

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 17 α* 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; Burdsal 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 TGF β -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 (Fig. 1, region I) of the early somite-stage embryo (E8.5) expresses the liver marker *albumin* in response to signals from adjacent cardiac mesoderm (Gualdi et al., 1996). Signals from cardiac mesoderm are, in part, mediated by FGFs, which can induce ventral foregut endoderm to express *albumin* (Jung et al., 1999). Another study focusing on pancreatic development in chicks demonstrated that the dorsal foregut/midgut endoderm (regions II and III, approx. 10-somite stage) receives permissive signals from the adjacent notochord resulting in expression of pancreatic genes such as *Pdx1* (Hebrok et al., 1998; Kim et al., 1997). The notochord factors, which can be mimicked by FGF2 and activin β_B , act to repress endodermal expression of *sonic hedgehog* (*Shh*) and thereby allow for 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 formation of 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-Onge 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 fates. 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). *Pdx1^{lacZ}* embryos were obtained from *ICR* \times *Pdx1^{lacZ}* mice (*Pdx1^{lacZ}* 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 Dulbecco's PBS containing 0.25% pancreatin, 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 *HNF3 β* .

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, TGF α , TGF β 1, TGF β 2, EGF, IGFI, IGFII, 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'-TGGCCA-GATCTTCAGACCAG-3' and 5'-GTAAGTTCAGGCACAGTGAG-3', amplify a product of 627 bp. *Somatostatin* (*SS*) primers (Gittes and Rutter, 1992), 5'-CCGTCAGTTCTGCAGAAGT-3' and 5'-CAGGGTCAAGTTGAGCATCG-3', amplify a product of 360 bp. *NeuroD* primers (Naya et al., 1997), 5'-CCTCTAATCGTGAA-AGATGGC-3' and 5'-CCTCGGACTTCTTGCCAG-3', amplify a product of 499 bp. *Insulin* primers (Gittes and Rutter, 1992), 5'-CAGCCCTTAGTGACCAGCTA-3' and 5'-ATGCTGGTGCAGC-ACTGATC-3', amplify a product of 346 bp. *Hesx1* primers (Thomas and Beddington, 1996), 5'-CGACCCAGAAGAGAATGTCTC-3' and 5'-AAAGAGCGTGTTCGGAGGCTG-3', amplify a product of 309 bp. *Fgf4* primers (Hebert et al., 1990), 5'-TACTGCAACGTG-GGCATCGGA-3' and 5'-GTGGGTTACCTTCATGGTAGG-3', amplify a product of 344 bp. *HNF3 α* primers (Ang and Rossant, 1994), 5'-TCTCACCCGGCTCACGGCC-3' and 5'-CTAGGAAGT-ATTAGCACGG-3', amplify a product of 305 bp. *NCAM* primers (Moos et al., 1988), 5'-ACAAGGAGGACACTCAGG-3' and 5'-GCTACTGCAGGATTGAGAG-3', which amplify a product of 282 bp. *IFABP* primers (Green et al., 1992), 5'-GGAAAGGAGC-TGATTGCTGTCC-3' and 5'-CTTTGACAAGGCTGGAGACCAG-3', amplify a product of 225 bp. *Pdx1* primers used for (Ohlsson et al., 1993), 5'-CCAAAACCGTCGCATGAAGTG-3' and 5'-TCTGGGTCCCAGACCCG-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 μ m 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, *Hesx1*, *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 E7.5-7.75 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

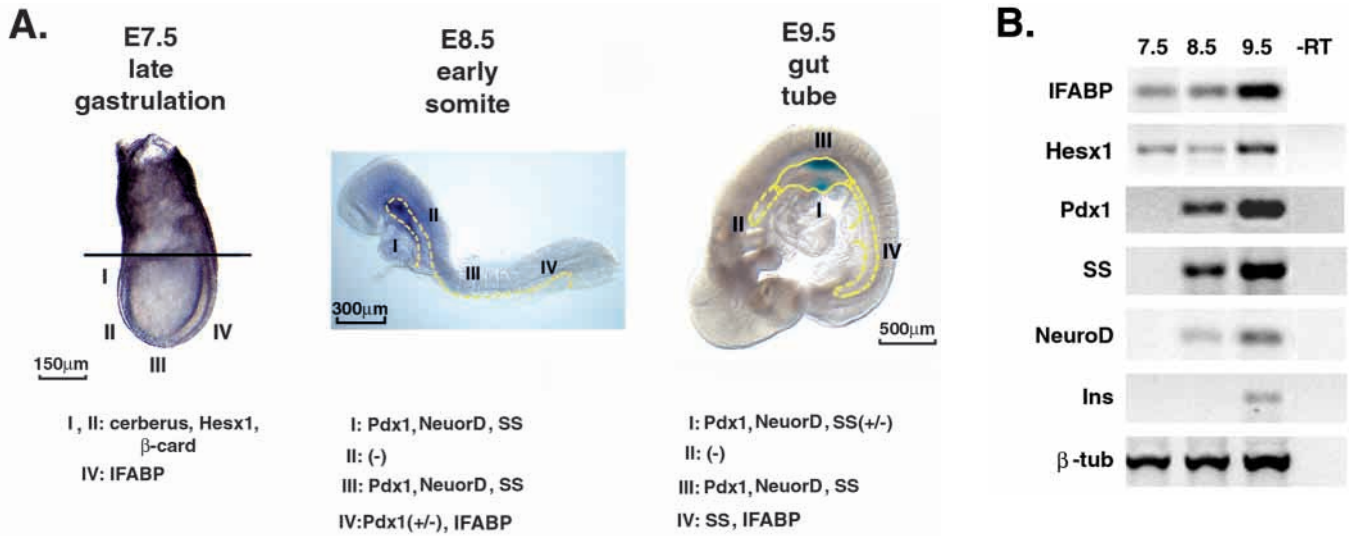
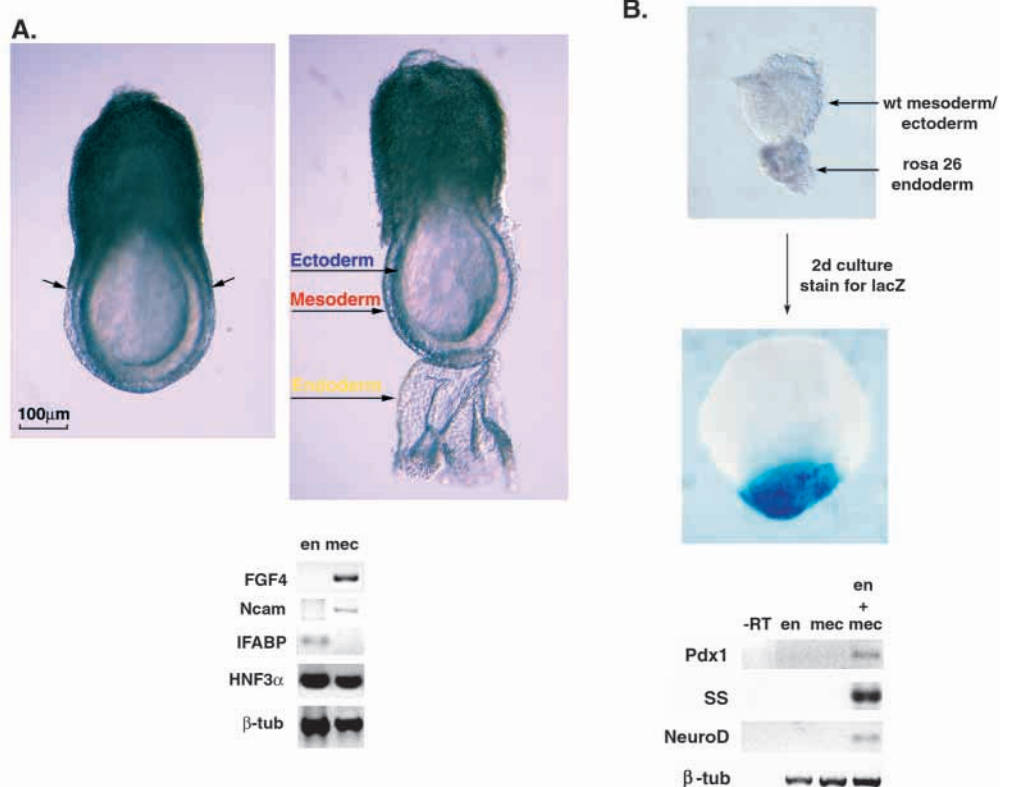


Fig. 1. Endodermal gene expression during gut tube formation in vivo. (A) Gene expression during morphogenesis of the gut tube. Endoderm regions I-IV, defined by fate-mapping studies (Lawson et al., 1986), were microdissected from each stage and gene expression was analyzed by RT-PCR (4-5 embryos pooled). 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.



requires additional instructions to express these genes. *HNF α* is expressed in several germ layers, and *β -tubulin* is ubiquitous.

We assayed mesoderm and ectoderm for their ability to induce gene expression in endoderm. Endoderm cocultured with mesoderm/ectoderm spreads out over these germ layers after 2 days of culture (Fig. 2B), as demonstrated using lac Z-expressing endoderm isolated from *rosa26* mice (Zambrowicz et al., 1997). Furthermore, the endoderm now expresses *Pdx1*, *SS* and *Neuro D*, suggesting that endoderm has received inductive signals for these genes (Fig. 2B). Whole-mount in situ analysis of recombinants using endoderm from *Pdx1^{lacZ/+}* animals (one *Pdx1* allele carries a lacZ reporter gene) verified *Pdx1* expression in endoderm. As in the E8.5 embryo, *Pdx1* expresses in several patches of endoderm cells rather than weakly throughout the endoderm (data not shown). Other genes induced in endoderm include *Pax6* and *insulin* (data not shown); however, these genes are also expressed in mesoderm/ectoderm cultured alone and are therefore not useful as markers in this recombination assay. These results show that E7.5 endoderm receives instructions from adjacent germ layers.

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 *IFABP* as an early marker of posterior endoderm (Fig. 3 lane 2) and *β -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 *IFABP* (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

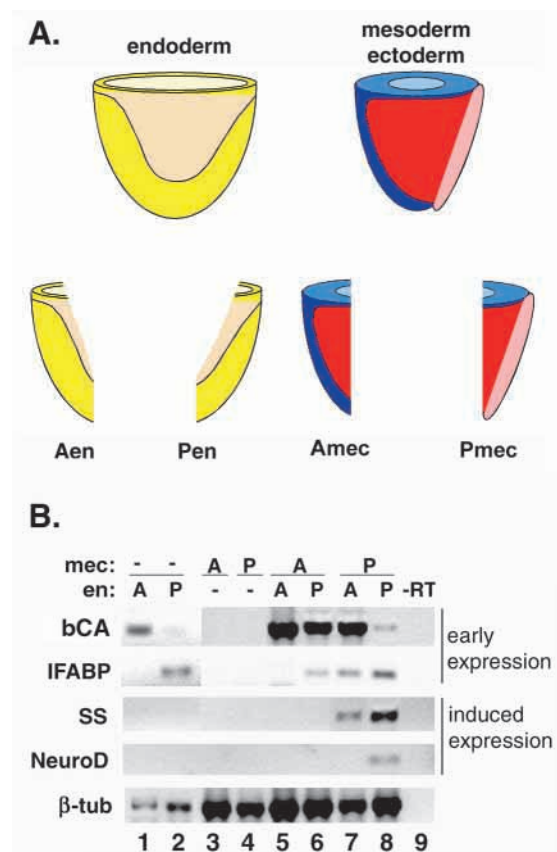


Fig. 3. Endoderm receives instructive signals from mesoderm/ectoderm. (A) Schematic illustration of anterior-posterior repositioning of E7.5 endoderm. Endoderm anterior (Aen) or posterior (Pen) to the node was isolated and cultured alone, or cultured with either anterior (Ame) or posterior mesoderm/ectoderm (Pme), for 2 days. (B) RT-PCR analysis of anterior/posterior endoderm recombined with anterior/posterior mesoderm/ectoderm. The marker for early anterior endoderm is *beta cardiac actin* (*bCA*, lane 1) and posterior endoderm is the *intestinal fatty acid binding protein gene* (*IFABP*, lane 2). These genes are not expressed in either anterior mesoderm/ectoderm (Ame, lane 3) or posterior mesoderm/ectoderm (Pme, lane 4) at this time (early expression). In contrast to *IFABP* and *bCA*, *SS* and *Neuro D* gene expression is induced after 1-2 days of coculture (induced expression). Experiments shown are representative of those that were repeated three times in triplicate.

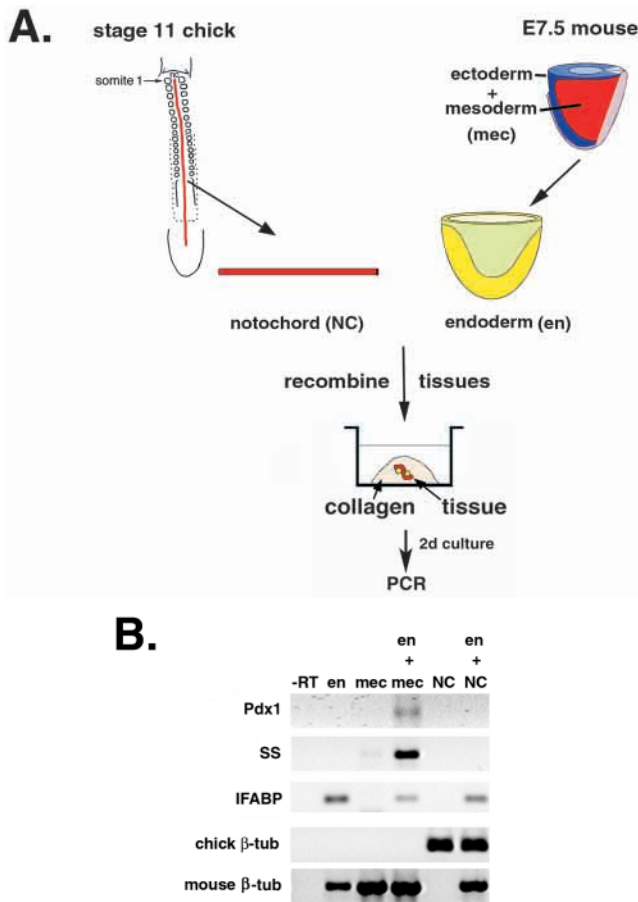


Fig. 4. Endoderm is not responsive to notochord. (A) Schematic representation of chick notochord recombined with E7.5 mouse endoderm. Notochord and endoderm were isolated as described in Materials and Methods, recombined in collagen so that they were in physical contact, and cultured for 2 days. (B) 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.

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*, *SS* or *NeuroD*. Also, pancreatic mesenchyme, which is able to regulate gene expression of the early pancreatic endoderm, does not induce gene expression in E7.5 endoderm (data not shown).

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

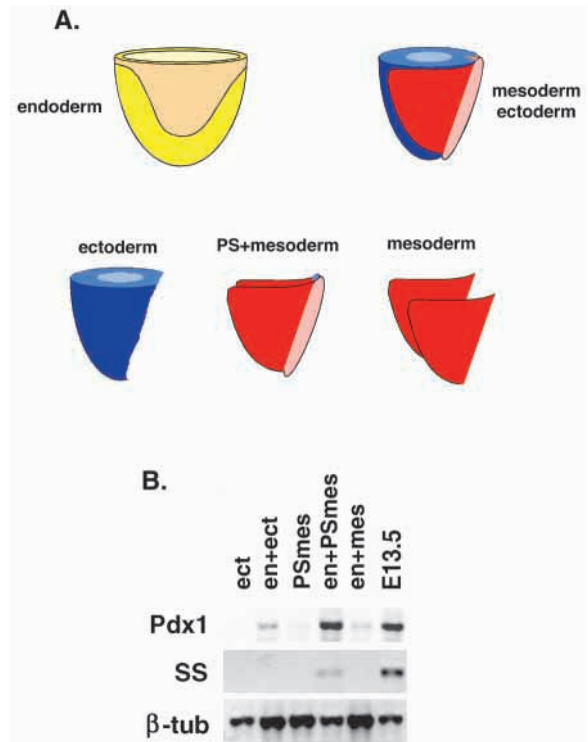


Fig. 5. Endoderm genes are differentially induced by isolated E7.5 mesoderm and ectoderm. (A) The primitive streak/mesoderm, mesoderm and ectoderm were separated as described in Materials and Methods. Isolated tissues were recombined in collagen and cultured for 2 days before analysis of gene expression 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.

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 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. 6. Soluble factors pattern E7.5 endoderm. (A) Endoderm alone in culture for 2 days, or placed on a membrane next to mesoderm/ectoderm. Endoderm alone will form often form a hollow cystic structure that resembles un-induced, squamous-like E7.5 endoderm (upper left panel). Endoderm placed adjacent to mesoderm/ectoderm condenses into a ball (lower left panel). H&E staining of sections of endoderm alone (upper right panel) show a hollow cyst-like structure, whereas endoderm cultured trans-membrane from mesoderm/ectoderm begins to form a thickened epithelium reminiscent of E9 gut epithelium (lower right panel). (B) RT-PCR analysis of endoderm alone (en) or endoderm placed on a membrane and cultured adjacent to mesoderm/ectoderm (mec). After 2 days in culture, endoderm was isolated separately, and assayed for marker expression. Expression of *Pdx1*, *SS*, *NeuroD* and insulin in endoderm was used as a measure of induction. In two separate experiments, five explants in each condition were assayed separately, and one representative example is shown. (-RT), control samples not treated with reverse transcriptase.

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, TGF β 1 and TGF β 2, whereas those expressed by mesoderm and/or primitive streak include BMP4, FGF4, HGF, EGF, TGF β 1 and TGF β 2. 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.

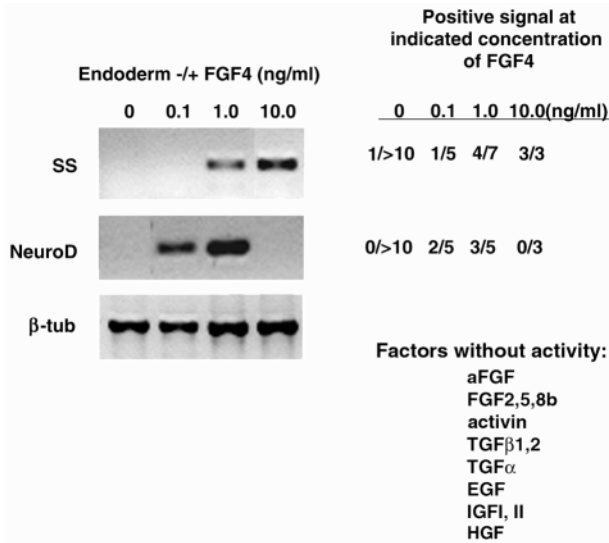


Fig. 7. FGF4 induces markers in isolated endoderm. E7.5 endoderm was isolated and cultured in medium containing increasing concentrations of peptide growth factors (see Materials and Methods). FGF4 (but not aFGF, FGF2,5,8b, TGF β 1,2, TGF α , Activin, HGF, EGF, IGF1 or IGFII) induces expression of *somatostatin* (*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-responsiveness to FGF4.

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 suggests 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 β 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 all along 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. FGFR1, 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, FGFR1 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

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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