Early mouse endoderm is patterned by soluble factors from adjacent germ layers

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SUMMARY

Endoderm that forms the respiratory and digestive tracts is a sheet of approximately 500-1000 cells around the distal cup of an E7.5 mouse embryo. Within 2 days, endoderm folds into a primitive gut tube from which numerous organs will bud. To characterize the signals involved in the developmental specification of this early endoderm, we have employed an in vitro assay using germ layer explants and show that adjacent germ layers provide soluble, temporally specific signals that induce organ-specific gene expression in endoderm. Furthermore, we show that FGF4 expressed in primitive streak-mesoderm can induce the differentiation of endoderm in a concentration-dependent manner. We conclude that the differentiation of gastrulation-stage endoderm is directed by adjacent mesoderm and ectoderm, one of the earliest reported patterning events in formation of the vertebrate gut tube.

Key words: Gut organogenesis, Gastrulation, Mesoderm/ectoderm, FGF4

INTRODUCTION

Endoderm organ development is an area of intense study, but little is known about how endoderm is initially formed and regionally specified in higher vertebrates. For example, it is unclear what signals and responding genes direct totipotent cells of the early mouse embryo to form a sheet of endoderm by the end of gastrulation (E6-7.5). It is also unknown how endoderm cells within the sheet obtain positional identity and form a primitive gut tube (E7.5-9). As the gut tube is subsequently transformed into a thick columnar epithelium, new gene expression marks territories for the esophagus, lungs, thyroid, thymus, stomach, pancreas, liver and intestines (Wells and Melton, 1999). While these and subsequent events in endoderm organogenesis are well described, the initial formation and early specification of the endoderm is poorly understood.

During gastrulation, endoderm, mesoderm and ectoderm all derive from the totipotent cells of the epiblast (E6-7.5). Endodermal cells migrate out of the primitive streak (PS) to form a cup on the outside of the embryo. In mammals, the genes directing endoderm formation are not yet identified, but several frog transcription factors (mixer, and sox 17α and β) can dictate the endodermal cell fate in a cell-autonomous fashion (Henry and Melton, 1998; Hudson et al., 1997). Generation of endoderm seems to be coupled with its initial anterior-posterior specification in that the first endoderm to migrate out of the PS becomes anterior endoderm. The first molecular evidence of endodermal anterior-posterior (A-P) specification is suggested by the expression of cerberus-like, Otx1 and Hesx1 in anterior endoderm and the intestinal fatty acid binding protein gene (IFABP) and Cdx2 in posterior endoderm (Beck et al., 1995; Bouwmeester et al., 1996; Rhinn et al., 1998; Thomas and Beddington, 1996). By the end of gastrulation (E7.5), overlapping domains of gene expression suggest that endoderm is subdivided into A-P regions (Fig. 1) and this is supported by fate-mapping studies (Lawson et al., 1991). For example, anterior endoderm cells of the late gastrula-stage embryo (E7.5) (Fig. 1, region I) end up in the ventral foregut of the early somite-stage embryo (E8.5), subsequently making the liver, lungs and stomach. Endoderm in regions II and III gives rise to dorsal foregut and midgut, eventually becoming the esophagus, stomach, dorsal pancreas and duodenum. The midgut and hindgut endoderm of the somite-stage embryo, which form small and large intestine, respectively, are derived from posterior region III and region IV.

It is not known how the early A-P patterns of gene expression are established, or how the fates of cells in different regions are specified to form a patterned tube. One possibility is that endoderm obtains its regional identity via an autonomous program, without regard to adjacent germ layers. For example, positional information within the endoderm could be specified by the time at which cells migrate through the primitive streak. Alternatively, other germ layers could provide positional identity by ‘stamping’ the endoderm. Both mesodermal and ectodermal germ layers express signaling molecules that could confer positional identity to endoderm (Beddington and Smith, 1993; Burdtsal et al., 1998; Conlon et al., 1994; Niswander and Martin, 1992; Yamaguchi and Rossant, 1995). Indeed there is precedent for this kind of
stamping in the context of neural specification where anterior visceral endoderm lacking the gene for the TGFβ-like growth factor nodal is unable to pattern anterior ectoderm (Varlet et al., 1997). In addition, it was shown in vitro that anterior mesendoderm induces expression of neural markers in adjacent ectoderm (Ang et al., 1994; Ang and Rossant, 1993).

After gastrulation, during somite and neural tube formation, the endoderm rolls up into a primitive gut tube. Studies have begun to uncover mechanisms by which regions of this tube are determined to form specific organs. The ventral foregut/midgut endoderm rolls up into a primitive gut tube. Studies have been genetically ablated in mice, producing defects in pancreatic development, the intercellular signals and pancreatic gene expression.

Some of the genes that regulate gut organogenesis in response to inductive signals have been identified. These include transcription factors, such as Pdx1 and NeuroD that have been genetically ablated in mice, producing defects in the pancreas, caudal stomach and duodenum. Other transcription factors necessary for proper gut development include Pax 4, 6, 8 and 9, Nkx 2.2 and Isl-1 (Ahlgren et al., 1997; Jonsson et al., 1994; Krapp et al., 1998; Mansouri et al., 1998; Peters et al., 1998; Sosa-Pineda et al., 1997; St-Offe et al., 1997; Sussel et al., 1998). How these transcription factors interact to generate specific cell types is as yet unclear, although some target genes of Pdx1, including insulin and somatostatin (Goudet et al., 1999), have been identified.

In the studies mentioned above, particularly those on pancreatic development, the intercellular signals and transcription factors act in a permissive way, revealing a predisposition or pattern in the endoderm that was presumably established by the start of gut tube formation. In this report, we address the origins of this early endoderm pattern using an in vitro assay that allows us to test tissues that specify endodermal fate. The results point to adjacent germ layers as the source of soluble signals that confer A-P pattern to endoderm. These signals are temporally specific, and appear to function in an instructive rather than permissive manner. Moreover, the growth factor FGF4 is expressed by the primitive streak and induces posterior endoderm markers in a concentration-dependent manner, suggesting that FGF4 may be a posterior morphogen for endoderm. Taken together, our findings suggest that instructive signals from the mesoderm and ectoderm act to regionally specify the late gastrulation-stage endoderm.

**MATERIALS AND METHODS**

**Mouse strains**

E7.5-7.75 mouse embryos were obtained from outbred ICR mice (Taconic, NY, USA) or ROSA26 mice (Jackson Laboratory, ME, USA) and staged according to established protocols (Downs and Davies, 1993). Pdx1lacZ/+ embryos were obtained from ICExPdx1lacZ/+ mice (Pdx1lacZ/+ mice were obtained from M. Gannon and C. Wright, Vanderbilt University, TN, USA), where 50% of the embryos carry a targeted Pdx1 allele.

**Embryo dissections and explant culture**

**Endoderm isolation**

Embryos were isolated and staged according to closure of the amnion and presence of an allantoic bud. Endoderm was manually isolated from early allantoic bud-stage embryos (E7.5-7.75), when endoderm is easily removed with only minimal mesoderm contamination. Embryos were immobilized by gently holding the extraembryonic region with a mouth pipette while a series of small tears were made around the endoderm at the embryonic/extraembryonic junction with polished tungsten needles. The endoderm was peeled away from the underlying mesoderm and ectoderm, and cut away from the node. This method was developed because endoderm isolated by proteolytic methods did not survive well in culture. Anterior and posterior endoderm was isolated from E7.5 embryos by first making a cut along the embryo anterior to the node or posterior to the node, then removing (as above) the respective region of endoderm. We specifically isolated endoderm on either side of the node to avoid contamination of endoderm with adherent node cells. Similarly, mesoderm/ectoderm lacking the node was isolated to avoid contamination with node endoderm.

**Mesoderm/primitive streak, and ectoderm isolation**

Embryonic regions were separated from extraembryonic regions with a tungsten needle and transferred to Dulbeco’s PBS containing 0.25% pancreatic, 0.5% trypsin and incubated at 4°C for 10 minutes (Hogan et al., 1994). Embryos were transferred to DMEM + 10% serum and gently blown through a mouth pipette to detach the endoderm, which was subsequently removed. The mesoderm was pulled away from the ectoderm, then cut from the primitive streak. The primitive streak and node regions were then separated from the anterior ectoderm.

**Chick notochord isolation**

Eggs (Preston, CT, USA) were incubated at 38°C and staged according to Hamburger and Hamilton (1951). For notochord isolation, tungsten needles were used to remove endoderm from the notochord of stage-11” (12 somites) embryos extending from the anterior intestinal portal (AIP) to the caudal limit of the somites. The notochord was cut at the level of the AIP and forceps were used to peel away the notochord along the length of the ventral neural tube.

**Recombination**

Endoderm was recombined with the various tissues and grown in collagen-matrix gel as previously described (Dickinson et al., 1995). Explants embedded in collagen were covered with 0.6 ml of DMEM + 15% FBS (Life Technologies, NY, USA) and cultured for 48 hours. Survival and health of explants were assessed by several criteria.

**Collagen explant morphology**

Endoderm cultured alone tends to form either hollow cysts, or balls of endoderm. Unhealthy or dying endoderm fragments and pulls away from the collagen. Healthy endoderm explants grow in size during culture.

**Cell morphology**

Healthy endoderm cells are refractile to light, where as dead cells appear dark under the microscope. Also, Hematoxylin and Eosin (H&E) staining of healthy endoderm explants (Fig. 6) show mitotic figures.
Gene expression

Only endoderm that appeared morphologically healthy had detectable expression of abundant genes such as β-tubulin and HNF3β.

In the transfilter assays, the mesoderm/ectoderm was placed on a collagen drop, the 0.4 mm Biopore membrane (Millipore, MA, USA) was put over the tissue, another collagen drop was placed over the membrane, and the endoderm was positioned in the collagen adjacent to the mesoderm/ectoderm. After 2 days of culture, the endoderm and mesoderm/ectoderm were separated for subsequent analysis by RT-PCR.

Endoderm assays with growth factors

Recombinant human aFGF, FGF2, FGF4, FGF5, TGFα, TGFβ1, TGFβ2, EGF, IGFl, IGFlI, HGF and murine FGF8b were from R&D Systems Inc. (Minneapolis, MN, USA), and murine Activin A was obtained as described (Sokol and Melton, 1991). The starting concentration of growth factors were as recommended by the manufacturer, and were increased up to 100-fold above this concentration.

RNA preparation and reverse transcription polymerase chain reactions (RT-PCR)

RT-PCR was performed on total RNA as described previously (Wilson and Melton, 1994). Primer pairs were used for PCR with the following conditions: 1 cycle of 94°C for 3 minutes; then 39 cycles of 94°C for 1 minute, 60°C for 1.5 minutes, 72°C for 1 minute; and finally 1 cycle of 72°C for 5 minutes. Primer sequences used are listed forward then reverse, 5’ to 3’. The PCR reaction was analyzed by electrophoresis in a 1.5% agarose gel and photographed after ethidium bromide staining. β-tubulin primers (Gittes and Rutter, 1992), 5’-GGGCCA-GATCTTCAGACCAGC-3’ and 5’-GTAAGTTGACAGCATGAG-3’, amplify a product of 627 bp. Somatostatin (SS) primers (Gittes and Rutter, 1992), 5’-CCGTCATGGTCTCGAGAAGT-3’ and 5’-CAGGTTCAAGTTGACAGCATG-3’, amplify a product of 360 bp. NeuroD primers (Naya et al., 1997), 5’-CTCTAATCGTAGAA-AGATGCCG-3’ and 5’-CCTCGAGGACTTTCTTGCCTG-3’, amplify a product of 499 bp. Insulin primers (Gittes and Rutter, 1992), 5’-CAGCCCTTATGACAGCACAGA-3’ and 5’-ATGCTGGTGGACGC-ACTGATC-3’, amplify a product of 346 bp. Hesx1 primers (Thomas and Beddington, 1996), 5’-CAAGCCAGAAGAAGATTCTC-3’ and 5’-AAAAGAGCGTGTTTCCAGGATG-3’, amplify a product of 309 bp. Fgf4 primers (Hebert et al., 1990), 5’-TACTGCAACGTGC- GGCATCGGA-3’ and 5’-GTGGAACCTTCCTCAGGTG-3’, amplify a product of 344 bp. HNF3α primers (Ang and Rossant, 1994), 5’-TCTCACCCGGCTCAGCCG-3’ and 5’-CTAGGAAGT- ATTACGGACG-3’, amplify a product of 305 bp. Ncam primers (Moos et al., 1988), 5’-ACAAGGGAGACACTCAGG-3’ and 5’-GCTACTGAGAGTGTCGAG-3’, which amplify a product of 282 bp. IFABP primers (Green et al., 1992), 5’-GGAAAGAGAC-GTGATGTCGTCG-3’ and 5’-CTTGTGACAGGCTGAGAGC-3’, amplify a product of 225 bp. Pdx1 primers used for (Ohlsson et al., 1993), 5’-CCAAAGCCCTCCTGAGAATG-3’ and 5’-TCTGGGTCCAGACCCG-3’, amplify a product of 305 bp.

LaCZ staining and histology analysis

Beta-galactosidase activity was detected in fixed whole tissue using the Histomark X-gal substrate system (Kireguard and Perry Laboratories, MD, USA). For H&E staining, 6 μm paraffin sections were dewaxed in xylene, rehydrated in PBS, and stained with H&E.

RESULTS

Endodermal gene expression during gut tube formation in vivo

Fig. 1 shows gene expression data from RT-PCR of dissected endoderm and in situ hybridization during gut tube formation (E7.5-9.5). Fate-mapping experiments suggest that endoderm cells have A-P identity by the end of gastrulation (E7.5) (Lawson et al., 1986) and gene expression analyses of microdissected anterior and posterior E7.5 endoderm verifies that genes are regionally expressed along the A-P axis (Fig. 1A). For example, Hex1, cardiac beta actin and cerberus-like (data not shown) are expressed in anterior endoderm (regions I, II; region III was avoided because of the tight association of endoderm with the node in this region) whereas the intestinal fatty acid binding protein gene (IFABP) is in posterior endoderm (posterior region III, and IV). These data are consistent with in situ analysis of cerberus-like and Hesx-1 (Biben et al., 1998; Thomas and Beddington, 1996), which showed these genes expressed in an anterior to posterior gradient in E7.5 endoderm.

As the endoderm sheet folds to form a gut tube and cells condense into an epithelium (Fig. 1A,B, E8.5), the de novo expression of several genes including Pdx-1, NeuroD and somatostatin (SS) is observed (Gittes and Rutter, 1992; Jonsson et al., 1994; Naya et al., 1997). RT-PCR analysis of dissected gut tube regions shows that initial expression of Pdx1 occurs as early as the 3-somite stage, where as NeuroD and SS expression initiate slightly later (by 5 somites). By E9.5, these markers are expressed in endoderm, which gives rise to stomach, pancreas and duodenum (Fig. 1A,B; regions I and III). In situ expression of Pdx1 in E9.5 embryos containing a lac Z reporter integrated into a Pdx1 allele verifies that Pdx1 expression (in blue) is restricted to pancreatic regions I and III (Fig. 1A, E9.5). NeuroD and SS are also expressed in regions I and III; however, SS expression extends to the posterior gut tube that will give rise to the small intestine (anterior region IV) (Gittes and Rutter, 1992). IFABP expression persists throughout these stages and marks the posterior gut. Whole-mount in situ hybridization of E9.5 embryos shows that IFABP is strongly expressed in the hindgut (region IV; data not shown). Insulin expression is first detected by RT-PCR at E9.5 in region III; however, protein is not detectable in the pancreatic bud until E10-11 (data not shown).

These data verify that Pdx1, NeuroD, SS and insulin become expressed in endoderm during gut tube formation. Since these genes mark different regions of the endoderm, we can use their expression to test for signals that regulate the regional and temporal specification of endoderm.

E7.5 endoderm requires signals from adjacent germ layers to differentiate

Separation and recombination of germ layers has been used to study the role of anterior mesendoderm in early anterior neural patterning (Ang and Rossant, 1993). We have adapted this technique to identify signals that induce gene expression in endoderm. Endoderm mechanically isolated from E7.5-9.5 embryos (see Materials and Methods) is free of lateral mesoderm and ectoderm, as measured by fgf4 and Ncam expression, respectively, and has marginally detectable axial mesoderm (Tbra, data not shown) (Fig. 2A). Endoderm cultured for 2 days in DMEM + 15% FBS survives in culture (as assayed morphologically and by gene expression, see Materials and Methods), and continues to express the early endoderm marker IFABP (Fig. 3B). However, Pdx1, SS and NeuroD are not expressed, suggesting that E7.5 endoderm...
Fig. 1. Endodermal gene expression during gut tube formation in vivo. (A) Gene expression during morphogenesis of the gut tube. Endoderm regions I-IV, defined by fate-mapping studies (Lawson et al., 1986), were microdissected from each stage and gene expression was analyzed by RT-PCR (4-5 embryos pooled). In the late gastrulation embryo (E7.5), the line separates the embryo proper (bottom) from the extraembryonic tissues (top). The embryonic endoderm is a sheet of cells surrounding the embryo proper. At E8.5, early somite stage, gut tube formation begins at the anterior foregut (regions I and II) and posterior hindgut (region IV) (endoderm outlined by dotted yellow line). Whole-mount in situ hybridization with albumin antisense RNA shows expression in the ventral foregut endoderm that is adjacent to the heart tube (signal in upper foregut is from trapped probe when compared to embryos probed with sense RNA). By E9.5, turning of the embryo closes up the midgut (E8.5 region III) and forms the gut tube (outlined in yellow). Pdx-1 (LacZ) expression in the E9.5 embryo defines the dorsal and ventral pancreas and the duodenum (domains I and III). (+/-) indicates weak gene expression, and (-) indicates no gene expression. (B) Endoderm gene activation between E7.5 and E9.5. Endoderm/gut tubes were isolated from the indicated stage embryos and gene expression analyzed by RNA extraction and RT-PCR. No signal was detected in control samples (not treated with reverse transcriptase; -RT). β-tubulin was amplified separately for 30 cycles (within the linear range) as a control for overall level of reverse transcription.

Fig. 2. E7.5 endoderm requires signals from adjacent germ layers to differentiate. (A) Endoderm was mechanically separated from the underlying mesoderm and ectoderm at the position indicated by the arrows (left) and gene expression was assayed by RT-PCR. Isolated endoderm (right) expresses IFABP, but not the mesodermal marker fgf4 or the ectodermal marker Ncam (bottom). (B) Isolated endoderm was cultured alone or in contact with mesoderm/ectoderm (mec) in collagen matrix for 2 days (top). Endoderm expressing beta-galactosidase (rosa 26 endoderm) spreads out along the mesoderm/ectoderm after 2 days of coculture (middle). Unlike endoderm cultured alone, endoderm cultured in contact with mesoderm/ectoderm expresses Pdx1, NeuroD and somatostatin (SS) (bottom). No signal was detected in control samples of RNA from samples not treated with reverse transcriptase (−RT). These experiments were done with three separate recombinants, and repeated three times.
Endoderm receives instructive signals from mesoderm/ectoderm

To ascertain whether the above signals are instructive or permissive, we swapped anterior and posterior endoderm relative to mesoderm/ectoderm. For example, if these signals are permissive, anterior endoderm will not adopt a posterior fate when cultured next to posterior mesoderm/ectoderm. If, however, these signals are instructive, anterior endoderm will become posteriorized, and express posterior genes when cultured next to posterior mesoderm/ectoderm. We used IF ABP as an early marker of posterior endoderm (Fig. 3 lane 2) and b-cardiac actin (bCA) as an anterior marker (lane 1). Curiously, bCA expression is predominantly in anterior endoderm, but not mesoderm (lanes 3 and 4) at this time.

When anterior and posterior endoderm are switched relative to mesoderm/ectoderm, anterior endoderm becomes posteriorized and expresses IF ABP (compare lanes 7 and 1), whereas posterior endoderm becomes more anterior and expresses bCA (compare lanes 6 and 2). We also observed that once these markers come on in endoderm, their expression persists even when the A-P position of endoderm is switched, suggesting that the endoderm retains some of its initial identity.

These data indicate that inductive signals from mesoderm/ectoderm can influence the A-P nature of endoderm. Somatostatin (SS) expression also indicates that endoderm has been partially respecified. Unlike in vivo expression of SS, which is in both anterior and posterior endoderm, in vitro we rarely observe anterior expression of SS, but rather routinely see expression in posterior endoderm cultured with posterior mesoderm/ectoderm (compare lanes 5 and 8). However, when anterior endoderm is cultured with posterior mesoderm/ectoderm, the number of explants expressing SS increases (Fig. 3B, compare lane 5 to lane 7). In vivo, NeuroD is expressed in both anterior and posterior endoderm, but in vitro is only induced in posterior endoderm cultured with posterior mesoderm/ectoderm. The observation that SS but not NeuroD is induced in anterior endoderm suggest that either certain cell types are subject to respecification, or that other signals are necessary for complete posteriorization of endoderm. Pdx1 is induced in both anterior and posterior endoderm (see below), and is therefore not useful for analysis of A-P specification.

Taken together, the results shown in Fig. 3 suggest that the A-P nature of endoderm is not irreversibly determined by E7.5, and can be altered by changing the spatial source of signal.

E7.5 endoderm is not responsive to notochord

The results presented above show that Pdx1 (a marker for pancreas and duodenum) is induced in gastrulation-stage endoderm (E7.5) after 1-2 days of coculture with mesoderm/ectoderm. Studies in chick have shown that notochord induces expression of Pdx1 in somite-stage endoderm (stage -11, approx. 10 somites; corresponds to E8.5+ in mouse) (Kim et al., 1997). It is possible that induction
of Pdx1 in earlier stage endoderm occurs via notochord precursor cells (notochord plate) present in the E7.5 mesoderm/ectoderm, which obtain competence to induce Pdx1 expression during the coculture period. To determine whether E7.5 endoderm is responding to notochord-like signals, we cocultured it with stage-11 chick notochord and assayed for gene expression. Results in Fig. 4B show that chick notochord does not alter expression of IFABP in cocultured E7.5 endoderm, nor does it induce expression of Pdx1 or SS when compared to mesoderm/ectoderm (en+mec). No signal is seen in samples not treated with reverse transcriptase (–RT). Experiments shown were done twice in duplicate.

Due to ease of isolation, we used chick rather than mouse notochord, and it is possible that a negative result is due to species-specific signaling. We believe this is unlikely since recombination of chick and mouse tissues is widely used to study inductive mechanisms (Keynes et al., 1997). Furthermore, mouse and human growth factors can substitute for chick notochord and induce gene expression in somite-stage chick endoderm (Hebrok et al., 1998). Although these data are preliminary, they suggest that E7.5 endoderm receives signals from mesoderm/ectoderm that make it competent to respond to subsequent notochord and mesenchyme signals. This is consistent with the observation that the A-P nature of gastrulation-stage endoderm is more plastic (Fig. 3) than somite-stage endoderm (Kim et al., 1997).

**Endoderm genes are differentially induced by isolated E7.5 mesoderm and ectoderm**

Endoderm of the E7.5 embryo is in contact with underlying primitive streak, axial and lateral mesoderm, and anterior ectoderm, all putative sources of endoderm patterning signals. In order to further identify the source of signals that instruct endoderm, we separated mesoderm/ectoderm into distinct germ layers and regions with gentle proteolysis and manual dissection (Fig. 5A). Endoderm was cocultured with isolated primitive streak (PS) plus mesoderm, mesoderm alone, or ectoderm. This analysis shows that the primitive streak is able to induce somatostatin and Pdx1 in endoderm (Fig. 5B). Pdx1 can also be induced, albeit less effectively, by lateral mesoderm and ectoderm, consistent with the fact that Pdx1 is normally

Fig. 4. Endoderm is not responsive to notochord. (A) Schematic representation of chick notochord recombined with E7.5 mouse endoderm. Notochord and endoderm were isolated as described in Materials and Methods, recombined in collagen so that they were in physical contact, and cultured for 2 days. (B) RT-PCR analysis of endoderm plus notochord recombinants after 2 days in culture. Notochord does not affect expression of the endoderm marker IFABP, nor does it induce expression of Pdx1 or SS when compared to mesoderm/ectoderm (en+mec). No signal is seen in samples not treated with reverse transcriptase (–RT). Experiments shown were done twice in duplicate.

Fig. 5. Endoderm genes are differentially induced by isolated E7.5 mesoderm and ectoderm. (A) The primitive streak/mesoderm, mesoderm and ectoderm were separated as described in Materials and Methods. Isolated tissues were recombined in collagen and cultured for 2 days before analysis of gene expression by RT-PCR. (B) RT-PCR analysis of endoderm recombined with isolated ectoderm (ect), mesoderm and PS (PS+mes), mesoderm alone (mes), or PS (PS) alone. Experiments shown were done twice in triplicate.
expressed in domains that are adjacent to lateral mesoderm and anterior ectoderm. This is consistent with data in Fig. 3, and data not shown, suggesting that ectoderm, mesoderm and primitive streak produce qualitatively different signals that confer regional identity to endoderm.

**Soluble factors instruct E7.5 endoderm**

To determine if endoderm is instructed by soluble factors, or requires cell contact, a 0.4 μm Biopore membrane was placed between endoderm and mesoderm/ectoderm during the culture period. After 2 days, endoderm and mesoderm/ectoderm were isolated separately and analyzed for gene expression. Endoderm cultured alone frequently forms a cystic, hollow structure with endoderm remaining a thin single layer of cells. In contrast, endoderm cultured adjacent to mesoderm/ectoderm differentiates, and begins to thicken into an epithelium (Fig. 6A). This morphological differentiation is reminiscent of in vivo differentiation, where the E7.5 flat, squamous endoderm (Figs 2A, 6A) differentiates into a thick columnar epithelium like that found in the E9 gut tube. Morphological differentiation of endoderm is accompanied by induction of the markers Pdx1, SS, insulin and NeuroD in endoderm (Fig. 6B). NeuroD is also occasionally induced in ectoderm by endoderm. This reciprocal induction is interesting, and not unexpected since NeuroD is expressed in motor neurons of the E9.5 embryo. Although we generally see endodermal expression of NeuroD before ectodermal expression, upon longer culture of explants we occasionally see ectodermal expression of NeuroD. While these results indicate endoderm is instructed by soluble factors, we do observe that survival of endoderm is greatly enhanced by cell contact (data not shown), demonstrating that the two processes may be coupled. These results prompted us to test known, soluble growth factors affects on endoderm differentiation.

**FGF4 patterns endoderm**

The E7.5 embryo expresses numerous growth factors. Growth factors expressed by ectoderm include FGF5, FGF8, TGFβ1 and TGFβ2, whereas those expressed by mesoderm and/or primitive streak include BMP4, FGF4, HGF, EGF, TGFβ1 and TGFβ2. To determine whether these soluble factors instruct endoderm, isolated endoderm was cultured for 2 days in medium containing different growth factors, and subsequently assayed for expression of Pdx1, SS and NeuroD. All growth factors were tested over a range of concentrations (see Materials and Methods). Of all factors tested, only FGF4 effected a concentration-dependent induction of SS and NeuroD in isolated endoderm (Fig. 7). Notably, SS is induced at higher concentrations of FGF4 than NeuroD, which is consistent with the A-P expression pattern of these genes relative to the source of FGF4. Specifically, SS is expressed in more posterior endoderm that becomes the duodenum and small intestine, and this endoderm is immediately adjacent to the primitive streak/mesoderm, the in vivo source of FGF4 (Niswander and Martin, 1992). NeuroD, however, is expressed in more anterior endoderm fated to become pancreas and duodenum and is therefore further away from the source of FGF4, and would likely respond to lower concentrations of this factor. It is also interesting that NeuroD is repressed by high concentrations of FGF4, suggesting that FGF4 could define the posterior boundary of NeuroD expression. Although NeuroD and Pdx1 have similar expression patterns in vivo, Pdx1 is not reproducibly induced by FGF4, indicating that Pdx1 expression requires additional signals. The fact that neither FGF2 nor activin reproducibly induced gene expression in E7.5 endoderm, but do induce Pdx1 expression in somite-stage chick endoderm (Kim et al., 1997), further highlights the differences in developmental competence between stages of endoderm.
hepatic endoderm expresses liver instruction from mesoderm/ectoderm. Similarly, somite-stage endoderm is only capable of acting on endoderm that has received prior signals from the adjacent notochord (Kim et al., 1997). However, these signals need not respond to subsequent inductive signals. For example, FGF2,5,8b, TGF\(\beta\)1,2, TGF\(\alpha\), Activin, HGF, EGF, IGF1 or IGFII induces expression of somatostatin (SS) and NeuroD in isolated endoderm. A representative example of the RT-PCR is seen on the left, and the number of recombinants is on the right. NeuroD and SS show a dose-responsive expression.

**DISCUSSION**

Endoderm appears early in gastrulation (E6-6.5), and by 7.5 days of development it forms a sheet of cells on the outside of the embryo. Regional expression of genes suggests that endoderm cells have an A-P identity by the end of gastrulation. The data presented here show that over the next day of development, endoderm receives instructions from adjacent germ layers, which induce new gene expression. Our data further suggest that these signals make endoderm competent to respond to subsequent inductive signals. For example, somite-stage endoderm responds to permissive signals from adjacent notochord (Kim et al., 1997). However, these signals are only capable of acting on endoderm that has received prior instruction from mesoderm/ectoderm. Similarly, somite-stage hepatic endoderm expresses liver albumin in response to signals from adjacent cardiac mesoderm (Gualdi et al., 1996). However, the pre-hepatic endoderm in these experiments was already specified or set aside prior to the induction by cardiac mesoderm. Also, the hindgut endoderm of the early somite-stage chick embryo has posterior identity, but through interaction with the adjacent mesoderm further differentiates into a posterior type (cloaca) of epithelium (Roberts et al., 1998). In each of these examples, the endoderm has received instruction prior to signaling from notochord, cardiac mesoderm or hindgut mesoderm, respectively. This process of sequential induction allows for broad regions of endoderm to become progressively determined towards the establishment of organ domains.

How is the endoderm determined before gut tube formation? Evidence pointing to a series of reciprocal inductions between germ layers that result in A-P specification is now emerging. For example, before gastrulation, Hesx1 is expressed in anterior visceral endoderm (Thomas and Beddington, 1996). These anterior cells are involved in specifying anterior ectoderm, which is mediated in part by the TGF\(\beta\) growth factor nodal (Varlet et al., 1997). Once this anterior pattern is established, it is reinforced in other anterior and ventral structures. For example, as gastrulation proceeds, cardiac mesoderm migrates from the posterior to reside next to the anterior endoderm, which is involved in differentiation cardiac myoblasts (Narita et al., 1997). Cardiac myoblasts then condense and begin to differentiate, and in the process signal back to the adjacent endoderm, resulting in activation of liver genes (Gualdi et al., 1996). Although it is not known how other regions of endoderm become specified, endoderm interacts with many different structures including the notochord plate, presumptive head ectoderm, the node, lateral mesoderm and the primitive streak. Our data support the idea that endoderm is involved in reciprocal induction along all the A-P axis.

The factors that mediate signals between germ layers are of general interest and our data show that FGF4, which is expressed in the posterior of the embryo (primitive streak) (Niswander and Martin, 1992), induces NeuroD and somatostatin (SS) expression in endoderm. Furthermore, FGF4 induces expression of these genes in a concentration-dependent manner, implicating it as a posterior morphogen. SS is expressed in more posterior endoderm next to the primitive streak, the main source of FGF4, and it is not therefore surprising that SS responds to higher concentrations of FGF4. NeuroD is expressed in more anterior endoderm, further away from the source of FGF4, and responds to lower FGF4 concentrations. In fact, it is possible that FGF4 may restrict posterior expression of NeuroD, since NeuroD expression is repressed by high concentrations FGF4. The A-P specification of endoderm and neural ectoderm is strikingly similar in that FGFs also act to posteriorize neural ectoderm (Cox and Hemmati-Brivanlou, 1995). The ability of FGF4 to posteriorize endoderm was tested by incubation of anterior endoderm with FGF4, and although FGF4 affects posterior gene expression, our results were inconclusive because these small pieces of isolated endoderm did not survive well in culture (data not shown).

It is remarkable that a few FGFs are able to mediate a wide variety of cellular responses throughout development. For example, of the FGFs tested (aFGF, FGF2,4,5,8), only FGF4 is able induce expression of SS and NeuroD. The potency of FGF4 over other FGFs could be explained by expression of different FGF receptors, or receptor isoforms. FGR1, which is expressed by endoderm at this time (data not shown), has several different splice variants that are differentially responsive to FGFs. In a cellular reporter assay, FGR1 isoform b is threefold more responsive to FGF4 than FGF2, and tenfold more responsive to FGF4 than either FGF5 or FGF8 (Ornitz et al., 1996). It is also noteworthy that FGF2 secreted by the notochord can induce somite-stage dorsal endoderm to express Pdx1 (Hebrok et al., 1998), whereas FGFs are not able to induce Pdx1 in late gastrulation-stage endoderm. This could mean that induction of Pdx1 in gastrulation-stage endoderm requires additional signals, whereas older somite-stage endoderm has already been exposed to these signals, and is now responsive to FGFs. We are testing combinations of FGFs to determine which are required for the induction of Pdx1.
growth factors for their ability to induce Pdx1 in E7.5 endoderm in vitro. FGFs are not limited to pancreatic induction, since FGFs secreted by cardiac mesoderm induce ventral foregut endoderm to express liver genes such as albumin (Jung et al., 1999). This finding is interesting since Pdx1 is also expressed by ventral foregut endoderm, implicating FGFs in the regulation of ventral Pdx1 expression. Since Pdx1 is expressed in several unique temporal and spatial patterns, its regulation is likely to be complex.

A role for FGF4 in endoderm patterning in vivo is undetermined since embryos that lack the fgf4 gene arrest prior to gastrulation and fail to make detectable mesoderm or endoderm (Feldman et al., 1995). In addition, embryos deficient for fgfr1 arrest during gastrulation (Deng et al., 1994; Yamaguchi et al., 1994). Unlike the fgf4−/− mice, fgfr1−/− embryos do make some lateral and axial mesoderm; however, these experiments did not address a role for FGFR1 in endoderm formation and patterning. Another study used a chimeric analysis with fgfr1−/− ES cells and demonstrated that these cells accumulate in the primitive streak, forming ectopic neural structures (Ciruna et al., 1997). These cells are greatly impaired in their ability to contribute to mesoderm. Furthermore, fgfr1−/− cells are almost completely absent in endodermal derivatives. These results could point to a role for FGFR1 in cell migration out of the primitive streak. Alternatively, FGF signaling may play a vital role in endoderm development, which would also result in a lack of fgfr1−/− cells contributing to endoderm. A genetic approach that targets the ability of endoderm to respond to FGF4 could elucidate a role for FGF signaling in endoderm patterning.

In vitro culture of germ layers has proven an effective way to study many cellular processes that are occurring in the late gastrulation-stage embryo. For example, in addition to results reported here, we observe that several of the growth factors tested promoted the survival and growth of early endoderm in culture (as assessed morphologically and by gene expression; data not shown), but did not induce endodermal differentiation. Also, it was seen that direct cell contact between germ layers promoted the best differentiation of endoderm. Using the explant recombination assay, we also verified previous observations where anterior endoderm is involved in both neural and cardiac induction (Ang and Rossant, 1993; Miralles et al., 1998; Schultheiss et al., 1995). In vitro recombination of germ layers may allow us to further clarify the reciprocal inductions that pattern the early endoderm.

In conclusion, our studies have identified inductive events involved in cell-fate specification of mouse endoderm. This addresses a part of the mechanism of endodermal organogenesis, but there is clearly much more to be learned. Of special interest to us is the specification of stem or precursor cells for various organs, and more investigations on the extracellular signals and cellular responses that underlie early endoderm differentiation may facilitate their identification and manipulation.

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REFERENCES


