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Coelom formation: binary decision of the lateral plate mesoderm is controlled by the ectoderm

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SUMMARY

Most triploblastic animals including vertebrates have a coelomic cavity that separates the outer and inner components of the body. The coelom is lined by two different tissue components, somatopleure and splanchnopleure, which are derived from the lateral plate region. Thus, the coelom is constructed as a result of a binary decision during early specification of the lateral plate. In this report we studied the molecular mechanisms of this binary decision. We first demonstrate that the splitting of the lateral plate into the two cell sheets progresses in an anteroposterior order and this progression is not coordinated with that of the somitic segmentation. By a series of embryological manipulations we found that young splanchnic mesoderm is still competent to be respecified as somatic mesoderm, and the ectoderm overlying the lateral plate is sufficient for this redirection. The lateral ectoderm is also required for maintenance of the somatic character of the mesoderm. Thus, the ectoderm plays at least two roles in the early subdivision of the lateral plate: specification and maintenance of the somatic mesoderm. We also show that the latter interactions are mediated by BMP molecules that are localized in the lateral ectoderm. Evolutionary aspects of the coelom formation are also considered.

Key words: Tissue interactions, Mesoderm subdivision, BMP4, Morphogen, Evolution

INTRODUCTION

The coelomic (body) cavity develops in the body of animals that belong to a variety of phyla including all the vertebrates. The cavity is a closed space in the trunk which is lined by two kinds of cell sheets, one underlying the body wall and the other one surrounding the gut. The coelomic cavity offers several advantages to the animals: (1) the body can become large in size because of the fluid-filled structure, (2) the tube-within-a-tube architecture frees the inner tube (gut) from the constraint of the outer body wall so that an individual organism can obtain high locomotive activity which is independent of digestive activity, (3) a large cavity allows for the development and expansion of new visceral structures, such as heart, liver and gonads (Gilbert and Raunio, 1997).

In vertebrates the coelomic cavity is derived from the lateral plate mesoderm (Fig. 1). The lateral plate is initially a homogeneous mesenchymal structure that is located lateral to the somitic mesoderm. Specification of the lateral plate and somite from the primitive paraxial mesoderm is known to be regulated by different concentrations of BMP-4 activity (Tonegawa et al., 1997; Tonegawa and Takahashi, 1998). Subsequently the lateral plate undergoes splitting into the somatic mesoderm (Smt-m) and splanchnic mesoderm (Spl-m). Thus, the subdivision of these two types of the lateral plate mesoderm is made by a binary decision. After splitting into the two layers, one is localized underneath the ectoderm and the other is attached to the endoderm (Fig. 1). At later stages, as an embryo is folded into a three dimensional structure, the lateral-most portions of the left and right lateral plates eventually meet at the ventral midline. This process produces a closed architecture of the body by leaving more laterally positioned structures, including blood islands, extraembryonic (Pardanaud et al., 1996; and references therein). Thus, in the trunk the coelomic cavity is lined by the somatopleure (lateral ectoderm + Smt-m) for its outer aspect, and by the splanchnopleure (endoderm + Spl-m) for the inner one (Fig. 1). The formation of the coelomic cavity is, therefore, a fundamental process that leads to a tube-within-a-tube structure. However, there has been no recent work which has dealt with the molecular mechanisms that establish the lateral plate splitting. The reason seems to be that in frogs and fish the two layers of the lateral plate are too fragile and thin to be identified and manipulated, and in mice this tissue is often torn away when embryos are dissected from the uterus.

We have studied the mechanisms by which the two subtypes are specified from the primitive lateral plate by taking advantage of a most simple morphological structure of chicken embryos. The lateral plate of avian embryos facilitates embryological manipulations because it is a solid and easily identifiable structure and is also completely flat. We first describe the progress of the splitting of the lateral plate in an anteroposterior (A-P) order. We then ask three essential

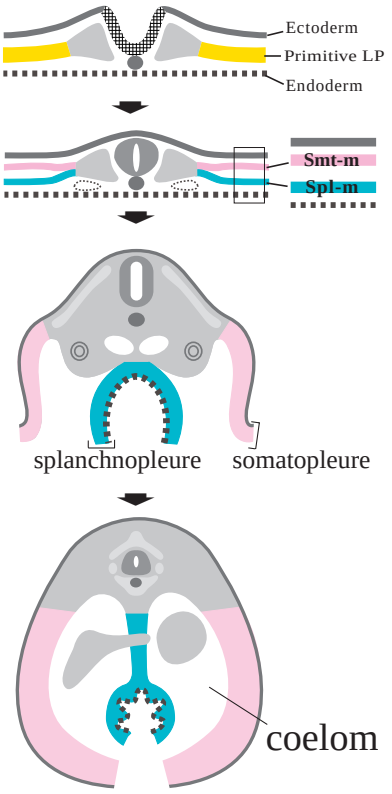


Fig. 1. Diagram illustrating coelom formation from the lateral plate. The primitive lateral plate (yellow) located lateral to the somite is a group of mesenchymal cells between the ectoderm and the endoderm. Subsequently it splits into the two cell layers: the somatopleural (somatic) mesoderm (Smt-m; pink) underlying the ectoderm and the splanchnopleural (splanchnic) mesoderm (Spl-m; green) closely associated with the endoderm. At later stages, the lateral-most portions of the lateral plates eventually close at the ventral midline. The somatopleure (lateral ectoderm + Smt-m) forms the outer body wall, whereas the splanchnopleure (lateral endoderm + Spl-m) develops to produce internal organs. The space between the somatopleure and splanchnopleure is the coelom.

questions. (1) What triggers the binary decision of the lateral plate? (2) To what extent is the lateral plate committed after this decision? (3) What molecules are involved in the binary decision? We demonstrate essential roles of the lateral ectoderm in the decision and also the involvement of BMP molecules in these tissue interactions. We also discuss evolutionary significances of the coelomic cavity that developed after emergence of the triploblastic animals.

MATERIALS AND METHODS

Histological preparation

For regular observations chicken embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin wax after dehydration with ethanol and xylene. Serial sections (15 μm) were prepared and stained with Mayer's hematoxylin solution (Wako) and mounted with Entellan (Merck).

Embryological manipulations in ovo

Fertilized White Leghorn chick and Japanese quail eggs from commercial sources were used throughout this study. For implantation of various tissues and substances, a slit was made in the ectoderm overlying the medial region of the lateral plate of a host embryo with a sharpened tungsten needle. A piece of either transplanted tissue, aluminum foil, nuclepore filter, or a COS cell aggregate was inserted in between the ectoderm and Smt-m. The operated embryos were incubated for 16 hours and then fixed with 4% paraformaldehyde in PBS for further analyses. For detection of quail cells the operated embryos were fixed with Carnoy's solution and stained as previously reported (Feulgen and Rossenbeck, 1924; Le Douarin, 1969, 1982).

The lateral plate tissue preparation

Spl-m was dissected from the region located between somite I and somite IV (Christ and Ordahl, 1995) of 3- to 25-somite stage embryos (Fig. 4 diagram) with a micro-feather blade (Feather) and electrically sharpened tungsten needles. Spl-m was isolated from the attached endoderm by treatment with 1.25% pancreatin in PBS for 10 seconds on ice. Fetal calf serum (FCS) was added to stop the pancreatin reaction and then the specimens were placed in a drop of HANK's solution (140 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 0.34 mM Na_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ pH 7.0) in which Spl-m was further divided into pieces about the size of one to two somites. Smt-m and the lateral ectoderm were prepared from the prospective flank region and the anterior-most level of the unsegmented somite of 18- to 20-somite embryos, respectively.

Cloning of chicken *HFH 8* and *Irx 3* cDNAs

HFH 8

Based on the amino acid sequence comparison of human and mouse HFH-8, and mouse HNF 3a, HFH 1, HFH 4 (Clevidence et al., 1994), a pair of degenerated oligonucleotides was designed as primers: 5′-GARATHTAYCARTTYYTNCA-3′ for 5′ end, and 5′-GTSACYT-GYTGRTGRTARTA-3′ for 3′ end of the gene (H: A or T or C, N: either of 4 nucleotide; R, purine; S, C or G; Y, pyrimidine). mRNA prepared from embryonic day 2.0 (E2.0) embryos was used as a template for reverse transcription with these primers. One μg of mRNA was heated at 65°C for 10 minutes, then chilled on ice. Ten picomoles of oligonucleotides was used for the reaction using First-Strand cDNA Synthesis kit (Pharmacia Biotech). After 1 hour at 37°C the enzyme was inactivated by 5 minutes at 90°C, and one fifth of the reaction mixture was amplified by PCR using the primers. The PCR reaction conditions were as follows. 10 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 50°C for 2 minutes, 72°C for 3 minutes and the last cycle was for an extension at 72°C for 10 minutes. A PCR product of 885 bp was cloned into pBluescript SK(–) (Stratagene). Four independent clones from RT-PCR were sequenced using Thermo Sequenase Cycle Sequencing kit (Shimadzu) (GenBank accession number: AB028627).

Irx 3

10^6 clones of a cDNA library prepared from the eyes of E3.0 chicken embryos were screened using *Caenorhabditis elegans Iroquois* cDNA which was PCR-amplified based on the GenBank sequence. One clone that contained 2 kb (ORF: 1446 bp) was isolated and analyzed further. The DNA sequence displays highest similarity to that of Xenopus *irx 3* (Bellefroid et al., 1998) and mouse *Irx 3* (Bosse et al., 1997). Particularly as shown in Fig. 3, the homeodomain shows 100 % match between these genes. We thus designated this clone, chicken *Irx 3* (accession number: AF157620).

RNA probes and in situ hybridization

Prx 1 (1045 bp) was kindly given by Dr T. Nohno. *Irx 3* of 1.5 kb and *HFH 8* of 885 bp were used. Dig-labelled RNA probe preparation and in situ hybridization of whole-mounted embryos followed by histological sectioning were as described by Takahashi et al. (1996). The in situ hybridization procedure for the recombination cultured specimens was modified as follows: the specimens were fixed with

4% paraformaldehyde in PBS for 30 minutes, treated with Proteinase K (5 mg/ml) for 5 minutes and with RNase I (33 mg/ml) for 15 minutes. The specimens were processed in a microcentrifuge test tube and then collected at the bottom of the tube by spinning at 3,000 rpm for 10 seconds at the end of each step.

COS 7 cell transfection

COS 7 cell transfection was performed as described previously using pCDM8 (in vitrogen)-derived vector in which elongation factor promoter drives mouse BMP or *Xenopus* Noggin cDNA (Takahashi et al., 1996; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998). Transfected COS cells were cultured for 24 hours and transferred to a dish coated with 1% agar to obtain cell aggregates.

Recombination culture

For a recombination culture the specimens were cultured on nuclepore filter (pore size 8 μm) (Costar) in a 4-well dish (Nunc) with DH 10 culture medium (1:1 mixture of Dulbecco's modified eagle medium (Nissui) and Ham's F12 medium (Nissui) containing 10% FCS). The nuclepore filter with the specimen floated on the surface of the medium.

RESULTS

Splitting of the primitive lateral plate progresses in an antero-posterior order

It has been thought that the splitting of the lateral plate progresses in an antero-posterior (A-P) order to form the two epithelial layers, the somatic mesoderm (Smt-m) and the splanchnic mesoderm (Spl-m). However, whether this process is coordinated with that of the development of the somites has not been well documented. We therefore performed serial histological sections of young chicken embryos at different developmental stages and we describe two representative stages, the 10-somite and 20-somite stages.

At the level of somite V (fifth somite anterior from the most recently formed somite; Christ and Ordahl, 1995) of 10-somite embryos, the lateral plate has already split into the two layers that are completely separated (Fig. 2, a-1). In contrast, at the level of somite I (newly formed somite), the two mesodermal layers are morphologically distinguishable but these epithelial cell sheets are partially attached to each other (Fig. 2, a-2). The coelomic cavity that separates the two layers is larger in the medial than the lateral region. At the level of the segmental plate, an epithelial alignment of the two distinct cell populations is obvious, but the coelomic cavity has not been formed (Fig. 2, a-3). At the level of the tail bud the lateral plate simply consists of a morphologically homogeneous population of mesenchymal cells and no sign of alignment of the two layers is visible (Fig. 2, a-4). By the 20-somite stage the lateral plate is split into the two layers all along the A-P axis. The coelomic cavity between the two mesodermal layers is more expanded at more anterior levels (Fig. 2, b1-4). Thus, the A-P progression of the splitting of the lateral plate is not

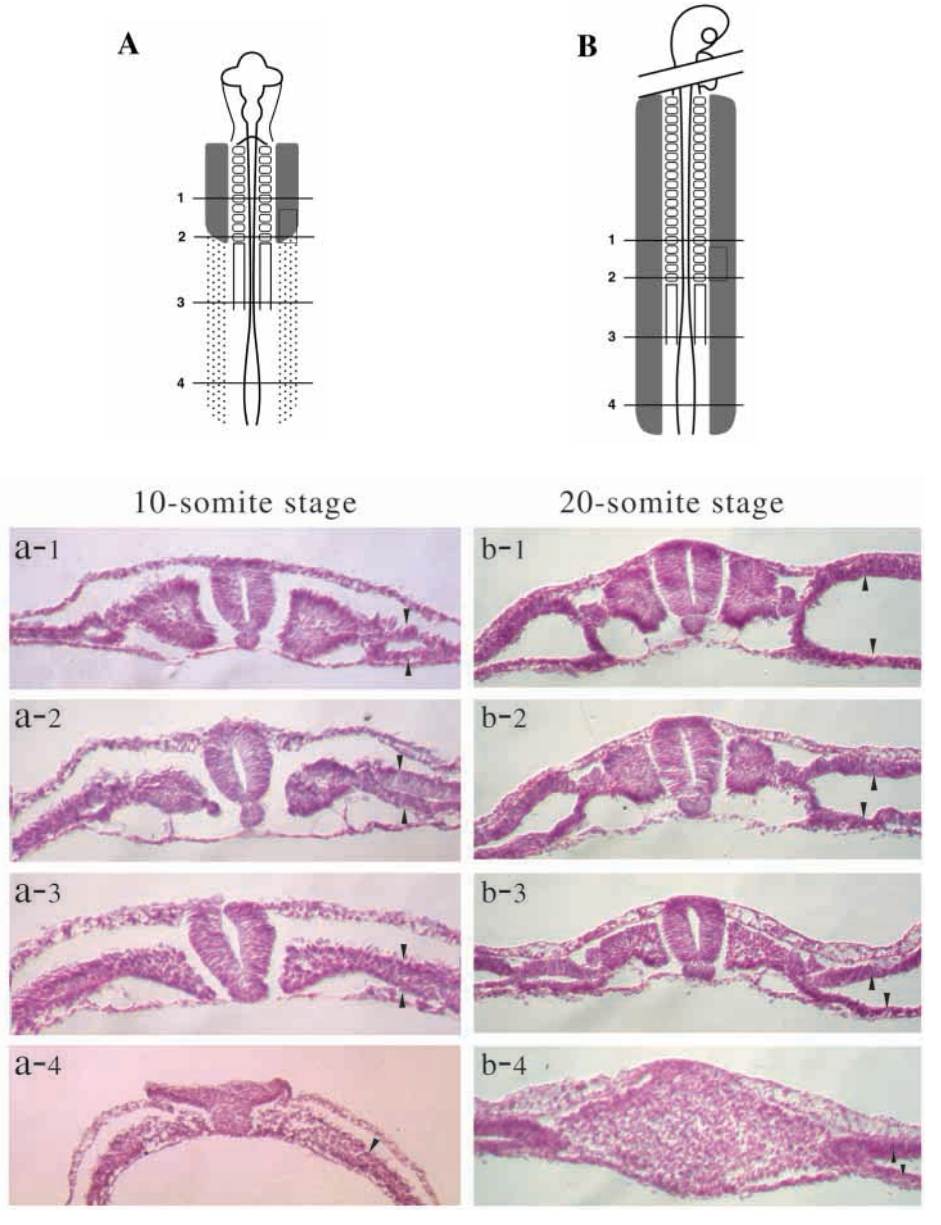


Fig. 2. The splitting of the lateral plate mesoderm progresses in an anteroposterior order. Transverse sections of a 10-somite embryo (A) and 20-somite embryo (B). Sections are at the levels of somite V (a-1, b-1), somite I (a-2, b-2), segmental plate (a-3, b-3), tail bud (a-4, b-4). In a 10-somite embryo the Smt-m and Spl-m (arrowheads) are morphologically distinct at the segmented levels (a-1, a-2), whereas in a region posterior to that the layers are barely observed (a-3, a-4). In a 20-somite embryo the splitting of Smt-m and Spl-m (arrowheads) is complete along the A-P axis (b-1 to b-4) and the coelom develops. Note that the A-P progression of the lateral plate splitting is not synchronous with that of the somite segmentation. In the top diagram the lateral plate mesoderm which has completed splitting is shown in gray and dotted areas represent the unsplit mesoderm.

synchronized with that of the somite segmentation. Accordingly, when compared at the same somitic level along the A-P axis (i. e. somite I) between 10- and 20-somite embryos, the lateral plate of the older embryo is morphologically more developed than that of the younger (compare Fig. 2, a-2 with b-2).

Molecular markers for the lateral plate

To investigate early events during the binary decision of the lateral plate, we first attempted to obtain molecular markers that are used to distinguish Smt-m from Spl-m since no markers had been reported for this purpose. We demonstrate that three genes, *Irx 3*, *HFH 8*, and *Prx 1* are useful (Fig. 3). The former two we have identified in this study and *Prx 1* had previously been reported for its global expression pattern in early chicken embryos (Nohno et al., 1993; Kuratani et al., 1994). Chicken *Irx 3*, found during expression cloning (Materials and Methods), is a homeobox-containing gene similar to mouse *Irx 3*, and *Xenopus iro* 3 (Bosse et al., 1997; Bellefroid et al., 1998). We obtained chicken *HFH 8*, possessing a winged helix motif with high similarity to human *HFH 8* (Pierrou et al., 1994), because a mouse homologous gene had previously been shown to be expressed in the visceral mesoderm surrounding the gut (Clevidence et al., 1994). Smt-m is identified by expression of *Prx 1* and *Irx 3*, whereas *HFH-8* is restricted to Spl-m (Fig. 3). Expression of these genes is localized in the respective cell layers when Smt-m and Spl-m are split and the expression remains in these tissues at later stages (Fig. 3). The lateral ectoderm is negative for *Prx 1* and *Irx 3*. Intensity of *Prx 1* signals in Smt-m is higher than that of *Irx 3*. A low level of *Prx 1* expression is also observed in the dermomyotome at E3.0.

Spl-m is respecified to Smt-m when relocated to the somatopleure

We performed transplantation experiments in which a piece of

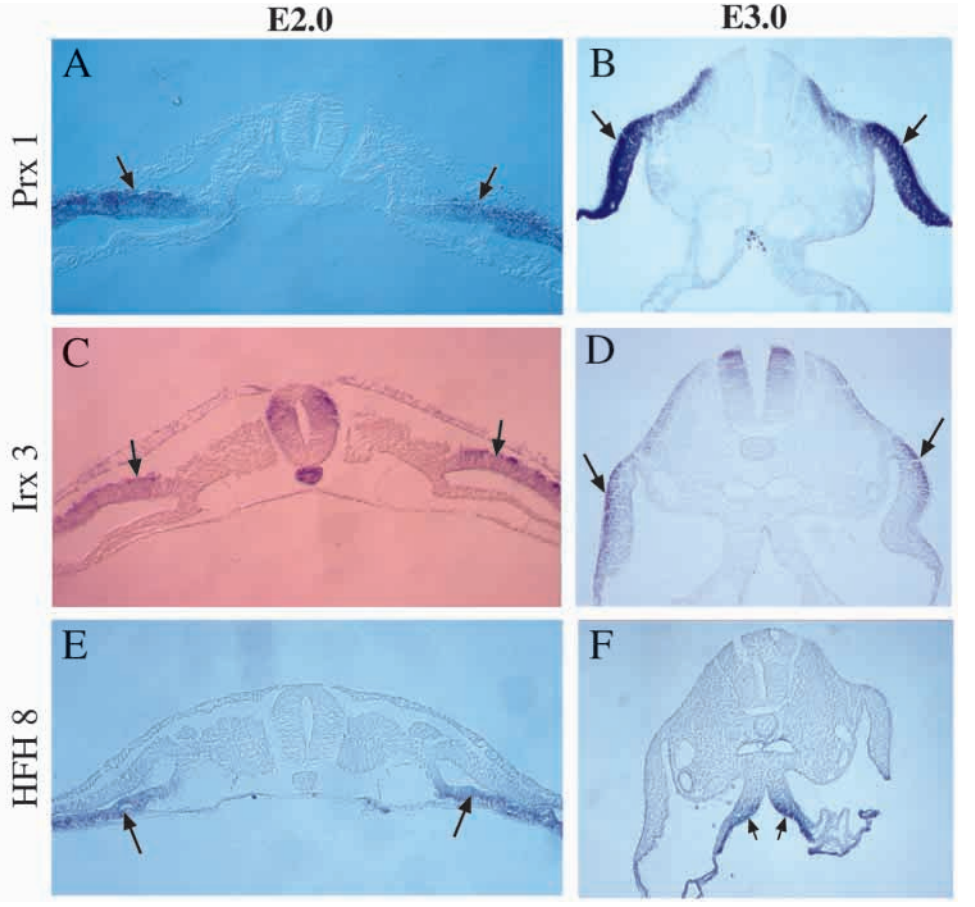
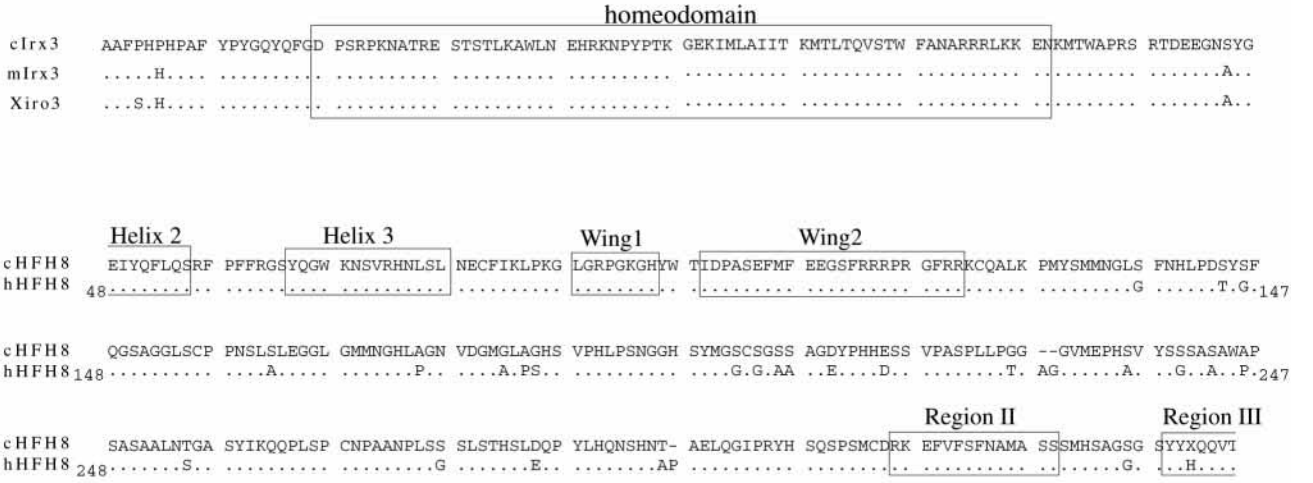


Fig. 3. Molecular markers of Smt-m and Spl-m. Transverse sections of the flank region of E2.0 (A,C,E) and E3.0 (B,D,F) embryos. Smt-m is identified by localization of *Prx 1* and *Irx 3* (arrows; A-D), whereas *HFH 8* is expressed specifically in Spl-m (arrows, E,F). (Below) Comparison of amino acid sequences of chicken Irx 3 and HFH 8 with respective counterparts in other vertebrates. The homeodomain of Irx 3, the winged helix motif, and the putative activation motif of HFH 8 are boxed.



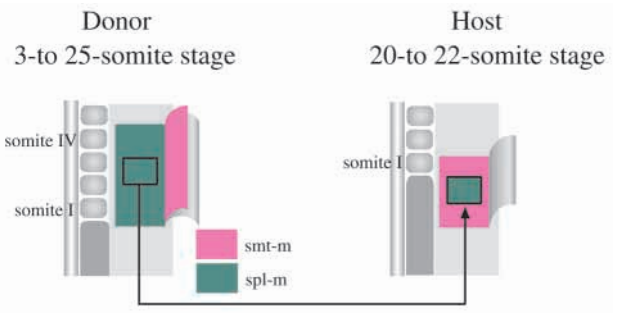
Spl-m was relocated to an ectopic position and examined whether the transplant was affected by the new environment. We focused on the lateral plate in the flank region positioned between the fore limb and hind limb along the A-P axis. As diagrammatically shown in Fig. 4, Spl-m that was dissected from the lateral plate between somite I and IV of different stages of quail or chicken embryos was inserted between the ectoderm and mesoderm of a host chicken embryo (20-somite stage) (see also Materials and Methods for details). Since at the 3- to 7-somite stages the lateral plate has not yet split into two layers, a piece of the primitive lateral plate was removed. Sixteen hours after the operation, grafted tissue taken from a donor no older than 22-somite stage remained as a cell aggregate on the operated site (Fig. 4C,D) and had turned on expression of the Smt-m markers (*Prx 1* and *Irx 3*) and extinguished the Spl-m character (*HFH 8*) (Fig. 4A,E,F; Table 1). Thus, Spl-m was redirected to Smt-m. In contrast, a grafted Spl-m dissected from embryos older than the 23-somite stage did not express the Smt-m markers (Fig. 4B; Table 1). Since *Prx 1* is also detected in the dermomyotome of normal embryos as mentioned earlier, we examined expression of *Pax 3*, a marker for the dermomyotome, in the transplanted Spl-m and found that the graft was negative for it (Fig. 4G), confirming that this showed Smt-m characteristics.

These results suggest that young Spl-m which has already separated and is morphologically distinct from Smt-m is capable of being respecified to Smt-m when relocated to the environment of the latter, and also that environmental cues which play important roles in Smt-m specification reside in the somatopleural components. As far as the lateral plate positioned adjacent to somite I to -IV is concerned, the developmental fate of Spl-m seems to be determined around the 23-somite stage of embryos.

The lateral ectoderm respecifies Spl-m to Smt-m

The experiments shown above demonstrated that the somatopleure, which is composed of the ectoderm and the mesoderm, respecified Spl-m to Smt-m. To determine which tissue or whether both are responsible for this respecification, we carried out in vitro tissue recombination culture. Tissue components of the lateral plate were dissected from the same region as above (adjacent to somite I to IV) of young embryos (16- to 22-somite stages). Tissues were cultured on nuclepore filter either in combination with another component or alone, followed by assessment for expression of *Prx 1*, *Irx 3*, and *HFH 8* (top diagram in Fig. 5; see also Materials and Methods for detail).

When Spl-m was cultured alone, it did not express *Prx 1*, *Irx 3* or *HFH 8*. In contrast, when Spl-m was recombined with the lateral (somatopleural) ectoderm, it turned on expression of *Prx 1* and *Irx 3* but not *Pax 3* or *HFH 8* (Fig. 5; Table 2), suggesting that Spl-m is respecified to Smt-m by the lateral ectoderm. When Spl-m and Smt-m were recombined and cultured together, neither tissue exhibited expression of *Prx 1* or *HFH 8* (Table 2). These results indicate that the lateral ectoderm alone is sufficient for Spl-m to Smt-m conversion but Smt-m is not. Interestingly, when Spl-m and its underlying endoderm were kept together and recombined with the lateral ectoderm, the tissue retained characteristics of Spl-m (*HFH 8* positive and *Prx 1* negative; Table 2). In addition, the surface



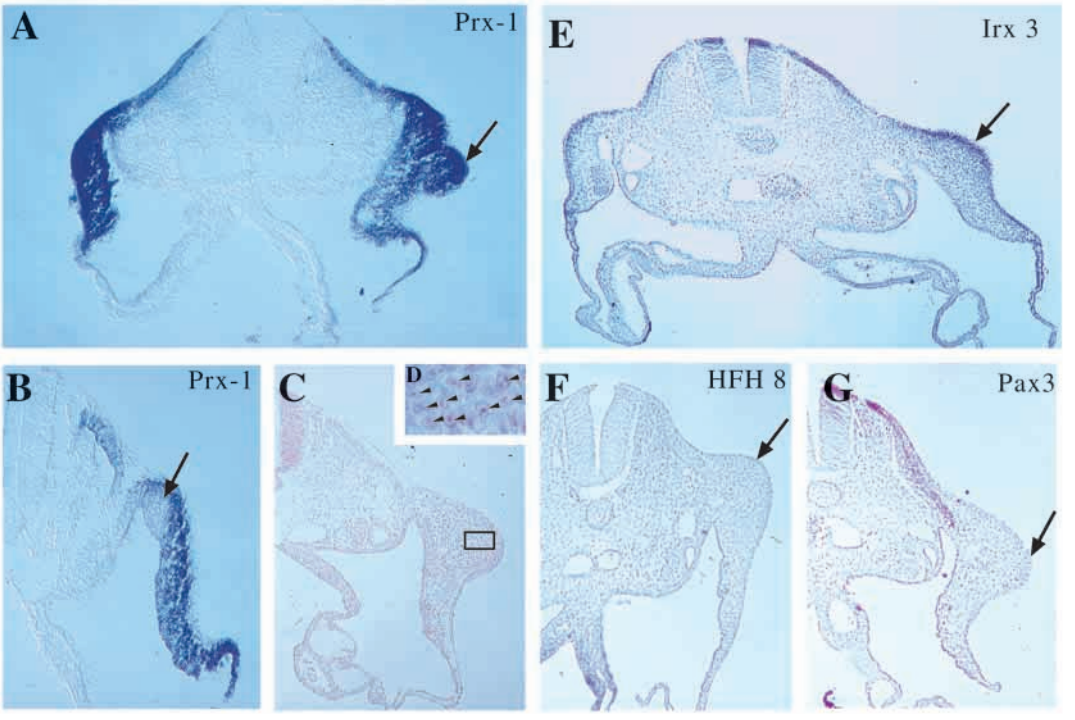


Fig. 4. Spl-m is respecified to Smt-m upon relocation. Diagram shows Spl-m at the A-P level of somites I-IV from 3- to 25-somite embryos of quail or chicken was transplanted inbetween the ectoderm and Smt-m of a host chicken embryo (20- to 22-somite stage). Transplanted Spl-m (arrows) from a young embryo expressed Smt-m markers, *Prx 1* (A) and *Irx 3* (E) but not the Spl-m marker, *HFH 8* (F) or dermomyotome marker, *Pax 3* (G). Spl-m from an older embryo did not express *Prx 1* (B). (C,D) Feulgen staining of a transverse section of a quail-chick chimera indicates the location of the transplanted Spl-m (arrowheads).

Table 1. Prx 1 expression in a grafted Spl-m into the somatopleure

Donor stage (Somite number)	Prx 1 in graft
3*	3/3‡
6*	2/2
7*	3/3
10	2/2
11	1/1
15	1/1
16	2/2
17	3/3
18	3/3
20	2/2
23	1/2
24	0/3
25	0/3

*Unsplit mesoderm was dissected.
‡Number of Prx 1-positive grafts/number of grafts examined.

ectoderm taken from the dorsal trunk region failed to induce *Prx 1* in the recombined Spl-m (Table 2). Thus, signals responsible for Spl-m to Smt-m respecification appear to be specific to the lateral ectoderm.

These observations suggest that during normal developmental processes the lateral ectoderm is required for specification of Smt-m from the primitive lateral plate and also that the signals for this specification are specific to the lateral ectoderm.

The lateral ectoderm maintains characteristics of Smt-m

We next studied whether after Smt-m has been specified, it maintains its identity independently or whether it requires interactions with other tissues. Two experiments were conducted. (1) A piece of aluminum foil or nuclepore membrane (pore size 0.1 μm) was inserted between the Smt-m and the ectoderm (Fig. 6A). The aluminum foil acts as a barrier blocking any interacting signals between these tissues whereas the nuclepore membrane allows diffusible secretory proteins to penetrate but not cell-cell contact. (2) An explant of Smt-m was cultured either alone or with the ectoderm (Fig. 6E).

Insertion of the aluminum foil resulted in a cessation of *Prx 1* expression in Smt-m (17/17, Fig. 6B). In contrast, when we implanted a piece of nuclepore membrane, mesenchymal cells of Smt-m underlying the filter retained *Prx 1* expression although the signal was slightly lower than the control side (12/12, Fig. 6C). We obtained an essentially identical result for *Irx 3* (data not shown). Thus, maintenance of the Smt-m character appears to require ectodermal signals and they are probably mediated by secretory factors acting close to the ectoderm.

For the explant culture, Smt-m was prepared from the prospective flank region. When Smt-m of younger than 20-somite stages was cultured alone, expression of *Prx 1* and *Irx 3*, which had been positive in this tissue, was down-regulated (Fig. 5G,I) whereas *HFH 8* remained negative in the explant (data not shown; n=15). In contrast, when Smt-m was recombined with the lateral ectoderm, it retained expression of the Smt-m markers (Fig. 5F,H). This ectoderm dependence of the somatopleural characteristics continued at least until the 22-somite stage, but after the 26-somite stage Smt-m no longer required the ectoderm (n=12, data not shown).

Table 2. Recombination culture of Spl-m with other tissues

	Markers			
Spl-m cultured with:	Prx 1	Irx 3	HFH 8	Pax 3
L* ectoderm	15/16‡	10/10	0/7	0/11
Smt-m	0/7	nt§	0/2	nt
L endoderm + L ectoderm	0/12	nt	11/11	nt
Dorsal ectoderm	0/47	nt	nt	nt
None	0/9	0/7	0/6	nt

*Lateral.
‡Number of positive cultures/number of examined.
§Not tested.

These observations led us to conclude that the lateral ectoderm plays at least two roles in the mesodermal subdivision of the lateral plate: one is to specify Smt-m from the primitive lateral plate, and the other is to maintain Smt-m after this subtype has been specified. It is likely that Smt-m maintenance signals are mediated by soluble factors emanating from the ectoderm.

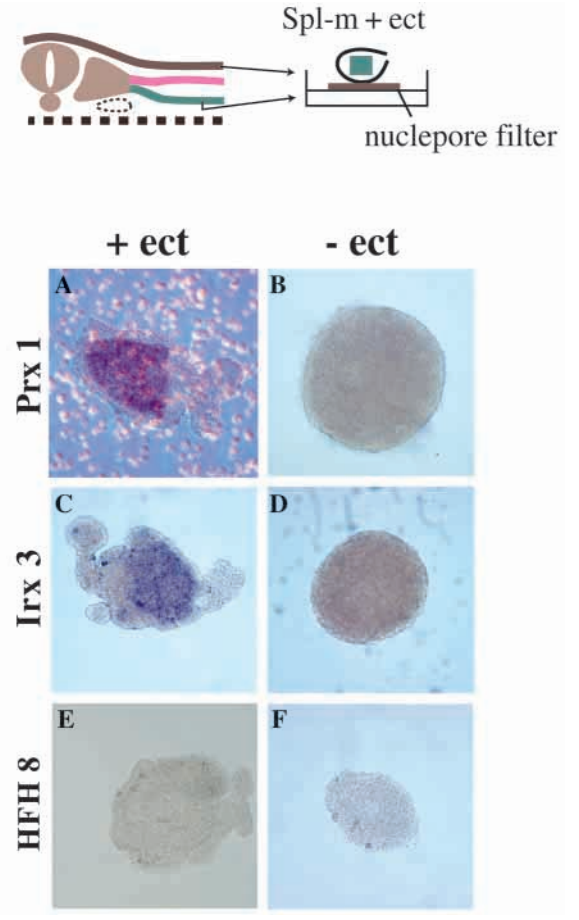


Fig. 5. The lateral ectoderm respecifies Spl-m to Smt-m. Spl-m of young embryos (16- to 22-somite stage) was cultured with or without the lateral ectoderm on nuclepore filters (diagram). Spl-m cultured in combination with the lateral ectoderm expressed *Prx 1* (A) and *Irx 3* (C) but not *HFH 8* (E) whereas when cultured alone, it displayed neither Smt-m markers (B,D) nor the Spl-m marker, *HFH 8* (F).

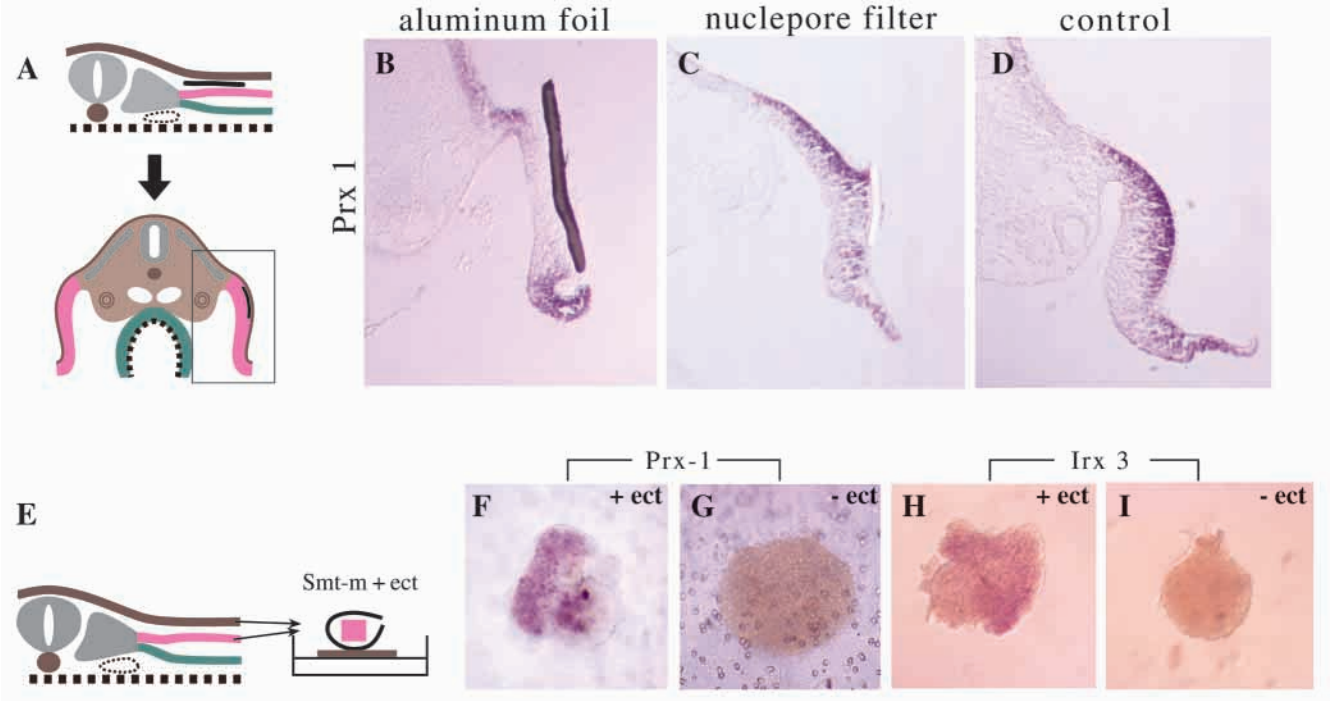


Fig. 6. The lateral ectoderm maintains characteristics of Smt-m. (A-D) Barrier insertion resulted in cessation of *Prx 1* expression. A barrier was inserted in between the lateral ectoderm and Smt-m (A). Insertion of a piece of aluminum foil extinguished *Prx 1* (B) whereas a nuclepore filter (pore size 0.1 μm) maintained the expression (C). (D) Control side. (E-I) Recombination culture experiments. A Smt-m explant cultured with the lateral ectoderm retained expression of *Prx 1* (F) and *Irx 3* (H), but when cultured alone, Smt-m markers were not maintained.

BMPs substitute for the lateral ectoderm in maintaining Smt-m identity

To elucidate the molecular cascade of the ectoderm-derived signals which are important for the Smt-m specification and maintenance, we looked at the roles of BMPs since these molecules have been reported to be expressed and to function in the early mesoderm (Pourquie et al., 1996; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998).

We examined expression patterns of BMP-2, BMP-4, and BMP-7 mRNAs mainly in the lateral plate region at the prospective flank level as shown above. BMP-2 and BMP-7 were detected in the ectoderm overlying both the dorsal (somites) and lateral plate regions. The signal for BMP-7 is almost uniform whereas BMP-2 expression is slightly more intense in the lateral ectoderm than in the medial one (Fig. 7C-F). In contrast, BMP-4 was expressed entirely in the lateral plate mesoderm including Smt-m and Spl-m but not in the ectoderm, and at later stages the signal regresses laterally particularly in Smt-m, thus leaving the medial-most portion of Smt-m devoid of BMP-4 (Fig. 7A,B). We could not detect the BMP-4 signal in the lateral ectoderm.

To study whether BMPs can substitute for the effect of the lateral ectoderm on the mesoderm, we recombined pieces taken from either Smt-m or Spl-m with BMP-producing COS cells, and cultured them on a nuclepore filter (Fig. 8A). Since biological activity of BMP-2 is indistinguishable from that of BMP-4, we used vectors expressing BMP-7 and BMP-4 cDNAs, respectively. We previously reported that COS cells transfected with BMP-4 cDNA (BMP-4/COS) exert the identical effect to that of recombinant BMP-4 protein (Takahashi et al., 1996). Moreover, every time we performed recombination culture, we confirmed the specific effect of BMP-4/COS by implanting them into the somite, a treatment known to convert the somite to lateral plate (Tonegawa et al., 1997).

When Smt-m was co-cultured with BMP-4/COS or BMP-7/COS, it retained expression of *Prx 1*, whereas expression was not retained with control COS cells (Fig. 8C-E; Table 3). Thus, BMP-4 and BMP-7 mimicked the effect of ectoderm on Smt-m. When Spl-m was co-cultured with COS cells transfected with BMP-4 or BMP-7, in contrast, it did not display the Smt-m marker expression (Table 3; Fig. 8B).

We previously reported that different levels of BMP-4 activity establish distinct subtypes of mesoderm (Tonegawa et al., 1997). However, in the present study we did not observe concentration-dependent effects of BMP-4 on Smt-m or Spl-m: relatively low levels of BMP-4 (1/5, 1/10; Tonegawa et al., 1997) were still capable of retaining *Prx 1* in Smt-m whereas these levels did not induce *Prx 1* expression in Spl-m (Table 3).

Thus, BMP-2 and BMP-7 substituted for the ectodermal signals on the Smt-m maintenance whereas these BMPs by themselves did not exert effects on the early specification of Smt-m in tissue culture experiments.

To further confirm that BMP-2 and/or BMP-7 are/is essential for maintaining the Smt-m identity in ovo, we ectopically implanted an aggregate of COS cells producing Noggin (Tonegawa and Takahashi, 1998), an antagonist of BMP-2 and BMP-7 (Smith and Harland, 1992; Zimmerman et al., 1996), inbetween the lateral ectoderm and Smt-m, and examined Smt-m markers. Expression of *Prx 1* and *Irx 3* was locally down-regulated (*Prx 1*, 19 out of 21; *Irx 3*, 33 out of 35; Fig. 8F-L), and *HFH 8* remained negative (n=11, not

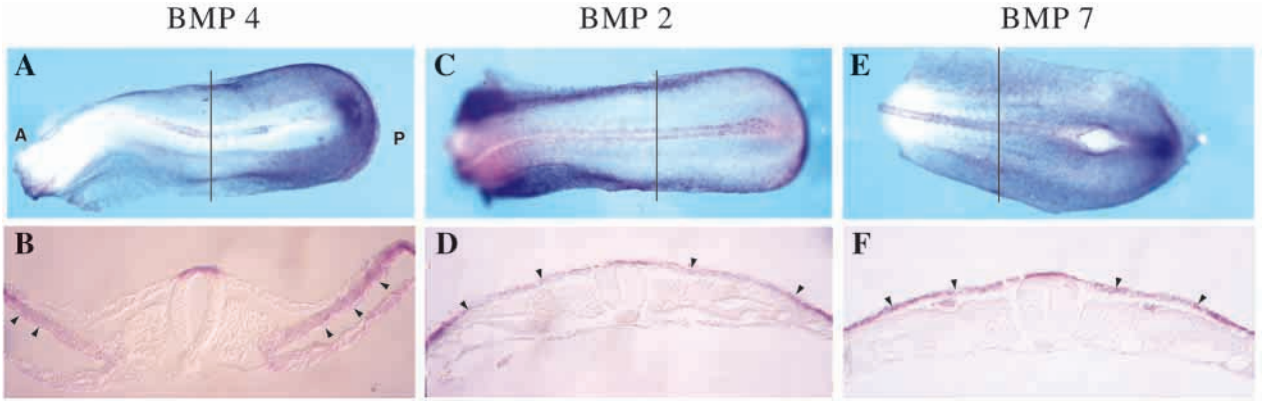


Fig. 7. Expression of *BMP*s in the lateral plate. Whole mount in situ hybridization of 19-somite stage embryos (A,C,E) and transverse sections at the levels indicated show that expression of *BMP-2* (D) and *BMP-7* (F) is localized in the ectoderm overlying the somites and the lateral plate regions (arrowheads). *BMP-4* (B) is detected in the lateral plate mesoderm (arrowheads).

shown) at the site of implantation, supporting the previous observations that these BMPs act in ovo to maintain the Smt-m characteristics.

We conclude that the ectodermal signals that play a role in maintaining the identity of Smt-m include BMP-2 and/or BMP-7, and also that specification of Smt-m from the primitive lateral plate requires interactions with the overlying ectoderm that are mediated by unidentified signals.

DISCUSSION

In this paper, we first emphasized that the lateral plate provides a most suitable model system for studying how complex structures of the vertebrate body are established from a primitive tissue because of its simple architecture composed of only two kinds of distinct cell layers. We investigated the molecular mechanisms of the coelomic cavity formation where

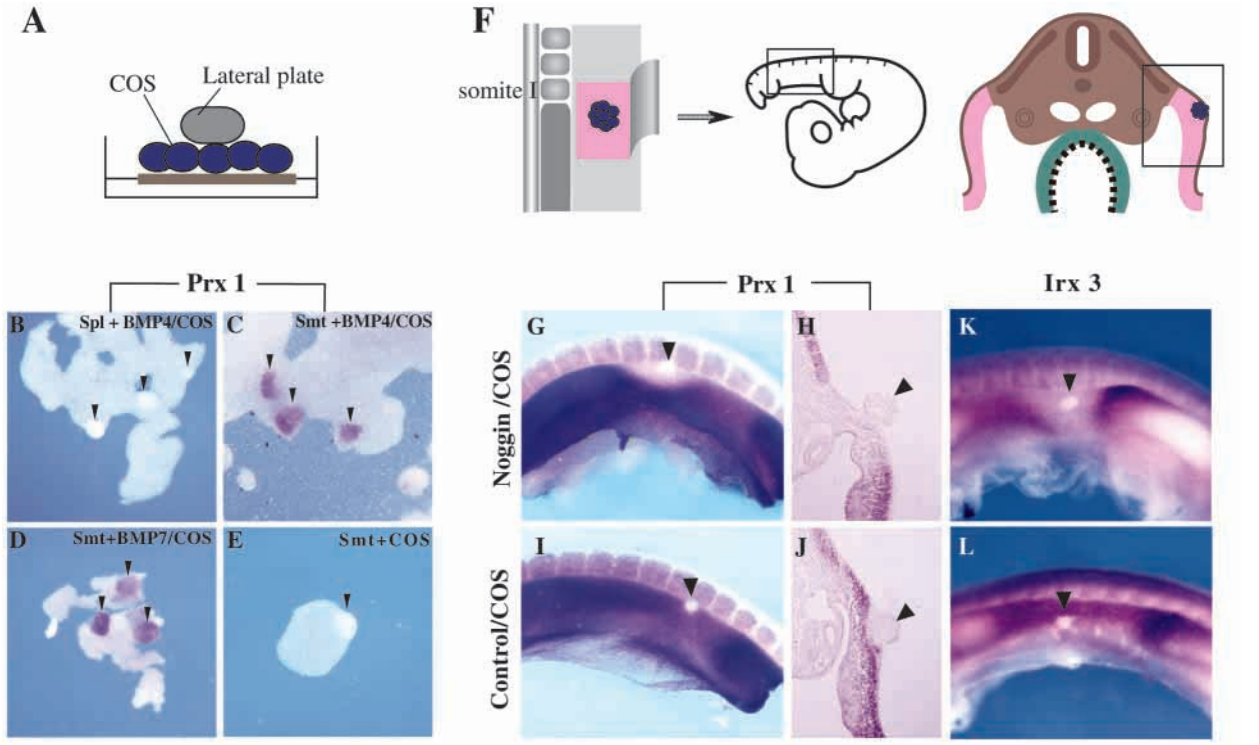


Fig. 8. Effects of BMPs on the lateral plate. (A) A piece of Smt-m or Spl-m was co-cultured with BMP-4- or BMP-7-transfected COS cells on a nuclepore filter. This combination did not induce *Prx 1* in Spl-m (B). In contrast, BMP-4/COS and BMP-7/COS cells maintained *Prx 1* expression in Smt-m (C,D). Non-transfected COS cells exerted no effect on Smt-m (E). (F-L) Noggin/COS cells locally down-regulated the expression of the Smt-m markers, *Prx 1* and *Irx 3*. An aggregate of Noggin-expressing COS cells was implanted between the lateral ectoderm and Smt-m. G-L correspond to the rectangles in F. Around the site of Noggin/COS expression of *Prx 1* and *Irx 3* was down-regulated (G,H,K). Control COS cells showed no effect (I,J,L). Arrowheads indicate position of COS cells.

Table 3. Effects of BMPs on Spl-m and Smt-m

Co-culture	Prx 1
spl* + control COS	0/6§
spl + BMP-4/COS ×1	0/29
spl + BMP-4/COS ×1/5¶	0/7
spl + BMP-4/COS ×1/10**	0/24
spl + BMP-7/COS	0/4
smt‡ + control COS	0/13
smt + BMP-4/COS ×1	42/42
smt + BMP-7/COS	15/15

*Spl-m.
‡Smt-m.
§Number of positive cultures/number of examined by WIH.
¶,**BMP-transfected COS cells (×1) were mixed with non-transfected cells at a ratio of 1:4¶ and 1:9**.

a binary decision is made during specification between the somatopleural and splanchnopleural components, which differentiate from the morphologically homogeneous cell population of the primitive lateral plate. We found that the somatopleural ectoderm plays an essential role in the formation of Smt-m and also that some of these interactions are mediated by BMP family molecules (Fig. 9).

Roles of the ectoderm in the coelomic cavity formation

During normal development the somatopleural ectoderm appears to control the underlying mesoderm in at least two ways: first it specifies Smt-m from the primitive lateral plate, and secondly it is also required for maintaining the characteristics of Smt-m after this component has been specified. We have observed that the latter effect of the ectoderm was substituted for by the action of BMPs whereas the former seems to be controlled by other molecules.

Specification of Smt-m

We have shown that young Spl-m, although morphologically distinct from Smt-m, is still competent to be respecified to Smt-m. This plasticity appears to last until the 23-somite stage as far as somites I-V are concerned, as revealed by in ovo transplantation, and in vitro tissue recombination experiments. We have also observed that the ectoderm is sufficient for this Smt-m to Spl-m conversion. Therefore, during normal embryogenesis the lateral ectoderm appears to be essential for Smt-m specification.

Maintenance of Smt-m

After Smt-m has been specified, this tissue component still requires the overlying ectoderm to be maintained as Smt-m. This has been demonstrated by insertion of a barrier between the ectoderm and Smt-m and also by tissue recombination cultures. The former experiment also suggests that the maintenance of Smt-m marker expression is regulated largely by soluble factors emanating from the ectoderm. In addition, these signals seem to act locally underneath the ectoderm, as it has been observed that in normal embryos signals for the Smt-m markers are more intense in the site juxtaposed to the ectoderm and are not detected in a region far from it, and also that an insertion of 0.1 μm nuclepore filter slightly decreased intensity of the marker signals. The possibility cannot be excluded that cell-cell contacts between the ectoderm and Smt-m are also required. It is conceivable that the endoderm which is tightly attached to Spl-m is important for the specification and formation of Spl-m. We showed, indeed, that down-regulation of Smt-m markers *(Prx 1, Irx 3*) is not necessarily concomitant with up-regulation of the Spl-m marker (*HFH 8*), suggesting that the splanchnic character is not a default state.

BMPs mediate ectodermal signals

We have found that BMPs are involved as a mediator of the ectodermal signals acting on Smt-m. BMP-2 and BMP-7 mRNAs are expressed in the lateral ectoderm whereas BMP-4 is localized in the mesoderm at the stages examined in this study. Smt-m co-cultured with the ectoderm was maintained as Smt-m and its ectodermal effects were substituted for by BMP-4- and BMP-7-producing cells. Since the biological activity of BMP-2 and BMP-4 are not separable, these observations suggest that during normal development BMP-2 and/or BMP-7 mediate(s) the signals emanating from the ectoderm. BMP-4 is expressed in Smt-m, so that one might argue that the intrinsic BMP-4 is acting for the self-specification. We propose, however, that external BMPs act directly on Smt-m because: (1) Smt-m that is positive for *BMP-4* mRNA cannot maintain itself when cultured alone; (2) when Smt-m displayed its characteristics in the recombination culture, the tissue itself did not express *BMP-4* mRNA (at least under the detection limit; data not shown). The roles of BMP-4 localized in the lateral plate mesoderm may, therefore, not be in the binary decision between Smt-m and Spl-m but, rather, that BMP-4 detected in Smt-m is a remaining signal after this molecule has played roles in dorsoventral patterning at earlier stages as previously reported (Pourquie et al., 1996; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998).

We were unable to convert Spl-m to Smt-m by implanting

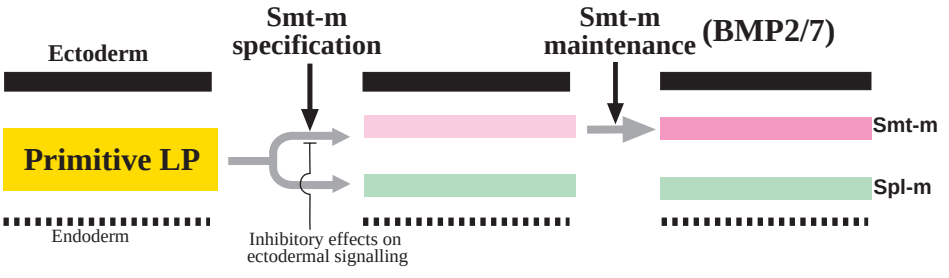


Fig. 9. Summary of the lateral plate subdivision. During normal embryogenesis when the primitive lateral plate generates Smt-m and Spl-m, the lateral ectoderm exerts signals for the Smt-m specification. The endoderm appears to act negatively on these interactions. After Smt-m has been morphologically established, this tissue requires the ectoderm to be maintained as Smt-m. These ectodermal signals are mediated by BMP-2 and/or BMP-7.

BMP-producing cells. Therefore, other signalling molecules mediate the ectodermal signals that specify Smt-m from the primitive lateral plate.

In this study we used three molecular markers for the lateral plate mesoderms, two of which we identified in this study. The amino acid sequences suggest that these genes encode DNA binding proteins. The roles of these genes in the coelom formation remain to be studied.

Summary and the coelomic cavity formation

The primitive lateral plate that consists of homogeneous mesenchymal cells is progressively subdivided and split into the two groups, somatic and splanchnic mesoderm. The splitting coincides with epithelialization of cell populations lining the ectoderm and endoderm, respectively. These two layers are ultimately detached from each other and the coelomic cavity between them expands. The cavity formation progresses in anteroposterior and mediolateral manner but does not coordinate with the A-P segmentation, suggesting that the coelomic cavity formation is independent of segmentation in avians. The specification of Smt-m is controlled by the somatopleural ectoderm by means of molecules yet to be identified. Even after the two sheets of cells are specified, their developmental fates are not committed. They keep receiving signals from the ectoderm and probably also from the endoderm, respectively, the former being mediated by BMP-molecules (Fig. 9). It should be determined what initiates the cavity formation that leads to the lateral plate splitting. It is worth noting that the solid embryonic ectoderm of mouse early embryos undergoes cavitation by tissue interactions between the ectoderm and its surrounding visceral (extraembryonic) endoderm (Coucouvanis and Martin, 1995, 1999).

Evolution of the coelomic cavity

The specification of the lateral plate is a requisite for the formation of the coelomic cavity. Recent knowledge that the basic mechanisms of body plan formation are highly conserved between species in vertebrates implies that similar interactions between the surface ectoderm and mesoderm are also required for the coelom formation in other vertebrate embryos. In amphioxus (cephalochordate) a coelom is already present in the paraxial mesoderm when it is segregated from the archenteron as an epithelial cell sheet, suggesting that the in-and-out segregation precedes the subdivision between the dorsal (somite) and ventral (lateral plate) mesoderms. In addition, the entire paraxial mesoderm undergoes segmentation, that is, the coelom is also segmented. Subsequently, each segmented coelom fuses to produce the antero-posteriorly extended cavity on either side of the body. Thus, new mechanisms of early mesodermal subdivision seem to have been acquired during chordate-to-vertebrate evolution. The coelom is found not only in deuterostome but also in protostome animals. The most primitive form of triploblastic animals such as planarians possess a loose mesenchymal structure that simply fills the space between the ectoderm and endoderm. It is interesting to know to what extent the protostome animals share the mechanisms of coelom formation with vertebrates.

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