and D.M. unpublished results). Identification of specific notochord factors that commit dorsal endoderm to a pancreatic fate should also allow characterization of the tissues and signals important for ventral pancreas development.

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