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Endoderm patterning by the notochord: development of the hypochord in *Xenopus*

Ondine Cleaver, Daniel W. Seufert and Paul A. Krieg*

Division of Molecular Cell and Developmental Biology, School of Biological Sciences, University of Texas at Austin, Austin, TX 78712, USA

*Author for correspondence at present address: Department of Cell Biology and Anatomy, University of Arizona College of Medicine, 1501 N. Campbell Avenue, PO Box 245044, Tucson, Arizona, 85724, USA (e-mail: pkrieg@u.arizona.edu)

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SUMMARY

The patterning and differentiation of the vertebrate endoderm requires signaling from adjacent tissues. In this report, we demonstrate that signals from the notochord are critical for the development of the hypochord, which is a transient, endodermally derived structure that lies immediately ventral to the notochord in the amphibian and fish embryo. It appears likely that the hypochord is required for the formation of the dorsal aorta in these organisms. We show that removal of the notochord during early neurulation leads to the complete failure of hypochord development and to the elimination of expression of the hypochord marker, VEGF. Removal of the notochord during late neurulation, however, does not interfere with hypochord formation. These results suggest that signals arising in the notochord instruct cells in the underlying endoderm to take on a hypochord fate during early neural stages, and that the hypochord does not depend on further notochord signals for maintenance. In reciprocal experiments, when the endoderm receives excess notochord signaling, a significantly enlarged hypochord develops. Overall, these results demonstrate that, in addition to patterning neural and mesodermal tissues, the notochord plays an important role in patterning of the endoderm.

Key words: Hypochord, Endoderm, Notochord, Induction, *Xenopus laevis*

INTRODUCTION

Although induction and patterning of the mesoderm and ectoderm have been intensely investigated in the last few decades (Ruiz i Altaba et al., 1993; Slack, 1993; Hemmati-Brivanlou and Melton, 1994; Kessler and Melton, 1994; Dale, 1997; Heasman, 1997), the molecular mechanisms underlying patterning and development of the endoderm have received relatively little attention. However, it is clear that patterning of endodermal cells along both the dorsoventral and anterior-posterior axes is necessary for the stereotyped development of visceral organs along the gastrointestinal tract.

Patterning of the vertebrate gut tube along the anterior-posterior axis depends upon extensive reciprocal signaling between the endoderm and adjacent mesodermal tissues (Ishizuya-Oka and Mizuno, 1984; Kedinger et al, 1986; Yasugi, 1993; Roberts et al., 1995; 1998). For example, experiments in rats have demonstrated that foregut mesoderm can induce the respecification of the midgut endoderm, causing it to take on foregut endoderm morphology (Kedinger et al., 1986). Patterning of the endoderm along the dorsoventral axis, on the other hand, is at least partly dependent on signals arising from the notochord. In the avian embryo, notochord signals have been shown to be necessary for development of the pancreas (Kim et al., 1997). In this particular case, activin-βB and FGF2 have been identified as candidate notochord signals that permit pancreas development by repressing *sonic hedgehog* (*Shh*) expression in the pancreatic primordium (Hebrok et al., 1998). This repression subsequently allows the expression of pancreatic genes, including *Pdx-1* and *insulin*.

The hypochord, or subnotochordal rod, is an endodermally derived tissue that exists transiently in the embryos of fish, lampreys and amphibians. It is a rod-like structure, consisting of a row of single cells lined up immediately ventral to the notochord (Hatta, 1893: Gibson, 1910; Löfberg and Collazo, 1997). Approximately 100 years ago, the development of the hypochord was investigated in a variety of organisms, leading to a description of the major landmarks of its origins and formation (Stöhr, 1895; Bergfeldt, 1896; Klaatsch, 1898; Reinhart, 1904; Gibson, 1910), and more recent work in urodeles has extended upon these earlier studies (Bose, 1964; Löfberg and Collazo, 1997). The intimate association between the hypochord and the dorsal aorta in fish and amphibian embryos has led to speculation that the hypochord is involved in patterning dorsal aorta development (Rückert, 1888; Hasse, 1892; Löfberg and Collazo, 1997). This hypothesis received direct support from recent experiments in *Xenopus*, which demonstrate that the hypochord expresses vascular endothelial growth factor, VEGF, a growth factor that apparently mediates migration of angioblasts to the

dorsal midline, where they organize to form the dorsal aorta (Cleaver and Krieg, 1998).

In recent years, the hypochord has been found to express a wide variety of different genes. These include transcription factor genes, such as the bHLH gene *twist* (Halpern et al., 1995; Schauerte et al., 1998), the forkhead genes, *XFKH2*, *fkd1*, *fkd2*, *fkd4* and *fkd7* (Bolce et al., 1993; Odenthal and Nüsslein-Volhard, 1998), and a number of extracellular matrix genes, such as a type II collagen gene, *col2a1* (Yan et al., 1995), the glycoprotein, SPARC (Damjanovski et al., 1994), the adhesion molecule, *F-spondin* (Ruiz i Altaba et al., 1993) and a spondin homologue, *mindin1* (Higashijima et al., 1997). Growth factor genes are also expressed in the hypochord, and these include the TGF-β family member *Radar* (Rissi et al., 1995), sonic hedgehog, *Shh* (Ruiz i Altaba, 1998) and vascular endothelial growth factor, *VEGF* (Cleaver et al., 1997; Cleaver and Krieg, 1998). None of these markers, however, are specific to the hypochord and expression is observed in other tissues such as the floorplate, the notochord and/or the somites.

In this report, we describe the morphogenesis and the subsequent disappearance of the *Xenopus* hypochord at the histological level, and we show that *VEGF* expression serves as a molecular marker of hypochord cells during early development. We find that hypochord precursor cells can first be detected in the endoderm at early neurula stages, some time after development of a morphologically distinct notochord. The juxtaposition of notochord and hypochord tissues in the embryo and the timing of hypochord development, raises the possibility that the notochord induces formation of the hypochord. Using embryological manipulations, we show that the notochord is required for the initiation of hypochord development and for hypochord marker expression. This requirement is restricted to early neurulation and the hypochord requires no subsequent maintenance signals from the notochord. Finally, grafting experiments demonstrate that increased notochord signaling can increase the number of cells expressing *VEGF* and increase the size of the differentiated hypochord. These experiments show that the notochord plays a critical role in patterning a group of endodermal cells to develop into the hypochord.

MATERIALS AND METHODS

Histology

For histological analysis of hypochord development, embryos were embedded either in Paraplast or in glycol methacrylate (JB-4, PolySciences). Embryos embedded in Paraplast were first stained for Type II collagen, as in Seufert et al. (1994), and then sectioned. Plastic sections were stained with either Brilliant Cresyl Blue or Toluidine Blue as in Hausen and Riebesell (1991). Paraplast embryos were sectioned at 6 μm and plastic embryos were sectioned at 4-5 μm. Sections, mounted with Permount, were then covered and photographed using Nomarski optics.

For sectioning of embryos subsequent to the whole-mount in situ hybridization procedure, embryos were dehydrated in ethanol, permeabilized in xylene (2×10 minutes), incubated in xylene/Paraplast (10 minutes), then in Paraplast (2×10 minutes, 1× overnight) at 60°C. The next day the embryos were embedded in Paraplast. 10 μm sections were dewaxed in xylene, mounted with Permount, covered and photographed using Nomarski optics.

Whole-mount in situ hybridization

Fertilized *Xenopus laevis* embryos were generated as described by Drysdale and Elinson (1991) except that adult females were not primed with pregnant mare serum gonadotrophin. Embryos were staged according to the tables of Nieuwkoop and Faber (1994). Digoxygenin-labeled RNA probes for whole-mount in situ hybridization were prepared according to manufacturers' recommendations (Boehringer-Mannheim). A 3.3 kb *VEGF* cDNA insert in Bluescript (Cleaver et al., 1997) was linearized using *Bam*HI and transcribed using T7 RNA polymerase. Whole-mount in situ hybridization (Harland, 1991) was performed with modifications as described by Cleaver et al. (1996). The temperature of both pre-hybridization and hybridization was 65°C for 14-18 hours each. Chromogenic reactions for all in situ hybridizations used NBT-BCIP (Boehringer Mannheim).

Nuclear morphology in *Xenopus* embryos

Embryos of stages 38-45 were fixed in MEMPFA overnight at 4°C and stored in methanol. Embryos were then embedded in Paraplast (as described above) and sectioned (10 μm sections). Sections were dewaxed in xylene (2×10 minute washes) and rehydrated through an ethanol series. Sections were then washed in PBS and stained with SYTOX Green (Molecular Probes) to visualize nuclear DNA by fluorescence microscopy. Post-staining washes were reduced to 3×2 minutes, to allow a slight background staining to outline cells.

Embryological manipulations

Embryos were staged according to Nieuwkoop and Faber (1994). Vitelline membranes were removed manually in NAM (Normal Amphibian Medium). Operations to remove portions of the notochord were accomplished using a tungsten knife and plastic Petri dishes coated with 1% agarose. Dissections were performed both with and without 0.01% trypsin or 0.01% collagenase. Notochords were removed from the posterior of the embryo, leaving a portion of the notochord intact in the anterior. An incision was first made either through the neural plate (stage 13-14 embryos) or to one side of the neural tube (stage 17-18 embryos) and the notochord was cut in two places and gently removed from the endoderm. For some experiments the notochord was not excised, but was displaced laterally using a tiny strip of modeling clay. In order to ensure that hypochord precursors remained intact, only embryos where the dorsalmost endoderm was undamaged by the dissection were selected for further analysis. Damage is obvious since the endoderm consists of a layer of a single cell thickness and any rupture causes an evident collapse of the archenteron roof. Those embryos whose dorsal endoderm remained intact were selected to continue with further manipulations. Embryos were then allowed to heal under glass bridges, in NAM/2 overnight at 13°C.

For notochord transplantation experiments, the extirpated notochords were placed in a recipient embryo. An incision through the neural tube was first made in the recipient embryo and the transplanted notochord was placed adjacent to the endogenous notochord, between the somite and over the endoderm. Embryos were allowed to heal under glass bridges, in NAM/2 overnight at 13°C. The embryos were then incubated in 20% Steinberg's solution until stage 28 or 34, when they were fixed in MEMPFA prior to assaying for VEGF expression by whole-mount in situ hybridization.

RESULTS

Histological analysis of hypochord development in *Xenopus laevis*

The development of the *Xenopus* hypochord was assessed by histological analysis of sectioned embryos. The specific region examined in these experiments lies in the trunk of the embryo,

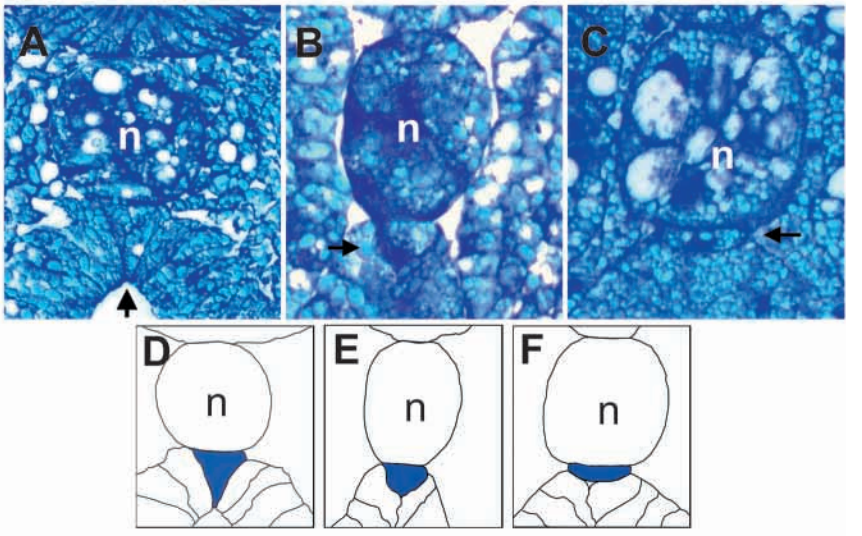

Fig. 1. Histological analysis of prehypochord cells in the early endoderm. All sections are transverse through the trunk of the embryo posterior to the developing pronephric tubules; (A) is stained with Brilliant Cresyl Blue and (B and C) with Toluidine Blue. (A) Stage 20. The dorsalmost endoderm cell has a distinguishable triangular shape. Surrounding endoderm cells are columnar. (B) Stage 22. The dorsalmost cell in the endoderm layer has taken on a more rounded appearance as it delaminates and is beginning to reorient within the endoderm. (C) Stage 24. The mostdorsal cell is now flattened against the dorsalmost periphery of the endodermal epithelium and lies at an angle to the adjacent columnar endoderm cells. (D-F) Traced outlines of sections A-C showing position of the prehypochord cell (blue) within the dorsal endoderm. n, notochord; arrow, prehypochord cell.

close to the posterior end of the developing pronephric tubules. Early during neurulation (stage 16), prehypochord cells can first be detected within the dorsalmost endoderm, immediately ventral to the notochord. These cells are distinguishable from adjacent endodermal cells by their close association with the notochord and their triangular appearance (data not shown; but see Hausen and Riebesell, 1991, plate 30). Later, during the early tailbud stage, the majority of endodermal cells organize into an epithelium and acquire a columnar appearance (stage 20). In contrast, the prehypochord cells located at the dorsalmost point of the endodermal epithelium, take on a more triangular shape (Fig. 1A; see also Hausen and Riebesell, 1991, plate 33). Slightly later, prehypochord cells undergo an epithelial-to-mesenchymal transition, during which they become more rounded and appear to lose close contact with other cells in the endoderm (Fig. 1B). Prehypochord cells then become flattened against the dorsalmost surface of the endoderm and increase the area of contact with the notochord (stage 24). The prehypochord cells now lie at a distinct dorsal position and have a different orientation relative to the other dorsal endodermal cells (Fig. 1C; see also Hausen and Riebesell, 1991, plate 41). At this point, the row of prehypochord cells is one, or occasionally two, cell diameters thick.

At stage 26, the flattened prehypochord cells begin to delaminate from the dorsal endoderm (Fig. 2A,B), and come to occupy a position between the dorsal surface of the endoderm and the ventral surface of the notochord. By stage 30, the hypochord has matured into a rod of single cells, which lies immediately under the notochord (Fig. 2C). Between stages 32 and 35, the dorsal aorta forms into a mature blood vessel immediately ventral to, and partly surrounding, the hypochord (Fig. 2D; Cleaver and Krieg, 1998). After the dorsal aorta matures, the hypochord cells flatten again, bringing them into close proximity to the ventral side of the notochord (Fig.

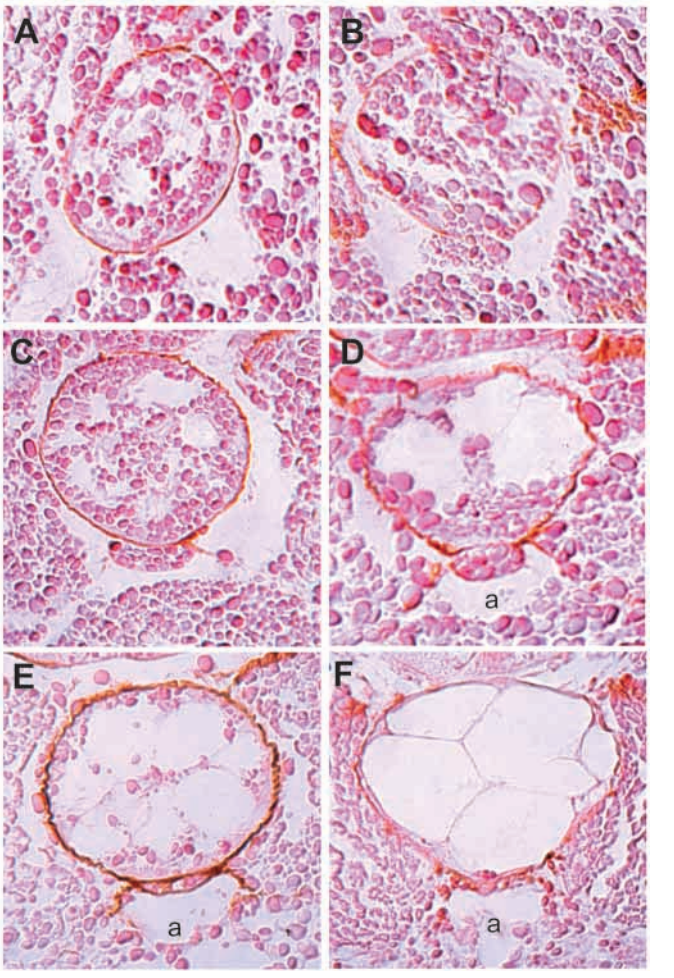

Fig. 2. Histological analysis of hypochord development. All sections are transverse sections through the trunk of the embryo posterior to the pronephric tubules and are stained using antibodies against Type II collagen and counterstained with Eosin. (A) Stage 26. Hypochord cells begin to delaminate from the dorsalmost endoderm. (B) Stage 28. Hypochord cells separate from the dorsal endoderm. (C) Stage 30. The hypochord lies adjacent to the ventral surface of the notochord and forms a mature organ. (D) Stage 34. The hypochord lies above the dorsal aorta (a) as circulation begins. (E) Stage 39. The hypochord begins to flatten significantly against the notochord. (F) Stage 41. The hypochord degenerates and is no longer detectable as a separate structure.

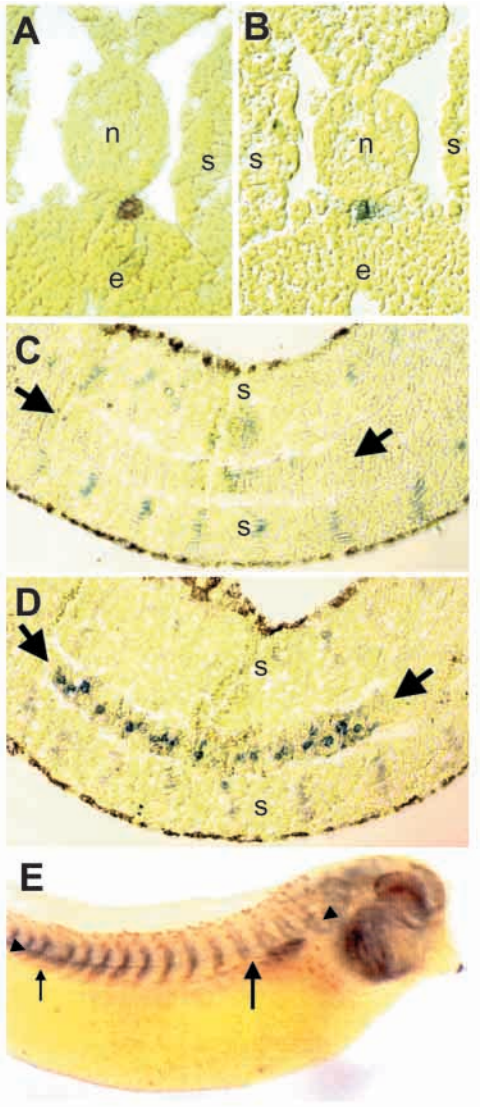

Fig. 3. *VEGF* expression is a marker for prehypochord cells. All embryos are assayed by in situ hybridization for *VEGF* transcripts. (A,B) Cross sections through stage-24 embryos at the level of the trunk, posterior to the pronephric tubules. Expression of *VEGF* can be seen in the nucleus of a hypochord precursor cell in the dorsalmost endoderm. n, notochord; s, somites; e, endoderm. (C) Frontal section through the ventral notochord (arrows) of a stage-24 embryo. No *VEGF* expression is visible in the notochord cells. Anterior to the right. (D) Same embryo as C, showing *VEGF* expression in the cells of the endoderm immediately ventral to the notochord (marked by arrows). Anterior is to the right. (E) Whole-mount in situ hybridization for *VEGF* in the mature hypochord (arrowheads). Notice declining expression in the anterior portion of the hypochord (large arrow), while expression in the posterior portion of the hypochord remains abundant (small arrow). Prominent staining is also visible in the somite nuclei and the pronephros.

2E). By stage 39, cells of the notochord become progressively vacuolated and mesenchyme is observed to accumulate around both the hypochord and the notochord. At this stage, although the hypochord remains histologically recognizable, it flattens significantly and associates closely with the ventral surface of the notochord. By stage 41, the hypochord is no longer distinguishable as a distinct structure (Fig. 2F).

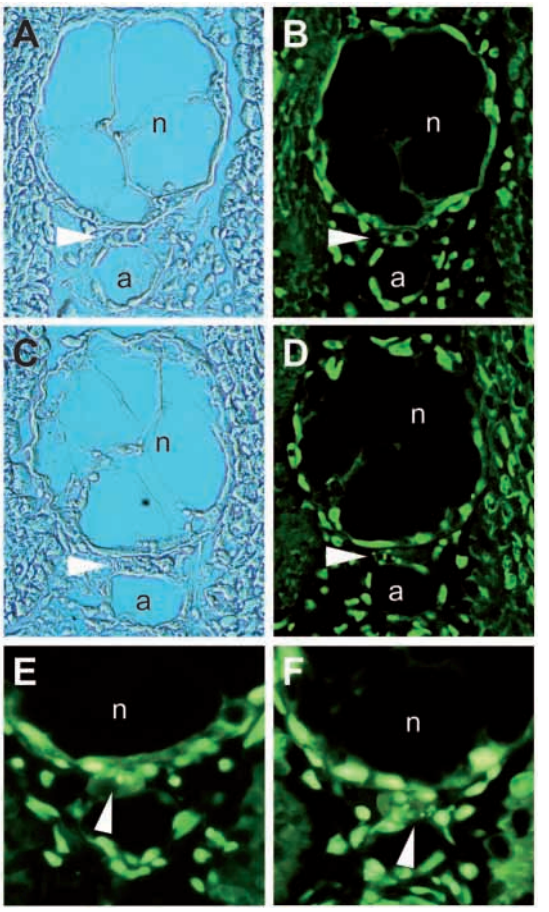

Fig. 4. Nuclei in the degenerating hypochord show characteristics of apoptosis. Nuclei are detected by SYTOX green staining. Highly condensed chromatin and nuclear fragmentation are evident along the hypochord of stage-41 and -42 embryos. All sections are transverse through the trunk. (A) Bright-field and (B) fluorescence views of section through the hypochord of a stage-38 embryo. An intact nucleus is evident in the hypochord in this section (white arrowhead) and nuclear morphology is similar to other embryonic cells. (C) Bright-field and (D) fluorescence views of a section through the hypochord of a stage-41 embryo. Notice the nuclear fragmentation evident in the hypochord cell nucleus (white arrowhead). (E,F) Higher magnification views of sections through the hypochord of stage-42 embryos. Note fragmented nuclei in the hypochord (white arrowheads). n, notochord; a, dorsal aorta.

VEGF expression in prehypochord and hypochord cells

The description of hypochord development, based on cellular appearance in histological sections, may be confirmed using *VEGF* expression as a molecular marker for prehypochord and early hypochord cells. Soon after prehypochord cells become distinct from adjacent endodermal cells by their histological appearance, transcripts for *VEGF* become detectable in the same cells, by in situ hybridization. For example, in stage-24 embryos, *VEGF* expression is clearly visible within the endodermal layer, in single cells that are in contact with the ventral surface of the notochord (Fig. 3A,B). Frontal sections reveal no VEGF staining in the plane of the notochord (Fig. 3C) but a line of VEGF expressing cells, one or two cells thick, is visible in the most dorsal endodermal tissue (Fig. 3D). We note

that VEGF transcripts are most clearly detected in the nuclei of the hypochord cells. In some cases, the nuclei do not lie exactly in the center of the hypochord cells and therefore the line of VEGF expressing nuclei can be seen to zigzag beneath the notochord (Fig. 3A,D). *VEGF* continues to be expressed in prehypochord cells as they delaminate from the endodermal layer. At stage 30, the hypochord becomes a mature structure and expression of *VEGF* reaches its highest levels (data not shown; but see Fig. 7C). It is at about this time that angioblasts migrate towards the dorsal midline and assemble to form the dorsal aorta immediately ventral to the hypochord (Cleaver and Krieg, 1998). As development proceeds (stage 34-36), *VEGF* expression in the hypochord declines rapidly, first at the anterior end and then progressively in more posterior regions (Fig. 3E). By the later tadpole stages, *VEGF* is no longer detectable in hypochord cells by in situ hybridization (data not shown).

The hypochord degenerates by apoptosis

To analyze the ultimate fate of the hypochord in the embryo, the morphology of hypochord cell nuclei was examined during the period when the structure degenerates. Using SYTOX green staining, single nuclei were observed in the hypochords of late tadpole stage embryos. At stage 38, nuclei in the hypochord are large and intact (Fig. 4A,B), having a similar morphology to the nuclei of adjacent mesenchymal and endodermal cells (nuclei in endothelial cells of the dorsal aorta are more flattened). By stage 41, however, pyknotic nuclei can be identified in the degenerating hypochord at numerous locations along the anterior-posterior axis (Fig. 4C-F). These nuclei show significant fragmentation, exhibiting approximately 3-8 visible fragments of variable size, consistent with the appearance of nuclei in apoptotic cells described in previous *Xenopus* studies (Anderson et al., 1997; Stack and Newport, 1997). Adjacent endothelial and mesenchymal cells were never observed to contain fragmented nuclei, although pyknotic nuclei could be seen in the neural tube, as reported by Hensey and Gautier (1998) (data not shown). By stage 43, the hypochord is no longer detectable as a distinct structure.

The notochord is required for hypochord development

To investigate the possibility that the notochord is required for formation of the hypochord, we removed the notochord from the posterior region of the embryo, at different times during early neurulation (stage 13-18). Embryos were then allowed to heal and to develop until the late tadpole stages (stage 30-35), when they were assayed for the presence of the hypochord, using in situ hybridization or histological analysis. It is important to note that extreme care was taken to leave the underlying endoderm intact when removing the notochord (see Materials and Methods). At these early stages, the endoderm is an epithelium a single cell layer thick and any damage to the endodermal layer results in the immediate and evident collapse of the archenteron. Examples of dissected embryos showing intact and damaged endoderm layers are illustrated in Fig. 5A-D. Only embryos with completely intact endoderm were assayed for hypochord development.

Transverse sections through the trunk of manipulated embryos reveal that, when the notochord is removed from the early neurula embryo (stage 13-14), the hypochord fails to develop in the operated region (see Table 1 and Fig. 5F,G).

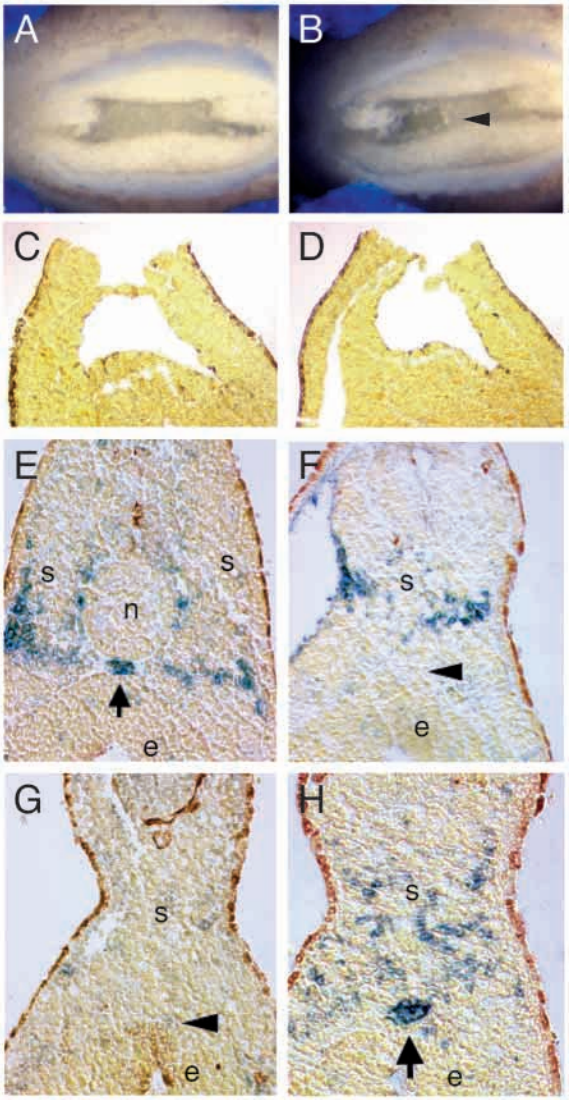

Fig. 5. Removal of notochord at stage 13-14 results in the failure of hypochord development. (A) Stage-14 embryo showing removal of notochord. Note that the endodermal cell layer is intact in this embryo. (B) Embryo with notochord removed showing clear damage to the endodermal layer (arrowhead). (C,D) Transverse sections through embryos following notochord removal, showing intact and damaged endodermal cell layers, respectively. Only embryos showing an intact endodermal layer were assayed in subsequent experiments. (E-H) Transverse sections through the posterior trunk of stage-30 embryos assayed for *VEGF* expression by in situ hybridization. (E) Section through an unmanipulated embryo. The hypochord is located ventral to the notochord. *VEGF* expression is evident in the hypochord (short arrow) and the somites. (F,G) Sections through embryos from which the notochord was removed at stage 13. Because of the absence of the notochord, the somites have fused at the midline, directly ventral to the neural tube. Notice the absence of *VEGF* staining at the midline and the absence of any recognizable hypochord structure. Horizontal arrowheads indicate the expected position of the hypochord, dorsal to the endoderm. (H) Section through an embryo from which the notochord was removed at stage 18. Notice the presence of a hypochord (vertical arrow) at the midline immediately ventral to the fused somites. s, somites; n, notochord; e, endoderm.

Table 1. Results of notochord removal and addition experiments

	n	Detectable hypochord	%	Ratio of cross sectional area of hypochord
Notochlord removal				
Removal of notochord at stage 13-14	20	0	0	
Removal of notochord at stage 17-18	20	19	95	
Notochord addition*				
Endogenous notochord only	20			1
Medial addition of notochord	20			3.3±0.58
Lateral addition of notochord	10			0

In notochord removal experiments, presence of the hypochord is assayed by VEGF expression and by histology.
In notochord implantation experiments, the ratio of cross-sectional area of the hypochord was determined relative to unmanipulated controls.
In lateral implantation experiments, no hypochord structure was visible by histology and no ectopic expression of VEGF was observced.
*All notochord addition experiments were assayed at stage 30.

Surrounding structures such as the endoderm and the neural tube show no evidence of damage, but the somites fuse medially, since they are no longer separated by the notochord. When the notochord is removed from the same relative posterior region of the embryo at a later stage of development (stage 17-18), the formation of the hypochord proceeds normally (see Table 1 and Fig. 5H). The results presented in Fig. 5 were obtained using VEGF expression as a marker for hypochord tissue, but identical results were obtained when later stage embryos were assayed histologically for the presence of the differentiated hypochord (data not shown). These experiments indicate that notochord tissue is essential for the induction of hypochord development, but that this requirement is complete by the late neurula stage.

Increased notochord signaling induces excess hypochord tissue

Since inductive interactions with the notochord are apparently necessary for hypochord formation, we examined the possibility that notochord signals are also sufficient for hypochord development. Experiments were conducted in which notochord tissue was placed adjacent to the endoderm, at ectopic locations in the embryo. Development of hypochord tissue was subsequently assessed using both histological analysis and in situ hybridization for *VEGF* expression. In these experiments, the notochord was removed from the posterior region of stage-13 to -14 donor embryos and implanted into a recipient embryo of the same stage. In initial experiments, the donor notochord was inserted at a medial location, between the endogenous notochord and the adjacent paraxial mesoderm, immediately dorsal to the endoderm. Manipulated embryos were then allowed to heal and develop to stage 26-35, when they were assayed for the presence of hypochord tissue.

In situ hybridization analysis of early tailbud embryos (stage 26) resulting from these transplantations demonstrated that placement of an ectopic notochord adjacent to the endogenous notochord (medial position) caused a significant increase in the amount of endoderm tissue expressing *VEGF* (Fig. 6). The region of increased *VEGF* staining extends laterally, from the normal location of expression under the endogenous notochord (Figs 3A, 6A), to the endoderm ventral to the ectopic notochord (Fig. 6B). Instead of a single nucleus expressing *VEGF* per transverse section, extensive *VEGF* expression can be detected in the endoderm beneath the paired notochords and sometimes up to 3-5 stained nuclei are distinguishable (Fig. 6B,D). This increased region of expression of VEGF in the dorsal endoderm was observed in 75% (15/20) of embryos examined. The anterior and posterior extremities of the excess *VEGF* expression correspond closely to the ends of the implanted notochord tissue. This additional *VEGF* expression is consistent with the induction of increased hypochord tissue within the dorsal endoderm. At present, we do not know whether the signal from the notochord that causes VEGF expression in the endoderm is directional. If it is, then correct orientation of the implanted notochord may be important for the success of the experiment. Although we have not pursued the matter further, this explanation could account for the 25% (5/20) of notochord implants that do not obviously express additional VEGF.

Sections through later embryos (stage 30), after overt hypochord formation, showed that implantation of ectopic notochord tissue resulted in the formation of clearly enlarged hypochords relative to controls (compare Fig. 6D,E). These enlarged hypochords developed from the region of the endoderm between the two notochords. Measurement of the cross-sectional area showed that these hypochords were approximately three times larger than hypochords present in unmanipulated embryos (Table 1). In no case, however, did we observe the presence of a distinct, second hypochord under the transplanted notochord. Occasionally, the endogenous and transplanted notochords fused into a single, large notochord (with twice the cross-sectional area of a normal notochord) and induced a wide region of *VEGF* staining in the endoderm (Fig. 6C). However, contrary to hypochord development in the manipulated embryos described above, these *VEGF*-expressing prehypochord cells did not appear to delaminate to form a mature hypochord in stage-30 embryos. Nor did these cells form a morphologically distinct hypochord, even when assayed at later stages of development. At present, the significance of this observation is not clear. It is interesting to note that embryos containing the fused, enlarged notochords also possess neural tubes that are much larger than those in control embryos.

To assess whether the entire endoderm is competent to form hypochord, experiments were carried out in which notochords were placed, adjacent to the endoderm, at ectopic locations in the embryo. This was achieved, either by lateral displacement of the

endogenous notochord from its normal position, or by grafting of extra notochords to lateral positions in the embryo. Assay of the displaced notochord experiments by VEGF expression showed that single, ectopic hypochords were able to form, immediately beneath the displaced notochord, at a range of positions in the dorsal endoderm (Fig. 7B). Analysis of adjacent transverse sections through ten embryos showed that competence to respond to notochord signaling appeared to diminish as the distance from the dorsal midline increased, but extended approximately half way along the region of endoderm contact with the somites. In the grafting experiments, notochords were inserted under the lateral plate mesoderm, immediately overlying the endoderm, ventrolateral to the somites. Examination of ten embryos with ectopic notochords implanted at lateral positions never revealed either ectopic *VEGF* expression or a histologically distinguishable ectopic hypochord (see Table 1 and Fig. 7C). Overall, our results suggest that the ability of endodermal cells to form hypochord is highest in the dorsal endoderm and decreases to zero in the lateral endoderm. The approximate domain of endoderm competent to form hypochord in response to notochord signaling is shown in Fig. 7D.

DISCUSSION

In 1895, Stöhr stated that the hypochord plays no role in directing the formation of any other embryonic structure. However, our recent results strongly suggest that the hypochord plays an essential role in the patterning of the dorsal aorta in fish and amphibian embryos (Cleaver and Krieg, 1998). In view of this important function of the hypochord in embryonic patterning, this study is aimed at understanding the origins of the hypochord, its morphogenesis, and the inductive signals responsible for its development. Our results indicate that the notochord plays a critical role in the specification and differentiation of the hypochord. This provides evidence that, in addition to patterning neural tissue and axial mesoderm, the notochord also patterns endoderm.

Hypochord development

Using a combination of histology and molecular markers, the *Xenopus* hypochord is initially detectable as a row of endodermal cells that have changed shape relative to their columnar neighbors. This row is no more than one or two cell diameters in thickness, as seen histologically (Fig. 1) or by staining for *VEGF* expression (Fig. 3). Prehypochord cells delaminate into the space ventral to the notochord, and soon after this time, the hypochord assumes its mature form: a rod of cells, a single cell diameter thick, immediately juxtaposed to the notochord. Later during development, following dorsal aorta development and maturation, the hypochord cells flatten significantly against the ventral surface of the notochord and begin the process of degeneration. Previous studies suggest that these cells may die by ‘karyolysis’, or apoptosis (Gibson, 1910; Löfberg and Collazo, 1997) and our observations of pyknotic nuclei in the degenerating hypochord (Fig. 4) strongly support the argument that hypochord degeneration proceeds via an apoptotic mechanism.

Notochord signaling is required for hypochord development

Notochord signals are important for the formation of adjacent neurectodermal and mesodermal tissues such as the floor plate, motor neurons, sclerotome, dermomyotome, and muscle pioneers in the myotome (van Straaten et al., 1988; Placzek et al., 1990; Yamada et al., 1993; Dietrich et al., 1993; Koseki et al., 1993; Pourquié et al., 1993; Münsterberg and Lassar, 1995; Bumcrot and McMahon, 1995; Ericson et al., 1997; Christ et al., 1992; Rong et al., 1992; Herrmann et al., 1990; Schulte-Merker et al., 1992; Halpern et al., 1993; Dodd et al., 1998). The notochord has also been shown to influence the left-right asymmetry of heart looping and the size of the heart field (Danos and Yost, 1995; Goldstein and Fishman, 1998). More recently, the notochord has been shown to be required for differentiation of the pancreas, an endodermally derived tissue (Kim et al., 1997). In this report, we demonstrate that the notochord is also involved in dorsoventral patterning of the endoderm, since it signals a subgroup of endodermal cells to develop into the hypochord.

Using embryonic manipulations, we have shown that the notochord is essential for hypochord development. Removal of the notochord during the early neurula stages leads to the complete failure of hypochord development (Fig. 5). Removal of the notochord during the late neurula stages, however, does not affect hypochord formation. This suggests that the notochord is required early for the development of the hypochord (stage 13-14), but that this requirement is complete by the late neurula stages (stage 17-18). At the cellular level, it is likely that the notochord sends an inductive signal to the dorsalmost endoderm, sometime between stage 13 and 17, which is responsible for inducing hypochord development. These results also indicate that hypochord development requires only this initial inductive signal, and that no maintenance signals from the notochord are required after stage 17. At later stages, therefore, the pathway leading to morphogenesis of the hypochord appears to be autonomously regulated.

The proposed role for the notochord in hypochord induction is supported by observations made in a number of zebrafish mutant embryos that are defective in notochord development. For example, *flh* and *ntl* homozygous mutants fail to produce differentiated notochord cells and, in embryos with these early-acting mutations, the hypochord either never develops or is severely disrupted (Marnie Halpern, personal communication; Rissi et al., 1995). However, in the zebrafish mutants *gup*, *bal*, *dop*, *sny* and *gno*, which are defective only in the maturation of the notochord (notochord cells fail to vacuolate), the hypochord appears normal as assayed by the expression of *collagen 2a* (Stemple et al., 1996). Once again, we can conclude from these observations that development of the hypochord depends on a signal from the early notochord, but not from the mature notochord. We predict therefore, that other zebrafish mutants, such as *mom* and *doc* (Odenthal et al., 1996), which completely lack a notochord in the trunk, will also lack a hypochord.

Notochord induces hypochord marker expression in dorsal endoderm

Notochord transplantation experiments have been used to further examine the inductive influence of the notochord on the dorsal endoderm. In these experiments, notochords were implanted adjacent to the endoderm of a recipient embryo, either medially, next to the endogenous notochord, or laterally,

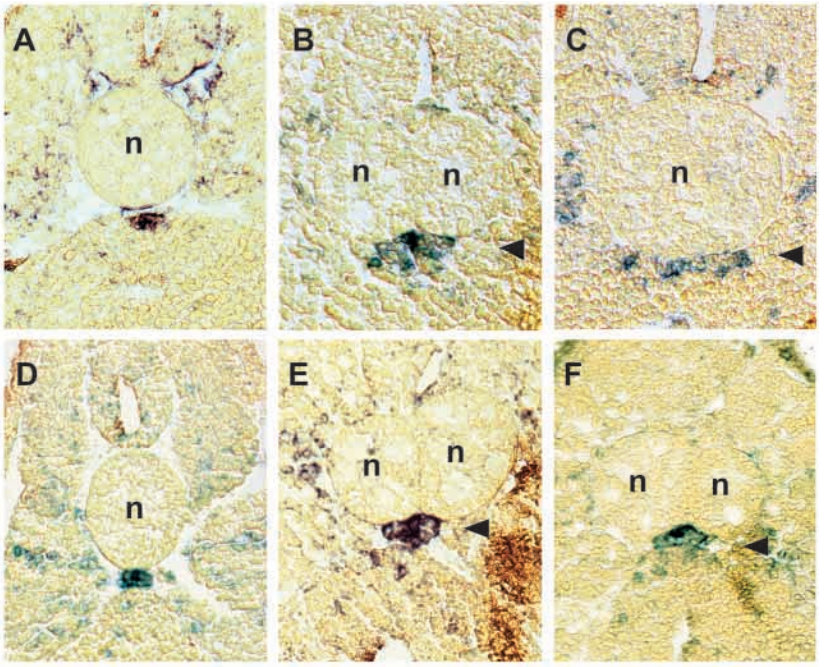

Fig. 6. Addition of notochord tissue results in increased *VEGF* expression and the development of enlarged hypochords. All panels show transverse sections through embryos that have been assayed for *VEGF* expression by in situ hybridization. n, notochord tissue. (A) Section through an unmanipulated stage-26 embryo showing *VEGF* expression in the hypochord precursor cell, located in the dorsal endoderm, immediately ventral to the notochord. (B,C) Sections through embryos that received homotopic notochord transplantations at stage 14. (B) Section through a stage-26 embryo that has received a transplanted notochord. The transplanted notochord is under the neural tube to the right and the endogenous notochord is under the neural tube to the left. Notice the significantly increased *VEGF* staining in the endoderm ventral to the notochords (black arrowhead). (C) Section through a stage-26 embryo in which the transplanted notochord has fused with the endogenous notochord. This notochord hybrid is now twice the size of a normal notochord. Notice the extensive *VEGF* expression in the endoderm ventral to the notochord (black arrowhead). (D) Section through an unmanipulated stage-30 embryo showing *VEGF* expression in the histologically distinct hypochord, located immediately ventral to the notochord and dorsal to the endoderm. (E,F) Sections through stage-30 embryos that have received a transplanted notochord. In both sections the transplanted notochord is on the right. Significantly enlarged hypochords (indicated by black arrowheads) are distinguishable both by histological appearance and by increased *VEGF* staining.

ventral to the somite. Our results indicate that a medially implanted notochord causes a significant increase in the region of *VEGF* expression in the dorsal endoderm in tailbud embryos. We interpret these results to mean that additional signals from the implanted notochord are causing a direct increase in the number of cells that contribute to the hypochord, and that this is detected by *VEGF* staining.

Implantation of a second notochord ventrolateral to the somites was never observed to induce ectopic *VEGF* expression or hypochord formation (Table 1). However, lateral displacement of the endogenous notochord revealed that an extended domain of endodermal tissue (from the midline to approximately half way beneath the somites; Fig. 7D) is competent to form hypochord in response to notochord signaling. The region of endodermal tissue that can form hypochord under experimental conditions is significantly broader than the extreme dorsal endoderm cells that normally form hypochord. These observations suggest that a dorsoventral prepattern already exists in the endoderm of the early neurula stage embryo, with respect to the ability to form hypochord. Although the factors that establish this endodermal competence remain unknown, gene expression patterns demonstrate that the dorsal endoderm is distinct from more

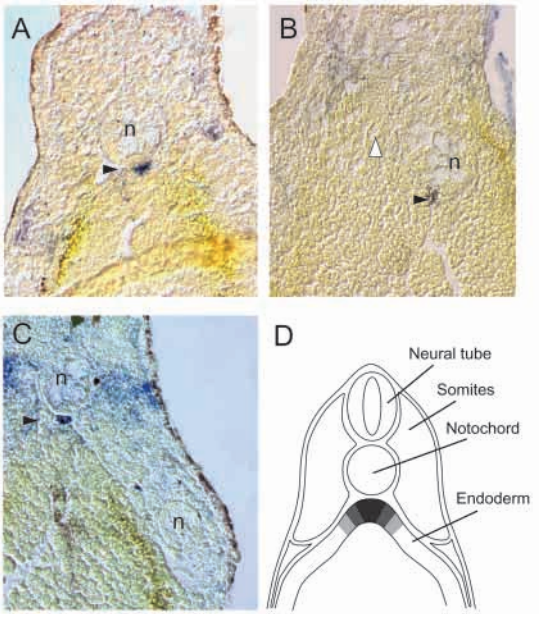

Fig. 7. The ability to form hypochord is restricted to dorsal endodermal cells. (A) Section through a stage-30 embryo showing *VEGF* expression (black arrowhead) in developing hypochord tissue, immediately ventral to the endogenous notochord (n). (B) Section through stage-30 embryo in which the endogenous notochord (n) has been displaced laterally at stage 14. The open arrowhead indicates the place where the notochord would normally be located. *VEGF* expression (black arrowhead) is visible in endodermal cells adjacent to the displaced notochord. (C) Section through a stage-30 embryo in which a second notochord was grafted lateral to the somite, immediately adjacent to the lateral plate mesoderm. The transplant was performed at stage 14. *VEGF* expression in hypochord tissue adjacent to the endogenous notochord is evident (black arrowhead). No ectopic *VEGF* staining or hypochord structure is visible adjacent to the ectopic notochord. Although the notochord in this example is slightly separated from the endoderm, examination of multiple embryos failed to reveal VEGF staining in lateral endoderm, even when the transplanted notochord was in immediate contact. (D) Diagram showing the approximate locations of endodermal cells capable of forming hypochord in response to notochord signaling. The shading indicates a diminishing ability to form hypochord in more lateral endodermal tissues.

ventrolateral endodermal tissues even at relatively early stages of development. Evidence of this prepattern includes the expression domains of Xsox17β (Hudson et al., 1997), the 4G6 antigen (Jones et al., 1993), XfrzA (Xu et al., 1998) and α2-macroglobulin (D. Kessler, personal communication).

When embryos are assayed at the mid-tailbud stage, after hypochord differentiation, we observe that secondary notochords can cause the development of abnormally enlarged hypochords. The development of two normal hypochords, however, is never observed. It seems possible that, as the cells of the enlarged hypochord field are delaminating, they associate with each other and are therefore prevented from forming two distinct organs. We favor the interpretation that the increased size of the hypochord results from increased recruitment of endodermal cells to the hypochord anlage, although we cannot exclude the possibility that hypochord precursor cells undergo increased proliferation.

Nature of the notochord signal

Our experiments clearly indicate that the notochord signals the endoderm to adopt a hypochord cell fate. However, it is not clear whether the notochord signal responsible for hypochord development is instructive or permissive. If instructive, implantation of a second notochord provides excess notochord signal, directing additional endoderm cells to take on a hypochord fate and resulting in an enlarged hypochord. If, on the other hand, the signal is permissive, the notochord may promote hypochord development by repressing gene expression within the dorsal endoderm cells, thereby allowing a hypochord cell fate. This mechanism would be analogous to the demonstrated role of the notochord in regulating pancreas development (Kim et al., 1997; Hebrok et al., 1998). Alternatively, it is possible that the normal function of notochord during hypochord formation, is to physically block an inhibitory signal from adjacent tissues, thereby permitting hypochord development. It follows that in our experiments, addition of a second notochord could cause an expansion of hypochord precursors by simply blocking endoderm exposure to an endogenous inhibitory signal from adjacent tissues, such as the somites or the neural tube.

At present, the molecular identity of the notochord signal remains unknown. In general terms, the signal could either be a cell-surface signaling molecule or a diffusible factor produced by the notochord. A number of diffusible factors expressed in the *Xenopus* notochord during early development are potential candidates for the hypochord inducing signal. These include Shh, eFGF, activin, chordin, noggin and follistatin (Ruiz i Altaba, 1998; Isaacs et al., 1995; Dohrmann et al., 1993; Sasai et al., 1994; Smith and Harland, 1992; Hemmati-Brivanlou and Melton, 1994). At first glance, Shh appears to be a good candidate for the hypochord inducing factor. Shh is known to be involved in the regulation of pancreas development and in controlling the regionalized expression of *Hox* genes in the gut mesoderm (Hebrok et al., 1998; Roberts et al., 1995, 1998) and there is strong evidence that specification of the floor plate and patterning of the somites is mediated in part by Shh from the notochord (Roelink et al., 1995; Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Hammerschmidt et al., 1996; Ericson et al., 1997). Furthermore, in both *Xenopus* and zebrafish, *hedgehog* genes are expressed in the early notochord during the time that hypochord tissue is induced (Ruiz i Altaba, 1998; Krauss et al., 1993; Currie and Ingham, 1996). Therefore, the location and timing of *shh* expression are consistent with a role in early hypochord patterning. However, genetic studies in zebrafish clearly indicate that the hypochord forms normally in zebrafish mutants such as *syu*, *yot*, *you* and *con*, which are defective in genes of the Shh signaling pathway (Schauerte et al., 1998). These observations do not exclude the involvement of other *hedgehog* family members, but it appears that additional experiments will be required to identify the notochord derived signaling molecules responsible for hypochord induction.

In summary, our experiments demonstrate that the notochord plays a critical role in patterning cells of the dorsal endoderm to form the hypochord. To the best of our knowledge, this report provides only the second example, in addition to pancreatic development in the chicken (Kim et al., 1997), of endoderm patterning by the notochord. Based on these initial examples, however, it seems likely that the notochord plays an important role in patterning of endodermal tissues, perhaps equivalent to its better understood function in regulating aspects of mesodermal and neural development.

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