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Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate

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Abstract

During embryonic development, organs arise along the gut tube as a series of buds in a stereotyped anterior–posterior (A-P) pattern. Using chick-quail chimeras and in vitro tissue recombination, we studied the interactions governing the induction and maintenance of endodermal organ identify focusing on the pancreas. Though several permissive signals in pancreatic development have been previously identified, here we provide evidence that lateral plate mesoderm sends instructive signals to the endoderm, signals that induce expression of the pancreatic genes *Pdx1*, *p48*, *Nkx6.1*, *glucagon*, and *insulin*. Moreover, this instructive signal directs cells to form ectopic insulin-positive islet-like clusters in endoderm that would otherwise form more rostral organs. Once generated, endocrine cells no longer require interaction with mesoderm, but nonendocrine cells continue to require permissive signals from the mesoderm. Stimulation of activin, BMP, or retinoic acid signaling is sufficient to induce *Pdx1* expression in endoderm anterior to the pancreas. Lateral plate mesoderm appears to pattern the endoderm in a posterior-dominant fashion as first noted in the patterning of the neural tube at the same embryonic stage. These findings argue for a central role of the mesoderm in coordinating the A-P pattern of all three primary germ layers.

Introduction

During embryonic development, the endoderm gives rise to the epithelium of the gut and associated organs, including the lung, liver, and pancreas. These organs arise along the gut tube as a series of buds in a stereotyped anterior–posterior (A-P) and dorsal–ventral pattern, and their budding is preceded by regionalized expression of organ-specific genes. The molecular mechanisms that specify the pancreas and other endodermal organs are not well understood. However, recent work has focused on directing the in vitro differentiation of embryonic stem cells into particular lineages, such as insulin-producing β cells of the pancreas. A better understanding of the extrinsic cues responsible for establishing organ identity in vivo would aid such in vitro differentiation studies. The aim of the present study is to elucidate the interactions governing the induction and maintenance of regional identity in the endoderm. Particular attention is given to the establishment of the prepancreatic domain.

Throughout its development, endoderm is influenced by signaling from mesoderm. During and just after gastrulation, endoderm receives initial A-P patterning information from adjacent mesectoderm. Mesectodermal signals are responsible for the induction of posterior identity within the endoderm (Wells and Melton, 2000). Subsequently, endoderm and adjacent mesoderm undergo a series of reciprocal inductions that specify cell fates in both tissues (Grapin-Botton and Melton, 2000). As an example, signals from the endoderm specify cardiac myoblasts (Marvin et al., 2001; Narita et al., 1997; Schultheiss et al., 1995) which in turn provide signals that induce the liver domain from ventral foregut endoderm (Gualdi et al., 1996; Jung et al., 1999; Le Douarin, 1964).

In the case of the pancreas, signals from notochord (Kim et al., 1997) and from blood vessel endothelium (Lammert et al., 2001), both mesodermal derivatives, are required for

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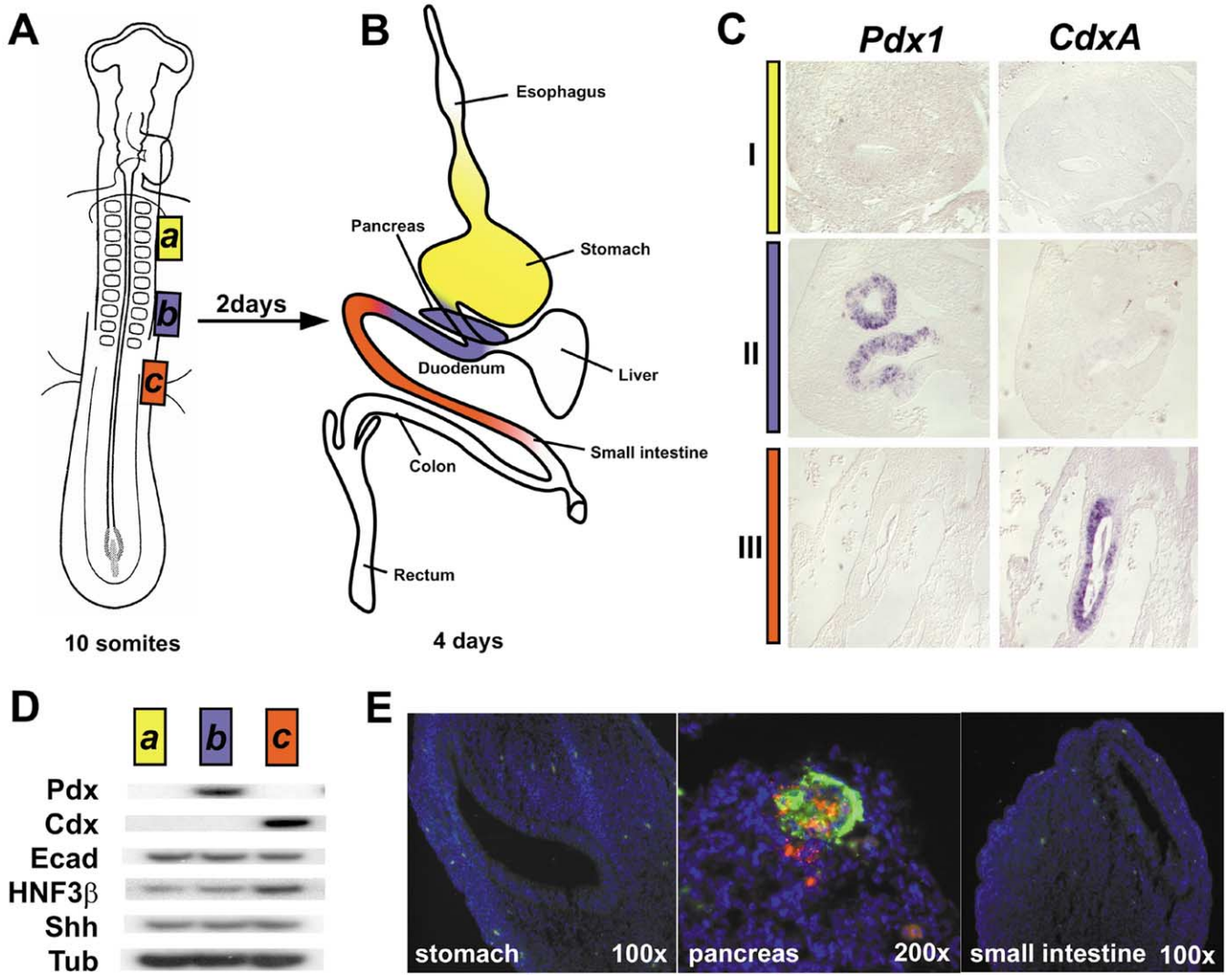
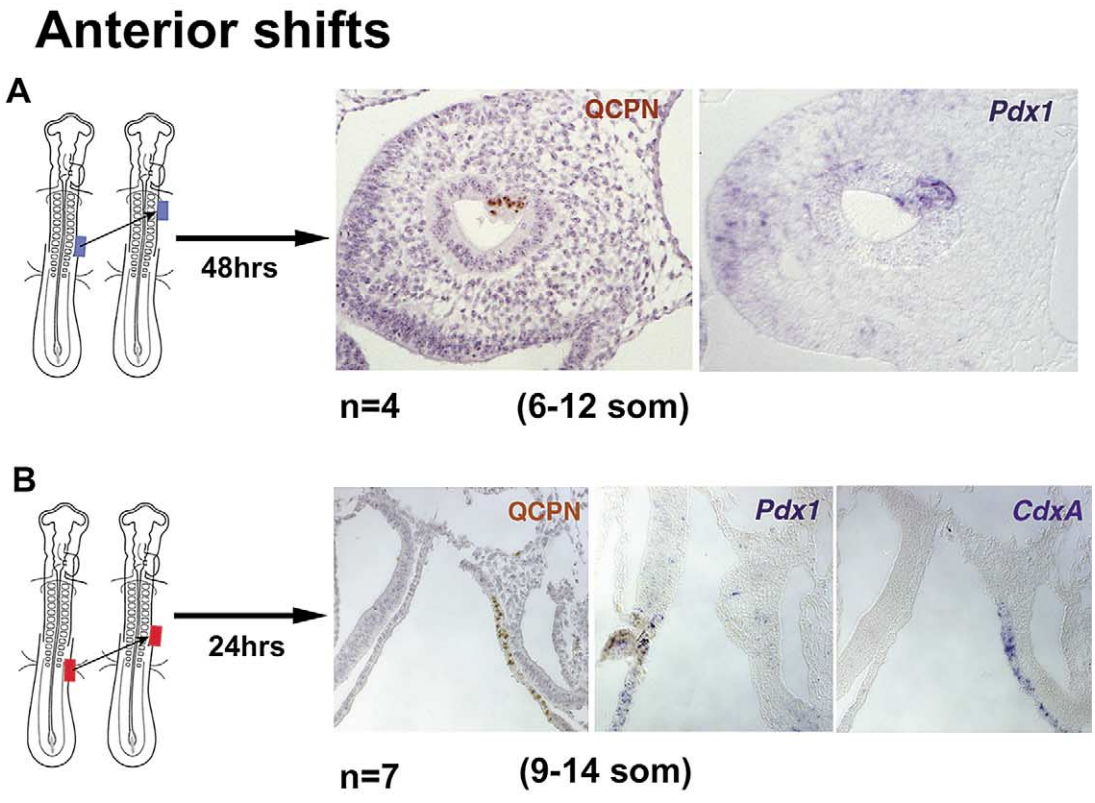


Fig. 1. Endoderm domains at the 10-somite stage. Schematic diagram of 10-somite-stage chick embryo (A) viewed from the ventral surface, anterior at top. Boxes indicate three lateral endoderm domains used in this study. Anterior domain (a, yellow), lateral to somites 2–4; medial domain (b, blue), lateral to somites 7–9; posterior domain (c, red), lateral to presumptive somites 12–14. The organs to which these endodermal blocks contribute after 48 h of development are indicated in 4-day embryo (B). Region a will contribute to stomach and esophagus; region b to pancreas and duodenum; region c to small intestine. In situ hybridization for *Pdx1* and *CdxA* on transverse sections through each region in a 4-day embryo are shown in (C). Section I, stomach, expresses neither *Pdx1* nor *CdxA;* section II, pancreas/duodenum, expresses *Pdx1* but not *CdxA;* section III, small intestine, expresses *CdxA* but not *Pdx1*. RT-PCR results on RNA isolated from indicated endoderm blocks dissected from 12-somite embryo (D). Region a expresses neither *Pdx1* nor *CdxA;* region b expresses *Pdx1* not *CdxA;* region c expresses *CdxA* not *Pdx1*. Controls show all regions express the endodermal and epithelial markers *E-cadherin* (E-cad), *HNF3β*, and *sonic hedgehog* (Shh). *β-tubulin* (Tub) serves as control for RNA recovery. Endoderm cells from region b labeled with Dil at the 10-somite stage contribute to the pancreas and not organs anterior or posterior to it by day 7 (E). Immunohistochemistry using antibody against insulin in green (FITC), CM-Dil fluorescence in red, nuclei in blue (DAPI).

proper differentiation of the dorsal pancreas. However, neither of these signals can induce pancreatic markers in endoderm that would not normally form the pancreas, suggesting that these signals are permissive rather than instructive. That is, they do not establish the pancreatic domain, rather they act upon endoderm that has already received a "pancreatic pre-pattern." Similarly, ventral foregut endoderm, which will give rise to liver and ventral pancreas, is composed of a population of bipotential precursors able to adopt gene expression patterns characteristic of each organ (Deutsch et al., 2001). In this model of liver development, FGF signaling from cardiac mesoderm is necessary for liver differentiation; abrogation of FGF signals allows the emergence of the endoderm's default pancreatic fate. To date, no signals have been identified that can act dominantly to convert nonpancreatic endoderm to form pancreas.

The pancreatic buds are marked by early expression of the gene *Pdx1*, a homeobox-containing transcription factor that is essential for pancreatic development. Genetic lineage labeling in mouse has shown that, early in organ formation (e10.5), *Pdx1* expression marks a pluripotent population of cells that gives rise to all cell types of the neonatal pancreas


Anterior shifts
A
48hrs
QCPN
Pdx1
n=4
(6-12 som)
B
24hrs
QCPN
Pdx1
CdxA
n=7
(9-14 som)
Posterior shifts
C
48hrs
QCPN
Pdx-1
n=4
(5-12 som)
D
48hrs
QCPN
Pdx1
CdxA
n=5
(5-12 som)

(endocrine, exocrine, and duct) and epithelium of the duodenum and posterior stomach (Gu et al., 2002). The presence of a rudimentary bud in *Pdx1* null mice indicates that *Pdx1* is not required for the initial specification of the pancreatic domain, but is rather a consequence of that specification. It is not known what turns *Pdx1* on in the domain that will form the pancreas.

We show here that the endoderm that will form the ventral pancreas has received sufficient information to initiate pancreatic gene expression by the six-somite stage in the chick, before gut tube formation or the initiation of organ budding. We further show that lateral plate mesoderm underlying the *Pdx1* expression domain sends instructive signals that are sufficient to induce pancreatic development in naive endoderm. This induction can be reproduced by using soluble growth factors of the BMP and activin families. The lateral plate mesoderm appears to pattern the endoderm in a posterior-dominant fashion analogous to the patterning of the neural tube, providing evidence for a central role of the mesoderm that coordinates the patterning of all three germ layers. In addition to inducing pancreatic gene expression, interaction with the mesoderm induces the endoderm to coalesce into hormone-expressing islet-like cell clusters. This is the first example of a signal capable of instructing ectopic pancreatic identity.

Materials and methods

Chick-quail chimera generation

Chick-quail grafting techniques have been previously described (Holland, 1993). Fertilized Japanese quail eggs (CBT Farms, MD) and white leghorn chicken eggs (SPAFAS, CT and Arare Farm, Geneva, CH) were incubated at 38°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (1951). Lateral endoderm blocks from donor quail embryos from HH stages 8–12 (5–14 somites) were dissected free of mesenchyme in 1.25 mg/ml dispase (Gibco BRL) in PBS by using tungsten needles electropolished in 2 μN NaOH or microscalpels made by sharpening steel needles. Blocks were taken from three positions along the A-P axis: between somites 2 and 4, 7 and 9, and 12 and 14. At the 10-somite stage, the level of the future somites posterior to the last somite was estimated by using the size of the last set of somites. Endoderm blocks were rinsed in PBS and immediately grafted into host chick embryos. Chick embryos were prepared by using a modified New culture technique (Sundin and Eichele, 1992) in which embryos are cultured ventral side up on agarose–albumin plates supported by a filter paper ring. Host embryos from HH stages 8–12 were used. A longitudinal incision in the endoderm just lateral to the somites was made with a microscalpel at one of the three A-P positions described above, and the donor endoderm was grafted into the hole in the host endoderm with the basal side of the graft epithelium contacting the splanchnic mesoderm of the host and the edges of the donor tissue tucked beneath the rim of the host endoderm to keep the epithelium in place during healing. Chimeric embryos were incubated in a humidified chamber in a tissue culture incubator (Queue StabilTherm or Coudelou) for 24 (HH stage 17) or 48 (HH stage 20) h. Embryos were fixed in FEA (3 vol formaldehyde, 6 vol ethanol, 1 vol glacial acetic acid) overnight at 4°C dehydrated in an ethanol series, soaked in xylene, embedded in paraffin, and sectioned at 6 μm. Alternate sections were collected on three sets of glass slides (SuperFrost Plus) for each embryo.

Immunostaining

One of the three sets of alternate 6-μm sections for each chimeric embryo was stained with the quail-specific antibody QCPN (Developmental Studies Hybridoma Bank) to identify cells derived from the grafted tissue. Sections were dewaxed in xylene and rehydrated in PBS. Endogenous peroxidases were blocked by incubation with 2.4% H_2O_2 in PBS. Slides were incubated overnight at 4°C with a 1:200 dilution of purified QCPN primary antibody (Developmental Studies Hybridoma Bank), rinsed in PBS, and then incubated for 2 h with a 1:200 dilution of biotin-conjugated anti-mouse lgG (Jackson ImmunoResearch). Signal was amplified by using the Vectastain ABC Kit (Vecto Labs) and stained with 3,3′-diaminobenzidine (DAB; Vector Labs). Slides were counterstained with Gill's hematoxylin (Poly Scientific), dehydrated to xylene, and mounted with Permount (Fisher). CM-Dil (Molecular Probes) was diluted to 2 mg/ml in DMSO and injected with a fine pulled pipet in the area just lateral to somite 8 in 10-somite-stage embryos in ovo. Cells of all three germ layers at the site of injection were labeled. Embryos were allowed to develop to day 7. Dissected guts were fixed in zinc formalin (Polysciences), equilibrated in 30% sucrose overnight, embedded in OCT (Tissue-Tek), and cryosectioned at 12 μm. Sections

Fig. 2. Endodermal commitment and plasticity. In vivo grafting of endoderm blocks from donor quail into host chick embryos. Origin and graft location are represented schematically at left. Age range of donors and hosts are indicated in parentheses. In situ hybridization on adjacent paraffin sections after culture is shown at right. QCPN anti-quail monoclonal antibody shows graft location. Probes as indicated. (A, B) Endoderm grafted anteriorly retains its posterior identity. Endoderm of the future *Pdx1*-expressing domain grafted anteriorly (A) shows *Pdx1* specification by the 6-somite stage. Endoderm of the future *CdxA*-expressing domain grafted into medial region (B) shows *CdxA* specification by the 9-somite stage. (C, D) Endoderm grafted posteriorly is respecified to adopt posterior fate. Anterior endoderm grafted into the *Pdx1*-expressing region (C) shows *Pdx1* upregulation in the graft, both before and after onset of *Pdx1* expression in the host tissue. Grafting of medial domain endoderm into the posterior domain (D) results in downregulation of *Pdx1* expression in the graft and upregulation of *CdxA*.

were immunostained by using guinea pig anti-insulin antibody (Dako) and visualized with a FITC-conjugated donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch).

In situ hybridization on paraffin sections

Sections (6 μm) on glass slides were hybridized with digoxygenin-labeled antisense probes as previously described (Wilting et al., 1997). Slides were dewaxed, treated with 1 μg/ml proteinase K for 7 min, and postfixed in 4% paraformaldehyde. Hybridization mix contained 1 μg/ml probe, and hybridization was done overnight at 70°C. Sections were washed in maleic acid buffer and blocked with 20% lamb serum/2% blocking reagent (Roche). Slides were washed again and developed with NBT and BCIP (Roche). Full-length cDNA in situ hybridization probes were used: *Pdx1* (Kim et al., 1997) and *CdxA* (Frumkin et al., 1994). Anti-sense probes were generated by using the MaxiScript in vitro transcription kit (Ambion) and digoxygenin–UTP (Roche).

Collagen and Matrigel explant culture

Lateral endoderm, lateral plate mesoderm pieces, or somitic mesoderm from three A-P positions (level of somites 2–4, 7–9, and 12–14) were dissected from HH stage 10 embryos in 1.25 mg/ml dispase (Gibco BRL) in PBS. Dissected pieces were rinsed in PBS and then cultured in a collagen matrix as previously described (Dickinson et al., 1995). Briefly, a 10 μl drop of collagen matrix was pipetted into each well of a 4-well tissue culture plate (Nunclon) and allowed to polymerize. Tissue was transferred onto this collagen pad with a minimum of liquid and sealed by placing another 10 μl drop of collagen matrix on top, leaving a completely encapsulated explant attached to the bottom of the well. Explants were cultured 48 h in low glucose DMEM (LifeTechnologies) supplemented with 15% chick embryo extract (Stemple and Anderson, 1992) and pen/strep. In explants where mesoderm and endoderm were recombined, the two tissues were carefully positioned with the basal face of the endodermal epithelium in direct contact with the cells of the splanchnic mesoderm, or somite mesoderm. Matrigel was used for explants cultured between 3 and 5 days. Explants were embedded in 10 μl pure Matrigel (BD biosciences), polymerized for 10 min at 37°C, and topped with culture medium. Endoderm explants were cultured in collagen droplets as described above with recombinant human growth factors or pathway inhibitors added to a base medium of low glucose DMEM/15% CEE. The following factors were assayed at the concentrations noted in parentheses: ActivinA (5 ng/ml; 50 ng/ml), BMP4 (10 ng/ml; 50 ng/ml), BMP7 (10 ng/ml; 50 ng/ml), bFGF (1 ng/ml; 10 ng/ml), FGF8 (1 ng/ml; 10 ng/ml), FGF10 (1 ng/ml; 10 ng/ml), noggin (10 ng/ml; 100 ng/ml), all-*trans* retinoic acid (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M), TGFβ2 (10 ng/ml; 50 ng/ml), and follistatin (10 ng/ml; 100 ng/ml) all purchased from R&D Systems, Inc., except the retinoic acid, which was obtained from Sigma. Cultures were incubated for 48 h, at which point total RNA was extracted and RT-PCR performed as described below. All embryos were at the 10-somite stage when explants were taken, except as noted.

RNA preparation and RT-PCR

Total RNA was prepared from explants by incubating the entire collagen droplet with embedded tissue in a simple lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris, pH 7.5, 50 mM NaCl) containing 250 μg/ml proteinase K at 42°C for 25 min. The lysate was extracted twice with phenol, precipitated overnight in the presence of 10 μg glycogen, treated with RQ1 DNase (Promega), precipitated again, and reverse transcribed using MMLV reverse transcriptase (RT; Life Technologies or Promega). The product of this RT reaction was used as the template for subsequent PCRs. Controls without RT were performed on the same original samples. Primer pairs were used for PCR with the following conditions: 94°C for 3 min; then 25–30 cycles of 94°C for 30 s, 60°C for 1.5 min, 72°C for 1 min; and finally, 72°C for 5 min. Reactions were performed in an MJ Research PTC-200 thermal cycler or a T3 thermocycler (Biometra). The PCR products were radioactively labeled by the incorporation of α^{32} P[dCTP] and resolved on a 6% polyacrylamide gel and analyzed by autoradiography (Kodak BioMax). Experiments were performed in replicates of between 5 and 20. A positive or negative call indicates that a consistent result was obtained in at least 75% of the replicates. β-Tubulin primers (Kim et al., 1997), 5′-AGATGCTGAACGTGCAGAACAAG-3′ and 5′-CCTTCCTCTTCAAATTCACCCTG-3′, amplify a product of 334 bp. *cPdx1* primers, 5′-CTTCAACAAGTACATCTCCCGACC-3′ and 5′-CGTCCCCGCTTTTTGTCTTC-3′, amplify a product of 135 bp (M. Gannon, personal communication). *CdxA* primers, 5′-AGGAACAAGAAGTGGAGGTGTCCC-3′ and 5′-TACACAAGCCCTGAGCTGAAGTCC-3′, amplify a product of 261 bp (Frumkin et al., 1991). *cShh* primers, 5′-ACAGCTCCCGAAAGCTCTTCTAC-3′ and 5′-TTGATGAGGATGGTGCCCTG-3′, amplify a product of 292 bp (Riddle et al., 1993). *cHNF3β* primers, 5′-TTTTGTTCCCTGCTGGCTCC-3′ and 5′-TCCTTGCGACAACGACAACG-3′, amplify a product of 201 bp (Berger and Sanders, 2000). *cE-cadherin primers*, 5′-GTGATCCGCAATGATGTGGC-3′ and 5′-TCGTTGAGGTAGTCGTAGTCCTGG-3′, amplify a product of 248 bp (Gallin et al., 1987). *Glucagon* primers, 5′-CCATTTTCCATGCTCTGGTGATC-3′ and 5′-TGGTGATAAGACAGAAAGTGCCTG-3′, amplify a product of 309 bp (Hasegawa et al., 1991; Kim et al., 1997). Insulin primers, 5′-TCTTCTGGCTCTCCTTGTCTTTTC-3′ and 5′-CGGCTTCTTGGCTAGTTGCAGTAG-3′, amplify a 308 bp product (Hasegawa et al., 1991; Kim et al., 1997). *Nkx 6.1* primers, 5′-AATAGCGCCCGTGTACGATA-3′ and 5′-

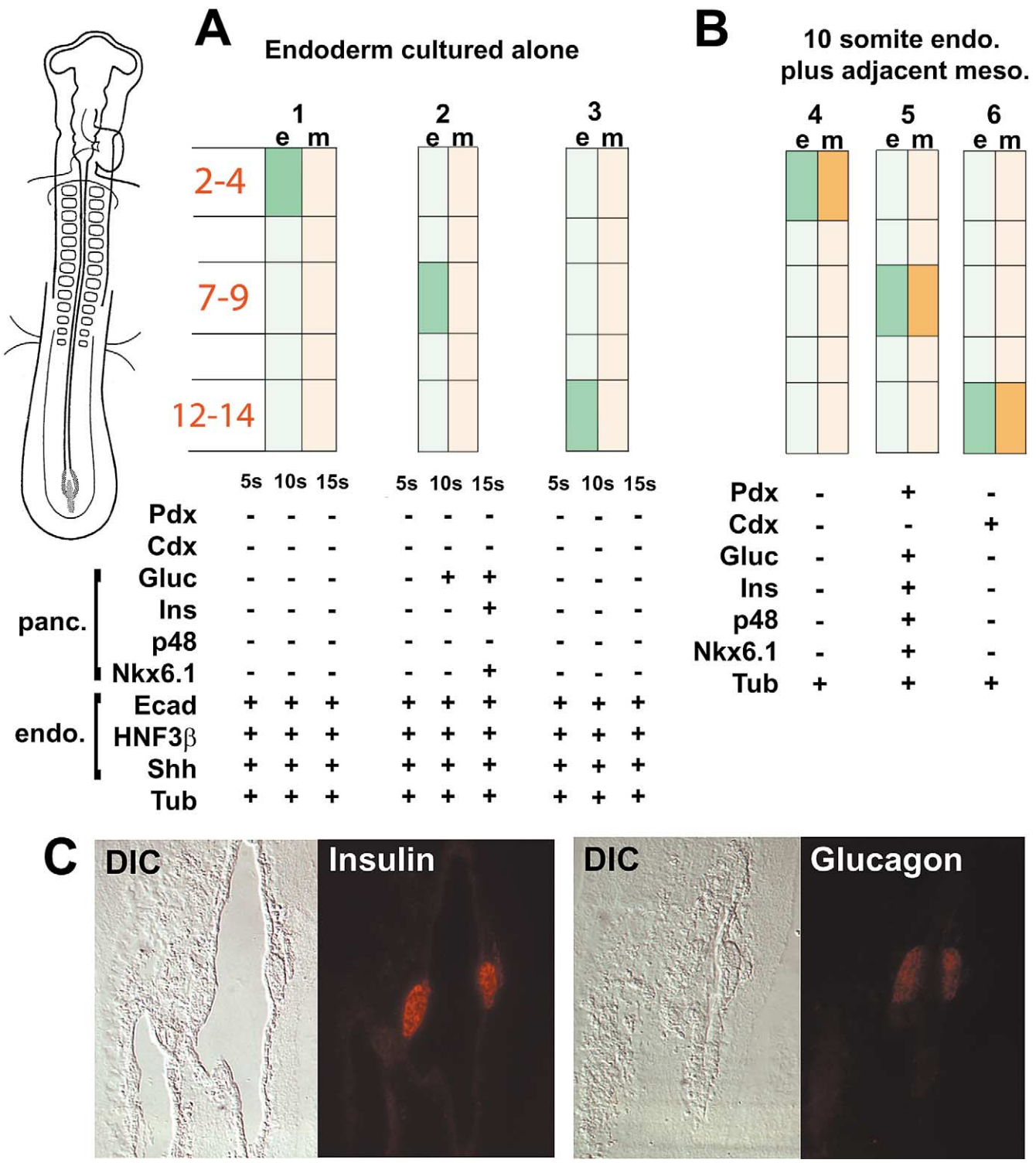


Fig. 3. Interaction with mesoderm is required to support regionalized gene expression. Blocks of endoderm (e, green columns) or lateral plate mesoderm (m, orange columns) from indicated A-P levels are cultured in a collagen droplet either alone (columns 1–3) or recombined as indicated (columns 4–6). Each numbered column represents a recombined explant, cultured for 48 h and then assayed by RT-PCR, as in Fig. 1. All experiments were done in replicates of between 5 and 20, a plus or minus sign indicates a result that is consistent at least 75% of the time. Endoderm cultured without mesoderm from three ages (5-somite stage, 5s; 10-somite stage, 10s; 15-somite stage, 15s) (A) loses *Pdx1* and *CdxA* expression, but maintains expression of endodermal and epithelial markers *E-cadherin* (Ecad), *sonic hedgehog* (Shh), and *HNF3β*. The pancreatic markers *glucagon* (Gluc), *insulin* (Ins), *p48*, and *Nkx6.1* are found only in the endoderm at the 7- to 9-somite level, and are specified progressively over time, with *glucagon, insulin*, and *Nkx6.1* expression specified by the 15-somite stage. Mesoderm cultured alone expresses neither endodermal nor pancreatic markers (data not shown). Endoderm from the 10-somite stage recombined with adjacent lateral plate mesoderm from the same axial position (B) maintains expression of predicted regional markers. Region a (column 4) expresses neither *Pdx1* nor *CdxA:* region b (column 5) expresses *Pdx1* not *CdxA*, as well as *insulin* and *glucagon;* region c (column 6) expresses *CdxA* not *Pdx1*. After 5 days culture in matrigel (C) recombinants of endoderm and mesoderm from region b have formed hormone-positive cell clusters, distinct from epithelium. Insulin and glucagon immunohistochemistry done on slides from different depths of same explant, DIC images show tissue morphology. Nuclear exclusion of staining reveals 10–13 hormone-positive cells per cluster in each plane of section.

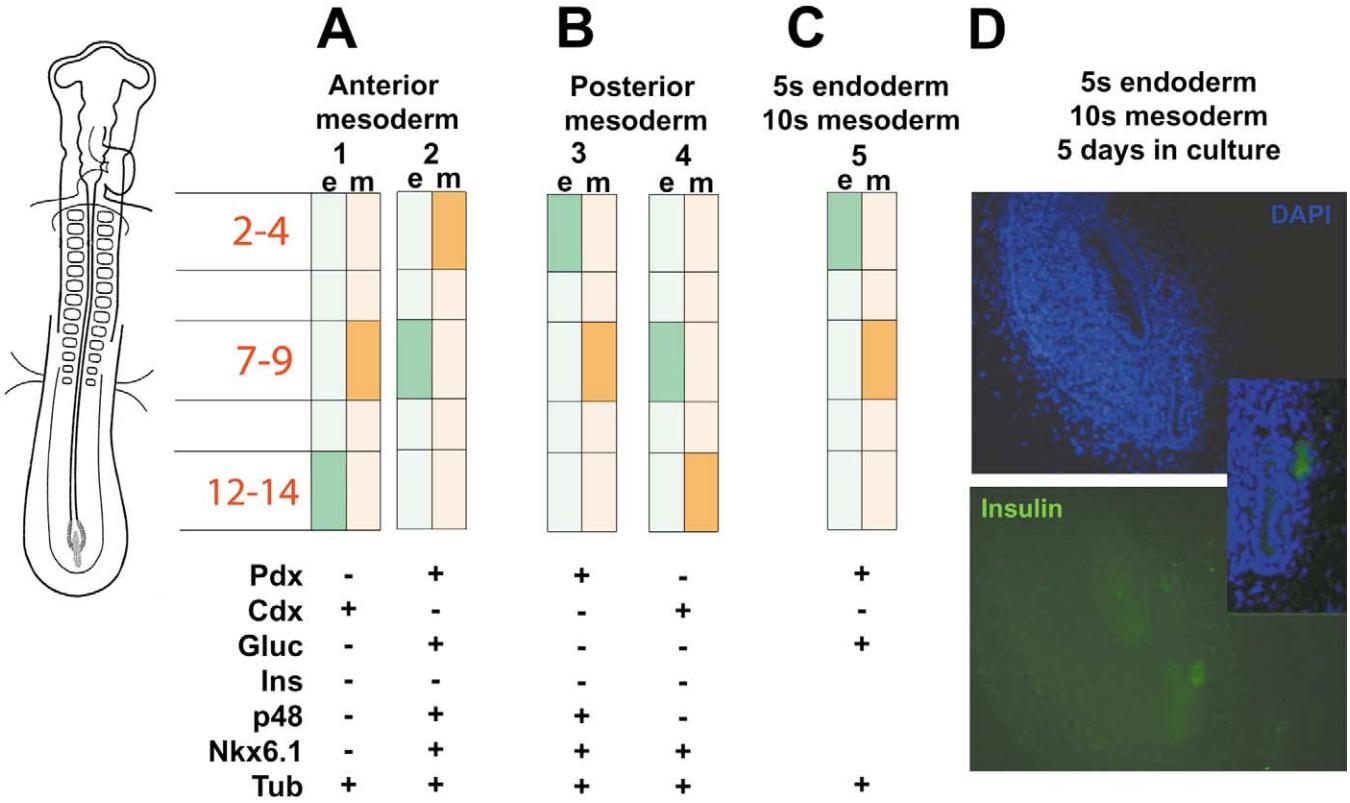


Fig. 4. Posterior mesoderm instructively patterns endoderm. Heterotopic recombinations between endoderm (e, green columns) and lateral plate mesoderm (m, orange columns) blocks from more anterior positions (A) support expression of *Pdx1, CdxA,* and most pancreatic markers (columns 1 and 2). Recombinations with lateral plate mesoderm from more posterior axial positions (B) respecify endoderm to express posterior markers. Region a endoderm recombined with region b mesoderm (column 3) expresses *Pdx1, p48,* and *Nkx6.1* but not the hormones *glucagon* and *insulin;* region b endoderm recombined with region c mesoderm (column 4) expresses *CdxA* and downregulates *Pdx1* and all pancreatic markers except *Nkx6.1.* Endoderm from young (5-somite stage) embryos recombined with region b LPM from 10-somite stage (C) is competent to express full panel of pancreatic markers, including *glucagon.* Region a endoderm from 5-somite-stage embryos recombined with region b mesoderm from 10-somite-stage embryos (same as column 5), cultured for 5 days in matrigel and assayed for presence of insulin protein shows formation of insulin-positive cell clusters delaminating from the endodermal epithelium (D). DAPI, blue, marks cell nuclei; FITC, green, marks insulin protein.

TTATATTCCGCCCGAAAGTG-3′, amplify a product of 200 bp (Qiu et al., 1998). *p48* 5′-CCAGCCCAAGAAAATCATCA-3′ and 5′-ACACTTCGGGTGAGGTCTGT-3′ primers amplify a 374 bp product (BBSRC Chick EST project Web site: http://www.chick.umist.ac.uk/).

Results

Pancreatic fate map

At the 10-somite stage, most of the endoderm is a flat sheet, beginning to fold into a tube at the anterior end. The genes *Pdx1* and *CdxA* mark distinct endoderm territories and are turned on at or a few hours after this stage (Frumkin et al., 1994; Ishii et al., 1997). *Pdx1* is detectable by RT-PCR at the 9- to 10- somite stage, and *CdxA* is detectable by RT-PCR at the 12- to 14- somite stage (Fig. 1D). We refined available fate maps, summarized in Fig. 1B, by grafting quail endoderm into chick hosts at the positions shown in Fig. 1A (data not shown). A combination of previous (Matsushita, 1996) and new fate mapping experiments shows that the three endodermal domains used in this study (colored blocks in Fig. 1) contribute to the esophagus and stomach (somite 2–4 level, block a, Fig. 1), duodenum and ventral pancreas (somite 7–9 level, block b, Fig. 1), and more posterior small intestine (somite 12–14 level, block c, Fig. 1). The ability of endoderm from block b to contribute to the ventral pancreas was confirmed by Dil labeling endoderm at the 10-somite stage and allowing the embryos to develop in ovo until pancreatic hormones could be detected by immunohistochemistry. Dil-positive cells costain with insulin antibodies (Fig. 1E), demonstrating that the endoderm lateral to somites 7–9 contributes to the mature ventral pancreas. In mouse, it is thought that the ventral pancreas territory is located on the sides of the anterior intestinal portal (AIP) at the 7- to 8- somite stage (Deutsch et al., 2001). Since the AIP's posterior progression is faster in mouse, the ventral pancreas territories we identify in chick have not yet been incorporated into the AIP. The

distinct A-P expression domains of *Pdx1* and *CdxA* persist and can be used to mark positional identity in the endoderm in day 4 embryos (Fig. 1C).

A commitment to express Pdx1 and CdxA is established by the 6-somite stage

Blocks of lateral endoderm from the three different positions along the A-P axis shown in Fig. 1A were grafted to new positions to test their determination state. These grafts were performed by using chick-quail chimeras, allowing the unambiguous identification of grafted cells (Selleck and Bronner-Fraser, 1995). Grafting was performed by using donors and hosts ranging from the 6- to the 14-somite stage; results were identical regardless of donor and host age. When endoderm of region b or c is grafted rostrally, the endogenous expression of *Pdx1* and *CdxA* is maintained in the transplanted endoderm (Fig. 2A and B). This commitment of the *Pdx1* domain can be demonstrated as early as the 6-somite stage (HH stage 8), approximately 5 h before the onset of *Pdx1* expression (9- to 10- somite stage; HH stage 10). Similarly, *CdxA* expression is maintained when endoderm from posterior regions is grafted rostrally before (6-somite stage) or after (14-somite stage) the onset of *CdxA* expression in the donor embryo at the 12- to 14- somite stage. We therefore conclude that the endoderm has received sufficient information to express region-specific genes by the 6-somite stage, which is at least 5 h before the onset of *Pdx1* or *CdxA* expression.

Posterior tissues can reprogram anterior endoderm

In contrast to results obtained when endoderm is grafted to more anterior positions, endoderm grafted to more posterior positions assumes the identity of its new location (Fig. 2C and D). When anterior endoderm is grafted into region b, *Pdx1* is induced in the graft (Fig. 2C). Similarly, when endoderm of region b is grafted into region c. *CdxA* is induced (Fig. 2D). In the latter case, we observe not only the induction of the posterior gene, *CdxA*, in response to signals coming from the posterior environment, but also a sharp downregulation of *Pdx1* expression in the graft (Fig. 2D). This indicates that the graft is changing its positional identity, rather than superimposing new gene expression on its original identity. This induction of posterior markers is seen in endoderm grafted both 5 h before and 5 h after the onset of detectable *Pdx1* expression in the donor embryo. Therefore, although the endoderm of region b has enough information to express *Pdx1* when shifted rostrally, it remains plastic and can change fate when moved to a posterior location.

Early specification of endocrine pancreas

The data above suggest that neighboring tissues influence gene expression and pancreatic specification in the endoderm. To directly address the question of which tissue interactions can reprogram anterior endoderm, we adopted an in vitro culture technique. Blocks of mesoderm from the three A-P domains defined above (a, b and c, Fig. 1A) were dissected from embryos at the 10-somite stage (HH stage 10) and recombined with blocks of endoderm from these same domains. After culturing the explants for 48 h, RT-PCR was performed to assess gene expression.

When endoderm from each of the three domains is cultured in isolation, regionalized gene expression is not detected at the 5- 10-, or 15-somite stage (Fig. 3A), consistent with previous reports in *Xenopus* (Horb and Slack, 2001). Although *Pdx1* and *CdxA* commitment is lost from these explants, they maintain expression of the endodermal and epithelial markers *sonic hedgehog* (Roberts et al., 1995), *HNF3β* (Dufort et al., 1998), and *E-cadherin* (Hatta et al., 1987; Thiery et al., 1984). *Pdx1* expression marks the posterior stomach and duodenum in addition to the pancreas. To focus more specifically on pancreatic fate, we assessed expression of the endocrine hormones *glucagon* and *insulin* (Slack, 1995), a marker of both the early pancreatic epithelium and later the exocrine lineage, *p48* (Kawaguchi et al., 2002; Krapp et al., 1996), and a marker of pancreas progenitors and β cells, *Nkx6.1* (Sander et al., 2000). In contrast to *Pdx1* and *CdxA*, the expression of particular pancreatic markers is consistently detected in endoderm blocks from region b cultured in the absence of mesoderm from the 10-somite stage onward (Fig. 3A, column 2). *Glucagon* expression is specified first, by the 10-somite stage, with *insulin* and *Nkx6.1* expression specified by the 15-somite stage. Specification of *p48*, *Pdx1*, or *CdxA* was not observed at any stage. Mesoderm cultured alone expressed neither endodermal nor pancreatic markers (data not shown).

When endoderm pieces are recombined with blocks of mesoderm from the same A-P position, appropriate expression of *Pdx1* or *CdxA* is recorded in the endoderm (Fig. 3B, columns 5 and 6). When endoderm and mesoderm from region b are recombined and cultured for 2 days, nearly all pancreatic markers, including *glucagon*, *insulin*, and *Nkx6.1*, are induced (Fig. 3B, column 5). If these recombinants are allowed to develop for a further 3 days in culture, islet-like cell clusters containing both insulin- and glucagon-positive cells begin to emerge from the endoderm (Fig. 3C). These results indicate that interaction with mesoderm is required for endoderm to maintain appropriate positional identity, and that in vitro culture can faithfully reproduce both the gene expression and morphological changes associated with pancreatic development.

Lateral plate mesoderm patterns the endoderm

Heterotopic recombinations demonstrate that the mesoderm is capable of instructing endodermal identity (Fig. 4). Mesoderm from region b induces *Pdx1* expression in anterior endoderm (Fig. 4B, column 3), and mesoderm from region c induces *CdxA* expression in endoderm from region

b while concomitantly downregulating anterior genes (Fig. 4B, column 4). This is consistent with the in vivo results shown in Fig. 2C and D. Further, we find that posterior endoderm retains its identity when recombined with anterior mesoderm (Fig. 4A) just as posterior endoderm maintains its original gene expression when grafted rostrally in vivo (Fig. 2A and B). From these data, we conclude that mesoderm from regions b and c is sufficient to induce the endoderm to express *Pdx1* and *CdxA*, respectively.

When anterior endoderm from a 10-somite stage embryo is cultured with mesoderm from region b, *Pdx1, p48,* and *Nkx6.1* are induced, though *glucagon* and *insulin* are not (Fig. 4B, column 3). To address whether this absence of endocrine markers was due to changing competence of the anterior endoderm, we cultured anterior endoderm from a younger embryo (5-somite stage) with region b mesoderm. Under these conditions, *glucagon* expression is detected after 48 h in culture (Fig. 4C). A further 3 days of culture reveals insulin-positive islet-like clusters emerging from the endodermal epithelium (Fig. 4D). This indicates that lateral plate mesoderm from region b is able to induce a range of pancreatic cell types in naive endoderm. These data also suggest that anterior endoderm has an early window of competence to respond to hormone-inductive cues that emanate from the LPM. This competence is restricted to the domain fated to become pancreas by the 10-somite stage. It is noteworthy that the mesoderm sends instructive information that is sufficient to organize cells into hormone-positive islet-like clusters similar to those seen in controls (Fig. 3C).

Retinoic acid, BMP and activin family members can induce posterior transformation of endoderm–mesoderm recombinants

We tested several soluble growth factors as candidates for the LPM-derived signal that induces *Pdx1* in anterior endoderm. Endoderm and mesoderm from region a were recombined in collagen droplets and grown for 48 h alone or with factors added to the culture medium (Fig. 5). These recombinants do not normally express *Pdx1* or other pancreatic markers (Fig. 3B, column 4). BMP7 (10 and 50 ng/ml), BMP4 (50 ng/ml), activin A (5 and 50 ng/ml), and retinoic acid (RA; 10^{-6} to 10^{-9} M), but not TGFβ2, were able to induce *Pdx1,* and less consistently *CdxA,* in anterior recombinants. However, these factors are not sufficient to induce *Pdx1* or *CdxA* in endoderm cultured in isolation ($n = 106$; data not shown). Therefore, these factors may act directly on the endoderm or indirectly via the mesoderm.

To confirm the role of BMP and activin signaling in the normal induction of *Pdx1,* soluble pathway inhibitors were added to cultures in which anterior endoderm was recombined with LPM from region b (Fig. 5, red arrows). In the absence of inhibitors, all recombinants induce *Pdx1* expression in the endoderm (Fig. 4B, column 3). Incubation with either follistatin, an inhibitor of the activin and BMP signaling pathways, or noggin, a BMP pathway inhibitor (Patel, 1998), was sufficient to block induction of *Pdx1* in recombined endoderm. These results suggest that the BMP and activin signaling pathways are active in *Pdx1* induction.

Discussion

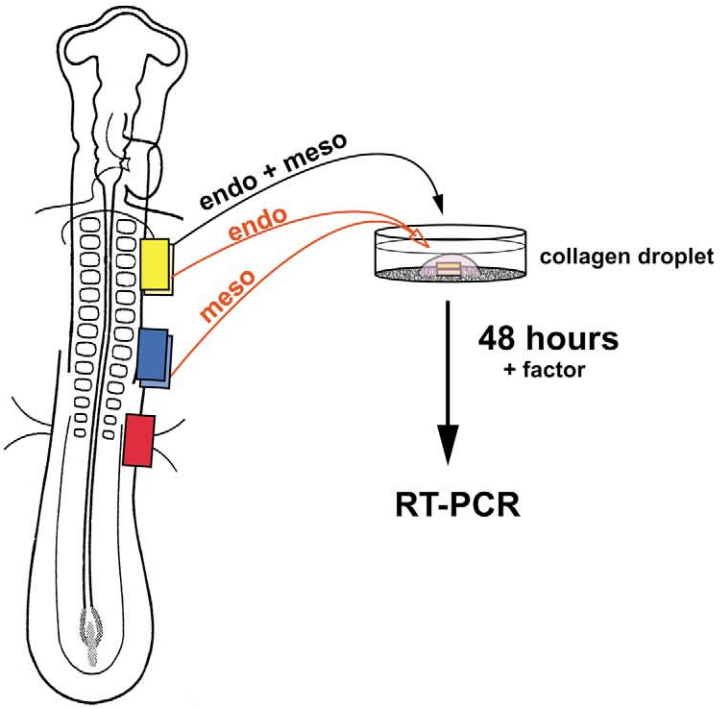
Instructive signals induce the pancreas

This report describes an inductive signal that establishes the ventral pancreatic domain. The experiments show that mesoderm that underlies the presumptive pancreatic domain is sufficient to induce Pdx1 and other pancreatic gene expression in nonpancreatic endoderm. The splanchnic mesoderm between somites 7–9 at the 10-somite stage is the source of these signals. In addition, this mesoderm is able to initiate the formation of ectopic islet-like clusters in anterior endoderm, endoderm that would normally contribute to the stomach and esophagus. The endogenous mesodermal signal is reproduced, at least in part, by BMP/activin signals and retinoic acid.

Sequence of pancreatic specification

Pancreas specification occurs in a stepwise manner. Between the 5- and 10-somite stages, the lateral plate mesoderm sends signals that induce multiple pancreatic markers in endoderm of regions a and b, including *glucagon, insulin,* and *Pdx1*. Competence to express *glucagon* and *insulin* becomes restricted to the pancreas primordium thereafter. The pancreatic endoderm does not require mesoderm after 10-somite stage to express *glucagon.* Expression of *insulin* becomes autonomous within the endoderm shortly thereafter, at the 15-somite stage. Pancreas progenitor and exocrine cell markers *Pdx1* and *p48* require continuous contact with mesoderm to maintain their expression. This finding confirms work in the frog that observed *XIHbox8* expression is dependent on contact with mesoderm at equivalent stages (Horb and Slack, 2001). Persistence of mature pancreatic markers after the elimination of *Pdx1* expression has also been observed in adult β cells (Ahlgren et al., 1998), though β cell identity is lost over time. In addition, some insulin- and glucagon-expressing cells do differentiate in the absence of *Pdx1* (Ahlgren et al, 1996). Our findings leave open the possibility that *Pdx1* or *p48* expression is required only transiently for cell type specification to occur, and that the emergence of cell type-specific expression can occur after the downregulation of early patterning genes. Our experiments suggest that the signals that maintain *Pdx1* expression are permissive since mesoderm anterior to the pancreas (level a) is able to maintain *Pdx1, Nkx6.1,* and *p48* expression in prepancreatic endoderm from level b, but not to induce *Pdx1* in endoderm from level a.

Previous work in the mouse has shown that the exocrine portion of the dorsal pancreas becomes mesoderm-independent at the 29-somite stage (Wessells and Cohen, 1967).



Factor (conc.)	Pdx1 (n)	CdxA (n)
activin A (5ng/ml)	+ (6/6)	+ (2/6)
activin A (50ng/ml)	+ (6/6)	+ (4/6)
BMP4 (10ng/ml)	+ (5/6)	- (6/6)
BMP4 (50ng/ml)	+ (6/6)	- (6/6)
BMP7 (10ng/ml)	+ (7/8)	+ (3/8)
BMP7 (50ng/ml)	+ (4/4)	+ (3/4)
bFGF (1ng/ml)	- (3/3)	- (3/3)
bFGF (10ng/ml)	- (3/3)	- (3/3)
FGF8 (1ng/ml)	- (3/3)	- (3/3)
FGF8 (10ng/ml)	- (3/3)	- (3/3)
FGF10 (1ng/ml)	- (3/3)	- (3/3)
FGF10 (10ng/ml)	- (3/3)	- (3/3)
RA (10-9 M)	+ (6/6)	+ (6/6)
RA (10-8 M)	+ (5/6)	+ (6/6)
RA (10-7 M)	+ (3/3)	+ (2/3)
RA (10-6 M)	+ (6/6)	+ (3/6)
follistatin (10ng/ml)	- (6/6)	- (6/6)
follistatin (100ng/ml)	- (5/5)	- (5/5)
noggin (10ng/ml)	- (4/4)	- (4/4)
noggin (100ng/ml)	- (3/3)	- (3/3)

Fig. 5. Soluble factors of the retinoic acid and BMP/activin families induce posterior transformation of the endoderm. Endoderm and mesoderm from the 2- to 4-somite level were recombined in collagen droplets and assayed by RT-PCR after 48 h in culture. Soluble growth factors (activin A, BMP4&7, bFGF, FGF8, FGF10, retinoic acid, or TGFβ2) were present in the culture medium at the indicated concentrations. Ectopic *Pdx1* induction is indicated by a plus sign. As in Fig. 3B, column 4, no *Pdx1* expression is detected in recombinants grown in control medium (data not shown). Recombinants of endoderm from the 2- to 4-somite level and mesoderm from the 7- to 9-somite level (red arrows) were cultured in the presence of soluble blockers of the activin and BMP signaling pathways (follistatin and noggin) and assayed by RT-PCR after 48 h. *Pdx1* induction is seen in explants grown in control medium (Fig. 4B, column 3; data not shown), but is blocked in the presence of either follistatin or noggin (red text).

Deutsch and colleagues (2001) observe *Pdx1* expression in the pancreas/liver endoderm field in the mouse at E8.5 after removing the mesoderm, but the presence of scattered fibroblasts in their cultures may be sufficient to sustain *Pdx1* expression. We find that any LPM tested can substitute for the original associated mesoderm, provided that it comes from an A-P position anterior to that of the endoderm. Other researchers have noted that nonpancreatic mesenchymes can substitute for pancreatic mesoderm after pancreatic induction has taken place (Percival and Slack, 1999; Wessells and Cohen, 1967). In all cases, these mesenchymes were derived from structures anterior to the pancreas.

At later developmental stages, mesoderm-derived signals are known to be essential for the maintenance of *Pdx1* expression in the endoderm and for the proper formation of the pancreas. Mice mutant in *Isl1* (Ahlgren et al., 1997) and *N-cadherin* (Esni et al., 2001) do not exhibit lateral plate mesoderm convergence around the dorsal pancreas and show a sharp downregulation of *Pdx1* expression as well as an arrest of organ development in the pancreas after initial budding has occurred. *FGF10,* a gene expressed in the mesenchyme around the pancreatic buds from the initiation of budding onward, is required for the maintenance of *Pdx1* expression (Bhushan et al., 2001).

The mesoderm patterns the endoderm in a posterior-dominant fashion

Our in vitro recombination and in vivo grafting experiments demonstrate that endoderm can assume a more posterior identity but not a more anterior one. Other researchers have found different results. Using tissue from day 6 embryos, after organ formation and cytodifferentiation is established, Ishii and colleagues (Ishii et al., 1997, 1998) exchanged endoderm and mesoderm from the small intestine and stomach and cultured for 6–8 days. They observed that posterior epithelium adopts anterior gene expression when cultured with anterior mesenchyme. Similar experiments in the rat show that endoderm from E14.5 colon cultured with mesoderm from the small intestine will assume an anterior identity after 2–4 weeks culture in a nude mouse (Duluc et al., 1994). Though these studies use tissue of a different developmental stage than the experiments reported here, it is perplexing that anterior transformations of the endoderm were observed in these cases. This discrepancy may be due to changes in gene regulation at later stages in development or the longer periods that the tissues were kept in culture. The primary concern of our experiments has been to investigate how the initial domains within

the endoderm are established in vivo, rather than to test endodermal plasticity at later stages.

Pdx1 and *CdxA* are both members of the ParaHox cluster of transcription factors (Brooke et al., 1998), and they may be subject to a cluster-wide regulation. It is conceivable that the posterior-dominant patterning we observe in these experiments is a consequence of this regulation and not a general rule governing the regionalization of the endoderm. However, in addition to members of the ParaHox cluster, we observe that expression of pancreas markers found outside the cluster, such as *glucagon, insulin Nkx6.1*, and *p48*, are nonetheless regulated by interaction with LPM. We also propose that the posterior-dominant patterning we observe does not simply reflect the gradient of maturation along the A-P axis. If endoderm from an anterior location is more mature, and therefore fixed in its identity, it should not be flexible in a recombination assay. Contrary to this prediction, we observe anterior endoderm repatterning after recombination with posterior mesoderm blocks.

Our observation of posterior-dominant patterning of the endoderm layer is strikingly similar to observations in the neural tube at the same stage. Neurectodermal segments transplanted to more posterior regions acquire a new posterior fate, whereas anterior shifts lead to maintenance of the original expression profile (Grapin-Botton et al., 1995). The somite mesoderm sends instructive signals to the neurectoderm (Grapin-Botton et al., 1997; Itasaki et al., 1996; Muhr et al., 1997). This points to a situation in which the mesoderm is the central tissue that instructs the two adjacent germ layers, endoderm and ectoderm. Indeed, we find that somite mesoderm from the same axial position as region c LPM is as efficient at instructing the endoderm to express *CdxA* (data not shown). LPM and somite mesoderm may, therefore, send the same signals to endoderm and ectoderm.

Possible mechanism of induction

In the neural tube, Fgfs (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Mathis et al., 2001; Storey et al., 1998), Wnts (Erter et al., 2001; Lekven et al., 2001; McGrew et al., 1997; Nordstrom et al., 2002) and retinoic acid (Maden, 1999) are known to be posteriorizing agents (Lumsden and Krumlauf, 1996). We show that retinoic acid, activin and BMPs have posterior transforming properties in the endoderm. Further, we show that inhibition of BMP and activin signaling is sufficient to block *Pdx1* induction. A striking observation is that these extracellular signaling agents induce both *Pdx1* and *CdxA* in anterior endoderm, demonstrating that a single signal is capable of inducing genes characteristic of multiple A-P regions. Therefore, these factors may provide a general posteriorizing effect rather than inducing expression of specifically pancreatic genes.

We show that retinoic acid shifts *Pdx1* and *CdxA* expression rostrally. This is consistent with a posterior-to-anterior gradient of retinoic acid proposed to be present in chick LPM at the 10-somite stage (Swindell et al., 1999). Previous reports have shown that RA has posteriorizing effects on the endoderm at earlier developmental stages (Bally-Cuif et al., 1995; Simeone et al., 1995). A recent report using zebrafish has shown a remarkable anterior expansion of pancreas and liver endoderm in response to exogenous RA, as well as a failure to generate pancreas when RA signaling is blocked with a chemical inhibitor (Stafford and Prince, 2002). In each of these cases, it is unclear whether RA is acting directly upon the endoderm, or indirectly via the mesoderm. Although our experiments show that retinoic acid is not sufficient for *Pdx1* or *CdxA* induction on endoderm cultured alone, we cannot rule out that it has a direct action on endoderm in conjunction with other factors that may be produced by mesoderm. The best evidence for direct action of RA upon the endoderm has been provided by an investigation of the *Hoxb-1* promoter, which showed that expression is regulated by RA in endoderm and ectoderm through two different promoter elements (Huang et al., 1998, 2002). No such elements have been identified in the *Pdx1* promoter. It is also possible that a consequence of RA treatment is activation of the activin/BMP signaling pathways in the adjacent mesoderm. Indeed, *BMP2* and *-7* promoters have been shown to be RA-responsive in cell lines (Helvering et al., 2000; Paralkar et al., 2002).

We show that treatment with BMP2, -4, and -7 as well as activin expands *Pdx1* and *CdxA* expression anteriorly. Tiso et al. (2002) have demonstrated that zebrafish *swirl* (*BMP2b*) mutants have a posterior expansion of the pancreatic domain and a general reduction in posterior endoderm identities. It is likely that such general signals result in a specific patterning response through a threshold mechanism (Gurdon and Bourillot, 2001) in which increasing quantity of signal received by the endoderm is translated into increasingly caudal fates. This model would explain why treatment with high concentrations of purified ligand (Fig. 5) is able to induce gene expression characteristic of multiple regions along the A-P axis rather than specifically inducing pancreatic or small intestine genes. One would then expect to see a graded expression of one or several BMPs or activin along the A-P axis at the stages studied here. The lateral plate mesoderm adjacent to the presomitic mesoderm strongly expresses BMP4 with expression becoming progressively weaker in the LPM adjacent to fully segmented somites (Pourquie et al., 1996; Reshef et al., 1998; Schultheiss et al., 1997). This dynamic expression pattern may represent a functional posterior to anterior gradient of exposure to BMP4 protein experienced by the endoderm. In addition, the expression of multiple BMPs has been reported in the mesenchyme surrounding the endoderm (Pourquie et al., 1996; Reshef et al., 1998; Roberts et al., 1995; Smith et al., 2000; Solloway and Robertson, 1999; Winnier et al., 1995), while the BMP receptors *BMPRIA* and *BMPRII* are expressed in endoderm (Mishina et al., 1995; Roelen et al., 1997). Furthermore, since noggin inhibits BMP and not activin signaling, the ability of noggin

to block the *Pdx1*-inducive activity of the mesoderm suggests that BMPs are principally required in this process.

Activin signaling has been previously implicated in *Pdx1* induction (Gamer and Wright, 1995) and can mimic the ability of the notochord to induce several pancreatic markers in the dorsal pancreas primordium (Kim et al., 1997, 2000; Miralles et al., 1998). In addition, activin receptor II inactivation in the mouse reveals a requirement for these receptors in A-P patterning in the stomach and pancreas (Kim et al., 2000). Though the tissues that interact are quite different, the same signaling pathways may be responsible for pancreatic induction in both the dorsal and ventral domains. The sequential interactions with mesoderm derivatives (mesectoderm, notochord, LPM, and endothelium) that characterize pancreatic development may be a method by which maintaining signaling through the same pathways sets the pancreatic fate.

There is considerable interest in driving primitive cells toward a pancreatic fate, a task that would profit from a better understanding of the extrinsic cues that cells require to become pancreas. It was recently shown that information gained from examining the crucial steps in motor neuron development in vivo could be used to induce motor neuron differentiation from embryonic stem cells in vitro (Wichterle et al., 2002). One of the necessary steps in this process was dictating A-P identity of the neural progenitors through treatment with exogenous factors. In this study, we have clarified the sequence of interactions with the mesoderm that are required to both initiate and maintain pancreatic cell fates, and have performed preliminary experiments that suggest a role for BMPs and activin in this induction. Our study shows that continuous presence of mesoderm is not required for endocrine differentiation, and that a short exposure to an inducing factor is sufficient. Experiments are underway to further dissect the pathways that control A-P patterning in the endoderm.

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