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# Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4

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## SUMMARY

**Molecular mechanisms by which the mesoderm is subdivided along the mediolateral axis in early chicken embryos have been studied. When the presomitic mesoderm (medial mesoderm) was transplanted into the lateral plate, the graft was transformed into lateral plate tissue, indicating that the primitive somite was not fully committed and that the lateral plate has a cue for mesodermal lateralization. Since the lateral plate expresses a high level of *BMP-4* mRNA, a member of the TGF-β family, we hypothesized that it is the molecule responsible for the lateralization of the somite. To test this, we transplanted COS cells producing BMP-4 into the presomitic region. Those cells locally prevented the presomitic cells from differentiating into somites, converting them instead into lateral plate mesoderm, which was revealed by expression of *cytokeratin* mRNA, a marker for the lateral plate. The effect was dependent on the level of effective BMP-4: with a high level of BMP-4, the somite was transformed completely to lateral plate; with a low level, the somite formed but was occupied by the lateral somitic component expressing *cSim 1*, a marker for the lateral somite. These results suggest that different thresholds of effective BMP-4 determine distinct subtypes of the mesoderm as a lateralizer during early development.**

Key words: mediolateral axis, mesoderm subdivision, commitment, BMP-4, pattern formation, chick

## INTRODUCTION

During early embryogenesis in vertebrates, one of the most dynamic events is gastrulation, which leads to the initial formation of the mesoderm (see review, Kimelman et al., 1992, and references therein). In chicken embryos, the mesoderm is derived from the cells that invaginate through the primitive streak, located along the midline. Subsequently, except for the axial-structure-forming cells, the mesodermal cells migrate laterally and anteriorly, and eventually those that are located medially differentiate into somitic mesoderm, and those located more laterally contribute to the lateral plate mesoderm (Selleck and Stern, 1991; Schoenwolf et al., 1992). Thus, as diagrammatically shown in Fig. 1, the mesoderm is subdivided in a stereotypic manner along the mediolateral (M-L) axis. This M-L axis is also considered to be a dorsoventral (D-V) axis since a flat chicken embryo is sequentially folded into a three-dimensional structure so that the medial-most structure will be located in the dorsal-most region and the lateral-most one will be in the ventral-most area. It is important to unveil the mechanisms by which this spatially determined subdivision of the mesoderm along the M-L axis is established.

The present experiments were designed to see how the subsets of the mesoderm are determined by taking advantage of the flat structure of a chicken embryo, which facilitates embryological manipulations at the molecular level. When we transplanted presomitic mesoderm into the lateral plate, the graft showed characteristics of the lateral plate. This indicates that the presomite is not yet committed and also that the lateral plate has a cue for lateralization. We hypothesized that BMP-4, a member of the TGFβ-family, which is normally expressed in the lateral plate (Roberts et al., 1995; Pourquie et al., 1996; Takahashi et al., 1996; Watanabe and LeDouarin, 1996), is a molecule responsible for this lateralization. It is known that BMP-4 is involved in the formation of the ventral mesoderm during gastrulation in *Xenopus* embryos (Jones et al., 1992; Fainsod et al., 1994; Harland, 1994; Schmidt et al., 1995). We investigated the effects of ectopic administration of BMP-4 on the presomitic mesoderm in chicken and observed that BMP-4 lateralized the presomitic mesoderm in a concentration-dependent manner. From these results, we propose that BMP-4 acts as a lateralizer during the mesodermal subdivision along the M-L axis.

## MATERIALS AND METHODS

### Microsurgery

Fertilized White Leghorn chick and Japanese quail eggs from commercial sources were used throughout this study. Microsurgery was performed on embryos at stage 14 (about 21-somite) according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) (about 50 hours of incubation in a humidified atmosphere at 38.5°C). A window was made in the eggshell and India ink diluted 1:5 in Hanks solution (140 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 0.34 mM

$Na_2HPO_4$, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$ pH 7) was injected into the sub-blastodermic cavity to make the embryonic structures more visible. A slit was made in the ectoderm overlying the segmental plate with a sharpened tungsten needle. An aggregate of COS 7 cells was then transplanted onto the anterior-most region of the presomitic region. In most cases, the operated embryos were fixed either at stage 18 (E3.0) or stage 22-23 (E3.5 to E4.0). Embryos were then treated for in situ hybridization (see below).

Quail-chick chimeras (Le Douarin, 1969, 1982) were made as follows. A fragment of the somite or somatopleure was isolated from quail embryos in 1.25% pancreatin (Gibco/BRL) in phosphate-buffered saline (PBS, $Ca^{2+}$, $Mg^{2+}$ free) for 5 minutes at room temperature followed by the addition of fetal bovine serum to stop the reaction. The fragment was then transplanted into chicken host embryos of an equivalent developmental stage. Three types of transplantation were performed in ovo. (1) The anterior-most region of the quail segmental plate was transplanted into the somatopleural region of a chicken embryo at the same level along the anteroposterior (A-P) axis. One day after the operation, the embryos were fixed with 4% paraformaldehyde (PFA) in PBS for in situ hybridization. The specimens were then refixed with Carnoy's fixative for Feulgen staining (Feulgen and Rossenbeck, 1924) to identify the quail cells among the chick cells in the chimera. Although the Feulgen staining following in situ hybridization decreases the clarity of the quail nuclear marker, the grafted quail cell population was still obvious (for example, see Fig. 1A-D). (2) A quail fragment of a somite of somite-stage I was transplanted between the somite (somite-stage I) and the overlying ectoderm of a chicken embryo. (3) The medial-most fragment of the somatopleural mesoderm of a chicken embryo was replaced by the equivalent portion of a quail tissue. In cases (2) and (3), the specimens were directly fixed with Carnoy's fixative 2 days after the operation and stained by the Feulgen method. For histological sectioning, the fixed embryos were dehydrated in ethanol and xylene and embedded in paraffin, followed by serial 7 μm sectioning.

### COS 7 cell transfection

COS 7 cells were grown until 70% confluent and transfected with 1 μg of pCDM8 (In Vitrogen)-derived vector, which contains the elongation factor promoter and the mouse full-length cDNA of *BMP-4* or the *lacZ* gene along with 6 μl of lipofectamine (GIBCO BRL) in a 35 mm culture dish for 5 hours according to the manufacturer's instructions. Transfected cells were cultured for 24 hours and then transferred to a dish coated with 1% agar to obtain cell aggregates. A cell aggregate of approximately 100 μm diameter was used for transplantation. For dilution of BMP-4/COS, the transfected COS cells were trypsinized and dissociated into single cells and mixed at a ratio of cell number, 1:1, 1:4, 1:9 and 1:24, respectively, with the cognate COS cells that had also been dissociated. Thus, a relative dose of BMP-4 secreted by a COS cell aggregate reflects each dilution level. At least four to twelve specimens were obtained at each dilution level.

### Probes

cDNA fragments for RNA probe preparation were as follows: (1) *Pax 3*, a 330 bp fragment of the chicken *Pax 3* cDNA subcloned in pBluescript $SK^+$(Stratagene) (given by Dr Kuroiwa, Nagoya); (2) *MyoD*, an *Eco*RI-*Eco*RI fragment of 1500 bp chicken *MyoD* (CMD-1) subcloned in SKII (–) (gift from Dr Fujisawa, Tokyo); (3) *Hox b-6*, an *Eco*RI-*Eco*RI fragment of 1100 bp of chicken *Hox b-6* subcloned in bluescript SK (given by Dr Kuroiwa, Nagoya); (4) a fragment of the chicken *cytokeratin* cDNA was obtained by RT-PCR according to the published sequences (Charlebois et al., 1990b); (5) *Msx 2*, an *Eco*RI-*Eco*RI fragment of 602 bp containing the 5′-half region of the homeobox, and *Bam*HI-*Eco*RI fragment of 795 bp at the 3′-most region of the non-coding region of the quail *Msx 2* gene (Takahashi and Le Douarin, 1990), were subcloned into pBluescript $KS^+$, respectively. The two cRNAs for *Msx 2* were mixed in the hybridization buffer; (6) Pax 1: an *Eco*RI-*Eco*RI fragment of 1526 bp chicken Pax 1 subcloned into KS II (Ebensperger et al., 1995).

Degenerate oligonucleotide primers directed to the sequences MKEKS and FDGCYQN from the sequence of the *mSim1* containing bHLH domain (Fan and Tessier-Lavigne, 1994; Pourquie et al., 1996) were used to isolate a 618 bp fragment of the chick *Sim1* gene by RT-PCR using mRNA derived from E2 chick embryos as a template. Digoxigenin-labeled RNA probes were prepared according to the instructions of Boehringer Mannheim. Both antisense and sense probes were prepared and specific signals with antisense probe were confirmed for all of the probes.

### Whole-mount in situ hybridization

Embryos were fixed for 2 hours in 4% paraformaldehyde in PBS, washed twice in PBT (PBS, 0.1% Tween-20) and then dehydrated by passing them successively through 25%, 50%, 75% and 100% methanol in PBT. The specimens were bleached in 6% hydrogen peroxide in methanol for 2 hours, followed by successive rehydration and washing twice in PBT. The specimens were treated with proteinase K (20 μg/ml) for 20 minutes for embryos at embryonic day 4 (E4), and 15 minutes for E2. They were refixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBS for 20 minutes at room temperature. After two PBT washes, the embryos were transferred to hybridization buffer (50% formamide, 5× SSC pH 4.5, 1% SDS, 50 μg/ml tRNA, 50 μg/ml heparin) and prehybridized for 1 hour at 70°C. Hybridization was carried out overnight at 70°C in the hybridization buffer containing a digoxigenin-labelled RNA probe. The embryos were washed three times for 30 minutes each time in 50% formamide, 5× SSC pH 4.5, 1% SDS at 70°C, then after a quick wash in RNase buffer (0.5 M NaCl, 10 mM Tris pH 7.5, 0.1% Tween-20), they were treated with RNase A (100 μg/ml) for 30 minutes at 37°C, followed by a quick wash in RNase buffer. The specimen were next washed three times for 30 minutes each in 50% formamide, 2× SSC pH 4.5 at 65°C, followed by washing in TBST (8 mg/ml NaCl, 0.3 mg/ml KCl, 2.5 mM Tris pH 7.6, 0.1% Tween-20). The embryos were preblocked in 10% fetal calf serum in TBST followed by incubation overnight in blocking solution containing alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) that had been preabsorbed with chicken embryo powder. After the embryos were washed extensively in TBST for at least 5 hours, they were processed to NTMT (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20). The alkaline phosphatase activity was visualized by incubating embryos in NTMT containing 0.45 mg/ml nitroblue-tetrazolium chloride (Boehringer-Mannheim) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphatase (Boehringer-Mannheim). After stopping the color reaction, embryos were post-fixed in 0.1% glutaraldehyde/4% paraformaldehyde in PBS for 30 minutes at 4°C to be processed for paraffin embedding.

### Histological sections

Hybridized embryos were embedded in paraffin wax after dehydration with ethanol and xylene. Serial sections of 15-25 μm thickness were prepared and mounted in Entellan (Merck).

### Skeletal preparations in toto

Embryos at the 9-day embryonic stage from which visceral organs and eyeballs had been removed were fixed in 80% ethanol, 20% acetic acid and 0.015% Alcian blue. After dehydration in 100% ethanol for 5 days, they were stained with 0.01% Alizarine red in 0.5% potassium hydroxide for 2 hours. Staining was stopped in 1% glycerol, 20% potassium hydroxide for 4 hours and cleared in 50 % glycerol.

## RESULTS

### Lateralization of the somitic mesoderm transplanted into the lateral region

Whether or not the differentiation of the presomitic mesoderm is irreversibly committed along the M-L (future D-V) axis when it becomes morphologically distinct from the lateral plate was

determined by transplanting a piece of a quail presomite taken from a 2-day embryo (E2) into the lateral plate region of a chicken host embryo (schematically shown in Fig. 1, see Materials and Methods for details). In this experiment, we examined expression of *cytokeratin* mRNA as an early marker for the lateral plate (see Charlebois et al., 1990a,b for expression at earlier stages, and see Fig. 1A for E3), and *Pax 3* expression for presomitic and somitic tissues (Williams and Ordahl, 1994). At present, *cytokeratin* expression is the only molecular marker that exclusively distinguishes the lateral plate from the presomitic region, because other molecular markers, such as expression of *BMP-4*, *Msx 2*, *Hoxb-6* and *Prx 1*, which were used as shown later, were supportive but not conclusive for identification of the lateral plate. We also localized grafted cells by the Feulgen method, which identifies the quail nuclear marker (Le Douarin, 1969, 1982). The transplanted cells ceased to express *Pax 3* (Fig. 1C,D; *n*=3), and instead turned on expression of *cytokeratin* mRNA (Fig. 1A,B; *n*=15). When old somitic mesoderm of somite-stage X (tenth somite anterior from the most recently formed somite; Christ and Ordahl, 1995) was transplanted into the same site, the graft continued to express *Pax 3* (Fig.1 F-H; *n*=3) and did not turn on *cytokeratin* expression (Fig. 1E; *n*=5). We conclude, therefore, that the presomitic mesoderm is not firmly committed along the M-L axis and also that cue signals reside in the lateral region to confer the lateral mesodermal characteristics.

### BMP-4 affects differentiation of the somitic mesoderm

It has recently been shown that the *BMP-4* mRNA is expressed in the lateral plate region (Roberts et al., 1995; Pourquie et al., 1996; Takahashi et al., 1996; Watanabe and LeDouarin, 1996; and also see Fig. 2A,B). Briefly summarized, expression of *BMP-4* mRNA is obvious in the presumptive lateral plate mesoderm by stage 10 (=10-somite) and the expression level becomes high by stage 13 in the lateral plate, particularly at the presomite level along the A-P axis. We, therefore, asked whether BMP-4 in the lateral plate has a role in specification of the mesoderm along the M-L axis. We transplanted COS cells transfected with *BMP-4* cDNA, which are known to exert specific BMP-4 activity (Takahashi et al., 1996), into the anterior-most region of the presomitic mesoderm of an E2 embryo (left diagram in Fig. 2 and also Materials and Methods for details). The molecular markers that we used to assess the

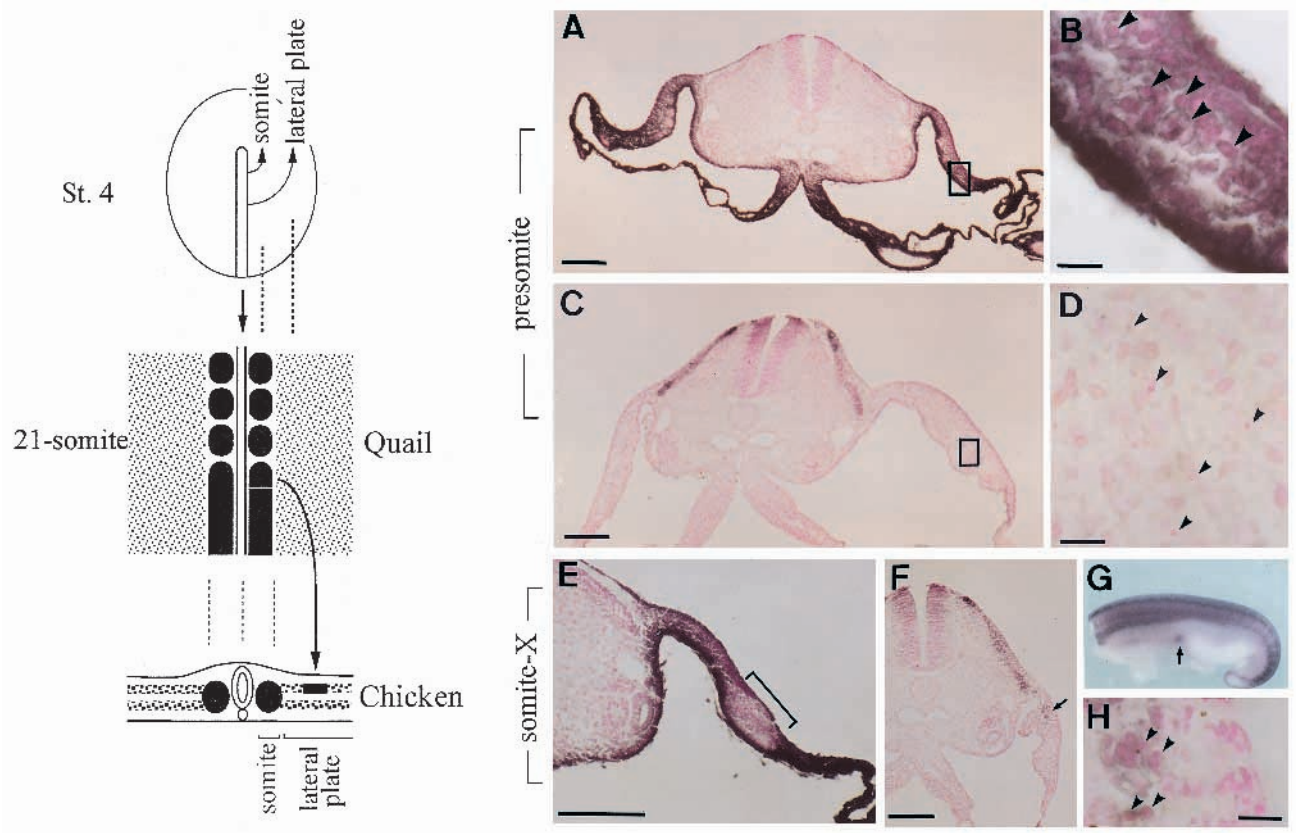


**Fig. 1.** Lateralization of the presomitic mesoderm when transplanted into the lateral plate region. Left panel: movement of the mesoderm from the primitive streak at stage 4 (gastrulation stage) and the transplantation experiment in which the quail presomite is grafted into the chicken lateral plate mesoderm are schematically depicted. (A-H) Embryos were assayed 24 hours after the graft. (A-D) Transplantation of the presomite to the lateral plate. (E-H) Control experiment in which stage X-somite was transplanted. (A) An operated embryo was hybridized in situ for detection of *cytokeratin* mRNA and Feulgen-stained to distinguish the grafted quail cells, which are shown in a rectangle. (B) A higher magnification of the rectangle in A demonstrates the presence of the quail cells (arrowheads), which started to express *cytokeratin* mRNA. (C,D) *Pax 3* expression. Grafted quail cells (arrowheads in D) stopped *Pax 3* expression. (E) *Cytokeratin* expression. The grafted old somite (bracket) remained negative for the ventral marker when transplanted into the lateral plate. (F-H) An operated embryo hybridized for *Pax 3* in situ (G) was sectioned and Feulgen-stained (F,H). An arrow indicates the transplanted somite, which retains the dorsal marker. Arrowheads in H are the quail cells shown in F by the arrow. Bar: A,C,E,F, 100 μm; B,D,H, 10 μm.

effect of BMP-4/COS were expression of *Pax 3* mRNA for the presomite and dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994), *MyoD* mRNA for the myotome (Weintraub et al., 1991; Pownall and Emerson, 1992) and *Pax 1* mRNA for the sclerotome (Deutsch et al., 1988) (Fig. 2, right diagram).

When examined at E4 (2 days after the transplantation), a region where the somite would be normally formed was largely smaller with BMP-4/COS than that in the control side (Fig. 2F,H). In such a region affected by BMP-4, the formation of the somite was locally perturbed. The transplantation abolished the expression of *Pax 3* (Fig. 2C,F, *n*=32) and *Myo D* (Fig. 2D, *n*=6), indicating that the dermomyotomal cells were absent. The signal of *Pax 1* mRNA was also significantly reduced in the cells deep around the notochord (Fig. 2H; *n*=6). COS cells transfected in the absence of DNA or with the *β-gal* gene showed no such activities (Fig. 2E for β-gal/COS).

Using *Pax-3* expression, the effect of BMP-4/COS was detected within 3 hours of the operation, when the implanted somite had developed to somite stage III. In the normal stage III somite, an epithelial dermomyotome has developed and expresses *Pax 3*. At this stage of a BMP-4/COS-implanted somite, however, neither the epithelial structure nor the *Pax 3* signal was detected. (Fig. 2G, *n*=8). β-gal/COS did not affect the dermomyotomal differentiation (*n*=16, data not shown). Thus, when BMP-4/COS was transplanted into the presomitic region, response of the mesoderm to BMP-4 was clearly

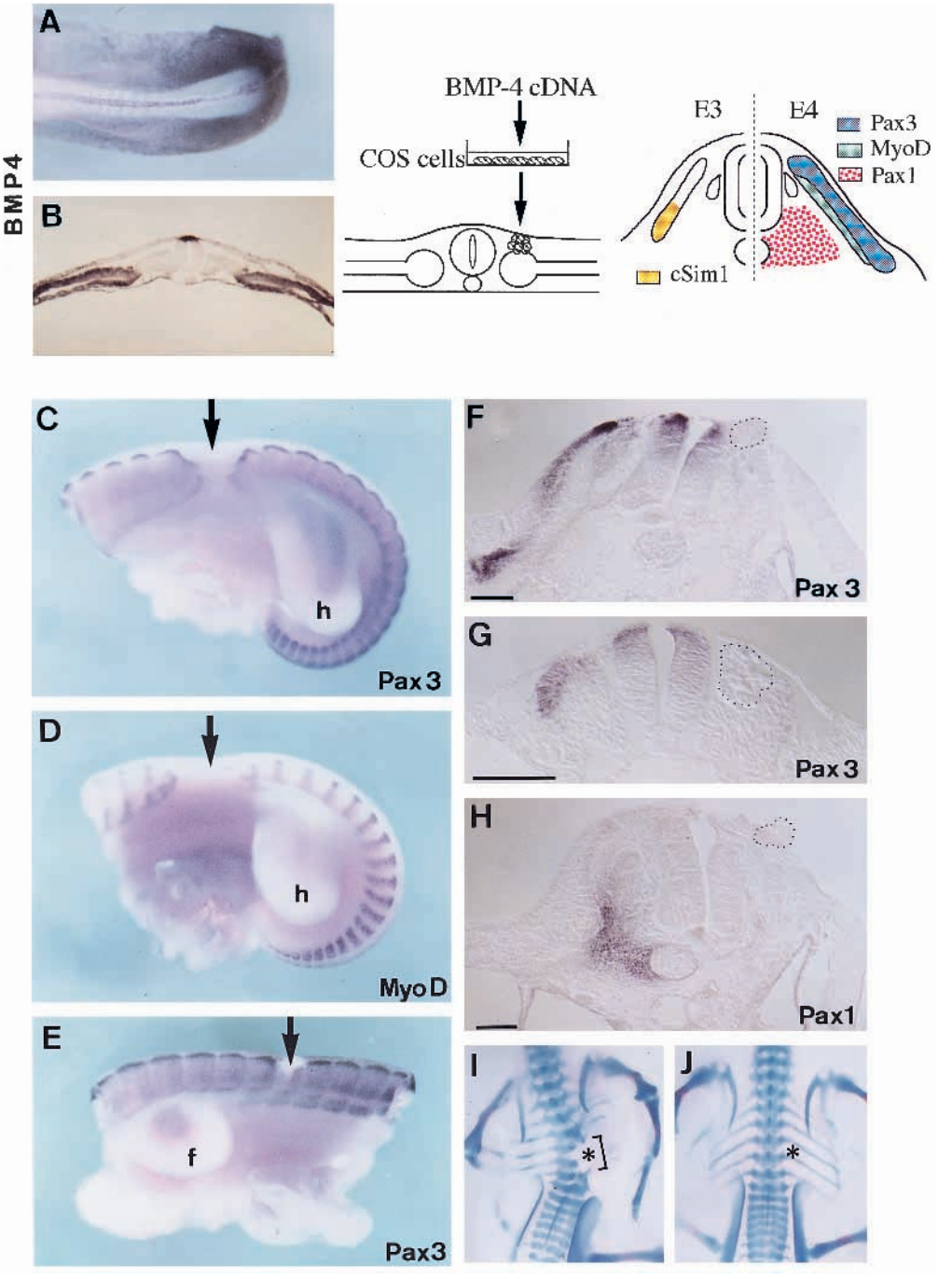


**Fig. 2.** BMP-4/COS affects differentiation of the somitic mesoderm. The left diagram shows a strategy for the transplantation of the BMP-4/COS. *BMP-4* cDNA subcloned in a COS-expression vector was transfected into the COS cells and an aggregate of these cells was then transplanted in between the presomite and skin at E2. The right diagram depicts summarized expression patterns of the marker genes used in this study. (A,B) *BMP-4* expression in a normal embryo at stage 14. *BMP-4* is expressed in the lateral plate and also along the dorsal midline. Anterior is to the left in A. (C-E). Whole-mount in situ hybridization of an E4-embryo that had received BMP-4/COS (C,D) and β-gal/COS (E), showing expression of *Pax 3* and *MyoD* mRNAs as markers for the dermomyotome. Anterior is to the left. Expression of *Pax3* mRNA (C) and *MyoD* mRNA (D) was lost in cells surrounding the site of the BMP4 /COS transplantation (arrow). (E) Expression of *Pax 3* in an embryo grafted with β-gal/COS as a control. (F-H) Transverse sections after whole-mount in situ hybridization. Grafted BMP-4/COS is shown in the dotted circle. (F) A section at the graft level in C. (G) *Pax 3* expression 3 hours after the graft. Whereas the non-operated side (left) showed morphological differentiation of the dermomyotome and expressed *Pax3*, the experimental side lacked both features. (H) *Pax 1* expression disappeared in the grafted side of an E4-embryo. (I,J) Skeletal preparation in toto at E9. The rib cartilages were not formed over two segments at the grafted site (asterisks) with BMP-4/COS (I), whereas β-gal/COS did not affect the formation (J). f, forelimb bud; h, hindlimb bud. Bar, 100 μm.

demonstrated. When transplanted into the somite of stage V or later, in contrast, expression of *Pax 3* was reduced to a lesser extent, and transplantation into the somite X did not result in any notable effect ($n$=6 and $n$=5, respectively, data not shown).

We also observed the loss of formation of the dorsal root ganglion in the experimental side (Fig. 2F,H). The central nervous system was not appreciably affected. Grafting BMP-4/COS into the intermediate mesoderm or the lateral plate region resulted in no gross effect on the morphology (data not shown).

The consequence of BMP-4/COS on somite-derived morphogenesis at later stages was further investigated by examining skeletal formation at E9.0 in toto. No rib cartilage was formed at the BMP-4/COS-grafted site over a two-somite length ($n$=9, Fig. 2I). The vertebral cartilage was also severely disrupted at the operated site. Such an effect was not observed for β-gal/COS ($n$=7, Fig. 2J).

BMP-2-expressing COS cells had the same effects as BMP-4/COS, when examined with *Pax 3* ($n$=4, data not shown). In contrast, COS cells transfected with *BMP-7* cDNA (Nishimatsu et al., 1992), cells secreting lefty, a BMP family member (Meno et al., 1996) and cells producing Wnt 1 or Wnt 3a (Parr et al., 1993) showed no such activities in our *Pax 3* experimental system, although these cells have been shown to exhibit specific activity of this molecule in a separate assay (Nishimatsu et al., 1992; Parkin et al., 1993; Meno et al., 1996; Kitajewski, personal communication). The effect on expression of *Pax 3* and *Myo D* is therefore elicited specifically by BMP-4 and BMP-2.

### Cell labelling analysis shows that BMP-4/COS converted the presomitic mesoderm to another cell type

Loss of the markers for the somitic mesoderm, particularly the dermomyotome, caused by the BMP-4/COS transplantation raised two possibilities: (1) the cells that would normally contribute to the dermomyotome died in response to BMP-4/COS, or (2) these cells survived but were transformed to another cell type. To distinguish between these possibilities, we transplanted a quail presomite in between the skin ectoderm and somite of a chicken host embryo in order to label the dermomyotomal cells (Aoyama, 1993). When quail tissue was transplanted together with β-gal/COS as a control, all the quail cells were found in the epithelial structure of the dermomyotome (Fig. 3A-C,G). When the quail cells were transplanted together with BMP-4/COS (Fig. 3D), in contrast, the quail-derived cells were found as an unorganized mesenchymal cell population underneath the skin near the original transplantation site (Fig. 3E-G). Thus, a substantial fraction of the cells that were exposed to BMP-4 survived and were located at the place where they would normally reside, but they never formed the epithelial structure of the dermomyotome.

We conclude, therefore, that BMP-4 transformed the cells that would otherwise differentiate into the dermomyotome to another cell type.

### The somitic mesoderm was converted to the lateral plate by BMP-4/COS

To investigate the action of BMP-4 on somitic mesoderm, in addition to *cytokeratin* expression, an exclusive marker for the lateral plate, mRNA expressions of *Msx 2*, *Hoxb-6* and *BMP-4* were used as supportive markers for the lateral plate. *Msx 2*, *BMP-4* and *Hoxb-6* mRNAs are normally expressed in the lateral plate and along the dorsal midline both in the ectodermal and mesodermal components (Takahashi et al., 1992, 1996; and this study).

As shown in Fig. 4A,B, BMP-4/COS elicited ectopic

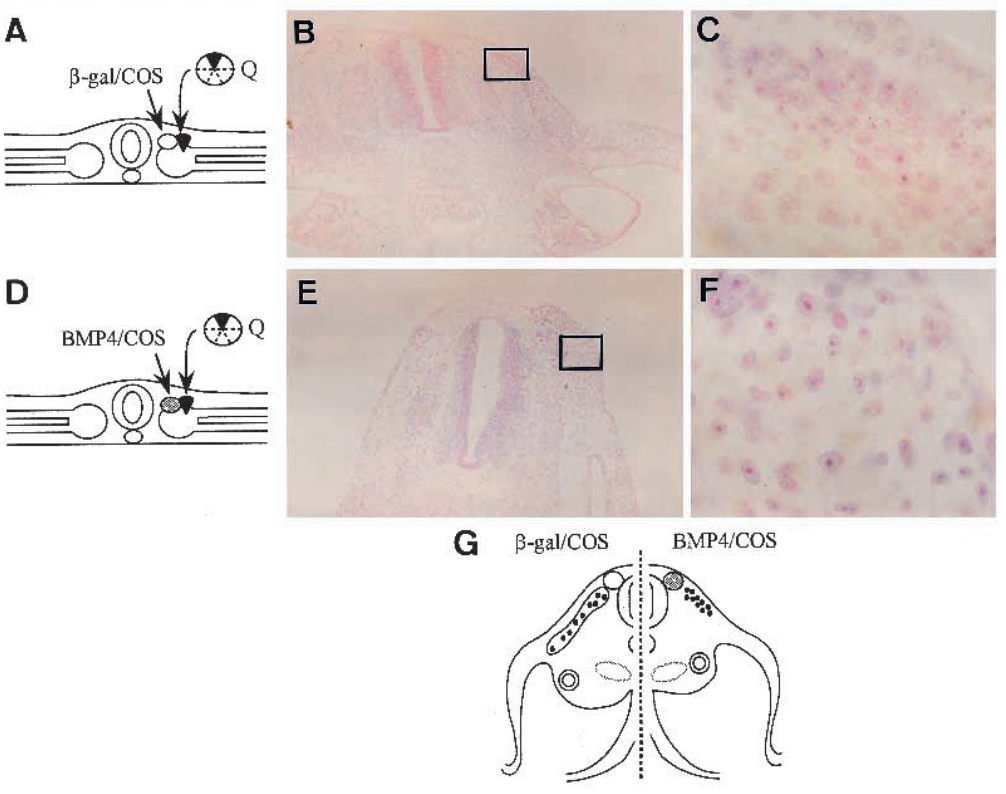


**Fig. 3.** The prospective dermomyotome was labelled by the quail-chick chimera technique. (B,C,E,F) The Feulgen staining at E4. Quail cells were identified at a higher magnification (C,F). (A-C) A control experiment. When a fragment of a quail stage I-somite was transplanted between the somite and skin together with β-gal/COS, the quail cells were distributed solely in the dermomyotome. (D-F) A similar transplantation together with BMP-4/COS. Grafted quail cells were detected in the vicinity of the transplanted BMP-4/COS, but they did not constitute an epithelial structure of a dermomyotome and remained as an unorganized mesenchyme. (G) A summary of the cell-labelling experiment. The somite-derived quail cells (black dots) were found exclusively in the dermomyotome in the control side. In the experimental side, BMP-4/COS inhibited the formation of the dermomyotome, but grafted quail cells populated the remaining mesenchyme.

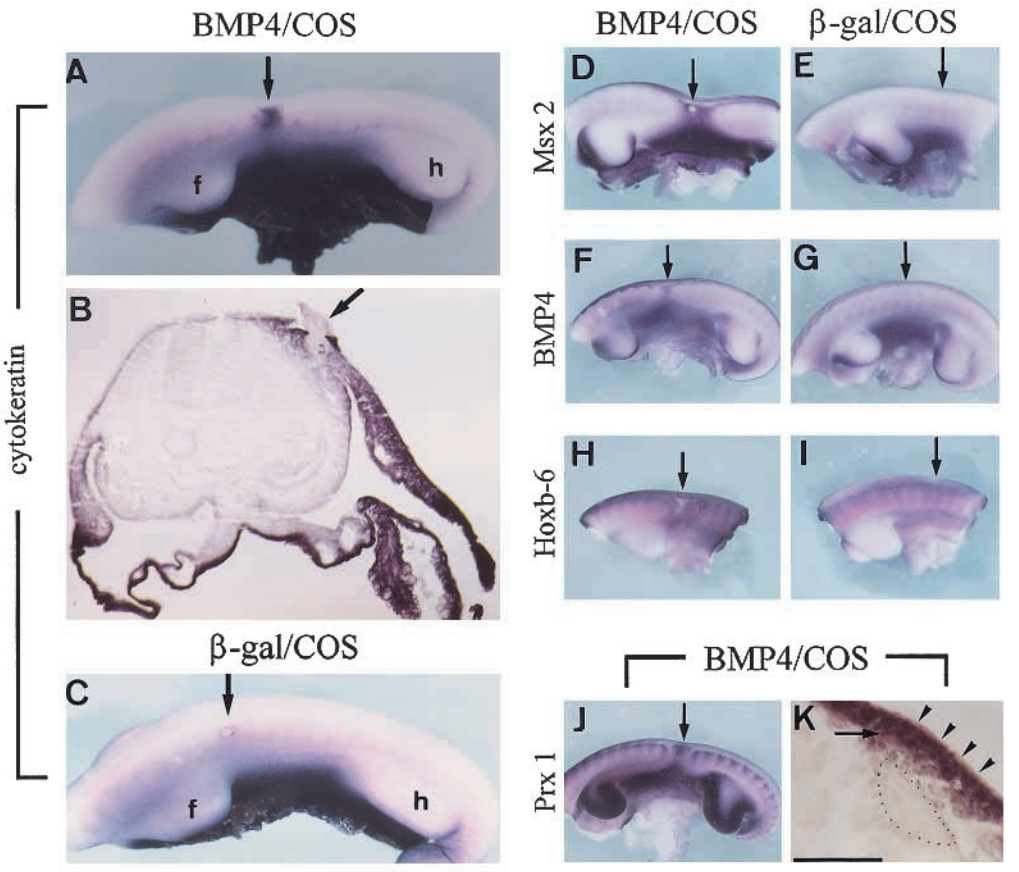


**Fig. 4.** BMP-4/COS converted the somite to the lateral plate mesoderm analyzed at E4. Probes: *cytokeratin* (A-C), *Msx 2* (D,E), *BMP-4* (F,G) and *Hox b-6* (H,I). Anterior is to the left for the whole-mount specimens. The arrow indicates the site of transplantation. (A,B,D,F,H,J,K) BMP-4/COS transplantation. (C,E,G,I) β-gal/COS transplantation did not affect the somite development. (A,B) BMP-4/COS induced *cytokeratin* expression in the cells surrounding the graft and the induced signal continued to the lateral plate. (D-I) BMP-4/COS also induced expression of *Msx 2* (D) *BMP-4* (F) and *Hoxb-6* (H) mRNAs, and these signals merged to those of the dorsal midline. (J,K) *Prx 1* expression upon BMP-4/ COS graft. A transverse histological section at the graft site after whole-mount in situ hybridization (J) is shown in K. Arrowheads show the epidermis, which is devoid of *Prx 1* expression. The arrow indicates induced expression of *Prx 1* by BMP-4 in the mesenchymal cells around the BMP-4/COS (dotted line), demonstrating that the *Prx 1*-positive cells are mesodermal. Bar is 50 μm f, forelimb bud; h, hindlimb bud.

expression of *cytokeratin* mRNA at the site of the graft ($n$=12). Observations of histological sections showed that the ectopic *cytokeratin* signal was localized in the superficial region including the mesenchyme and skin surrounding the BMP-4/COS (Fig. 4B). Induced expression of *Msx 2* ($n$=15, Fig. 4D), *BMP-4* ($n$=4, Fig. 4F) and *Hoxb-6* ($n$=5, Fig. 4H) was also detected in the operated side. These patterns for *Msx 2*, *BMP-4* and *Hoxb-6* were almost complementary to that of *Pax 3* as shown earlier (Fig. 2C). β-gal/COS transplantation did not induce expression of any of these markers (Fig. 4C,E,G,I, $n$>6 for each marker).

The induced expression of these markers was not due to the lateral plate cells having invaded the somitic area. This was shown by the fact that, when a piece of the lateral plate mesoderm was labeled by the quail-chick chimera technique together with the BMP-4/COS implantation into the presomite, the labeled cells stayed in the lateral plate where they would normally reside and did not migrate into the somite region ($n$=6, data not shown). The formation of the mesonephric tissue derived from the intermediate mesoderm located between the somite and the lateral plate mesoderm appears not to be grossly affected (Fig. 4B).

BMP-4 is known to induce the epidermis differentiation around the gastrulation stage, examined by the animal cap assay in early *Xenopus* embryo (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Although the stage of the somitic lineage focused in this study is much later than that studied in *Xenopus* embryos, we needed to exclude the possibility that the converted presomite by the effect of BMP-4 was the epidemis. To do this, we examined expression of the *Prx 1* gene, which is normally positive exclusively in the mesoderm of the lateral plate and dermomyotome (Nohno et al., 1993). As shown in Fig. 4J,K ($n$=6), the presomite-derived tissues affected by BMP-4 were clearly positive for *Prx 1*, indicating that the affected tissue was not the epidermis but the mesodermal mesenchyme.

Taking the patterns of all these markers together, we conclude that at least a part of the somite was transformed to lateral plate mesoderm by BMP-4.

### Concentration-dependent effect of BMP-4/COS on somitic mesoderm

It has recently been reported by Pourquie et al. (1996) that transplantation of the cells that produce retrovirus carrying the *BMP-4* gene converted the medial portion of the somite to the lateral somite rather than to the lateral plate. Expression of *cSim 1*, which is normally restricted to the lateral portion of the somite, was used in their analysis. We speculated that the apparent discrepancy in the resulting lateralization observed in this study and theirs reflects the difference in the effective concentration of BMP-4. To test this, we mixed BMP-4-producing COS cells with normal COS cells to give a dilution to 1/2, 1/5, 1/10 and 1/25 (see Materials and Methods) and examined their effects on expression of three markers, *Pax 3*, *cSim 1* and *cytokeratin* mRNAs over a range of dilutions. More than four embryos were assessed for each marker for every dilution level of BMP-4/COS.

The fraction of the dermomyotome expressing *Pax 3* as measured along the length of the mediolateral direction diminished as the concentration of BMP-4 increased (Fig. 5A-C). A

reduced size of the dermomyotome revealed by *Pax 3* expression was obvious at the dilution level 1/10, and the dermomyotome was almost lost at the level of 1/2 (Fig. 5A,C).

For induction of *cytokeratin* expression (index: *cytokeratin*-positive area induced ectopically/ normal somitic area along the M-L line, see Fig. 5B), BMP-4 was not effective at the level 1/10. At 1/5, the index was significantly higher than the control and it was almost 1.0 at the non-diluted level (the entire mesoderm along the M-L axis was converted to express *cytokeratin* mRNA; Figs 5A,C and 4B). The *cytokeratin*-positive area was observed in an area where the *Pax 3* signal was abolished. Thus, at the threshold level of 1/5, the characteristics of the lateral plate were induced. Levels higher than this threshold affected a larger area of the presomite converting it to lateral plate.

At the dilution level of 1/25, the occupation index for *cSim 1* (fraction of the *cSim 1*-positive dermomyotome/ remaining dermomyotome) was substantially increased from the control

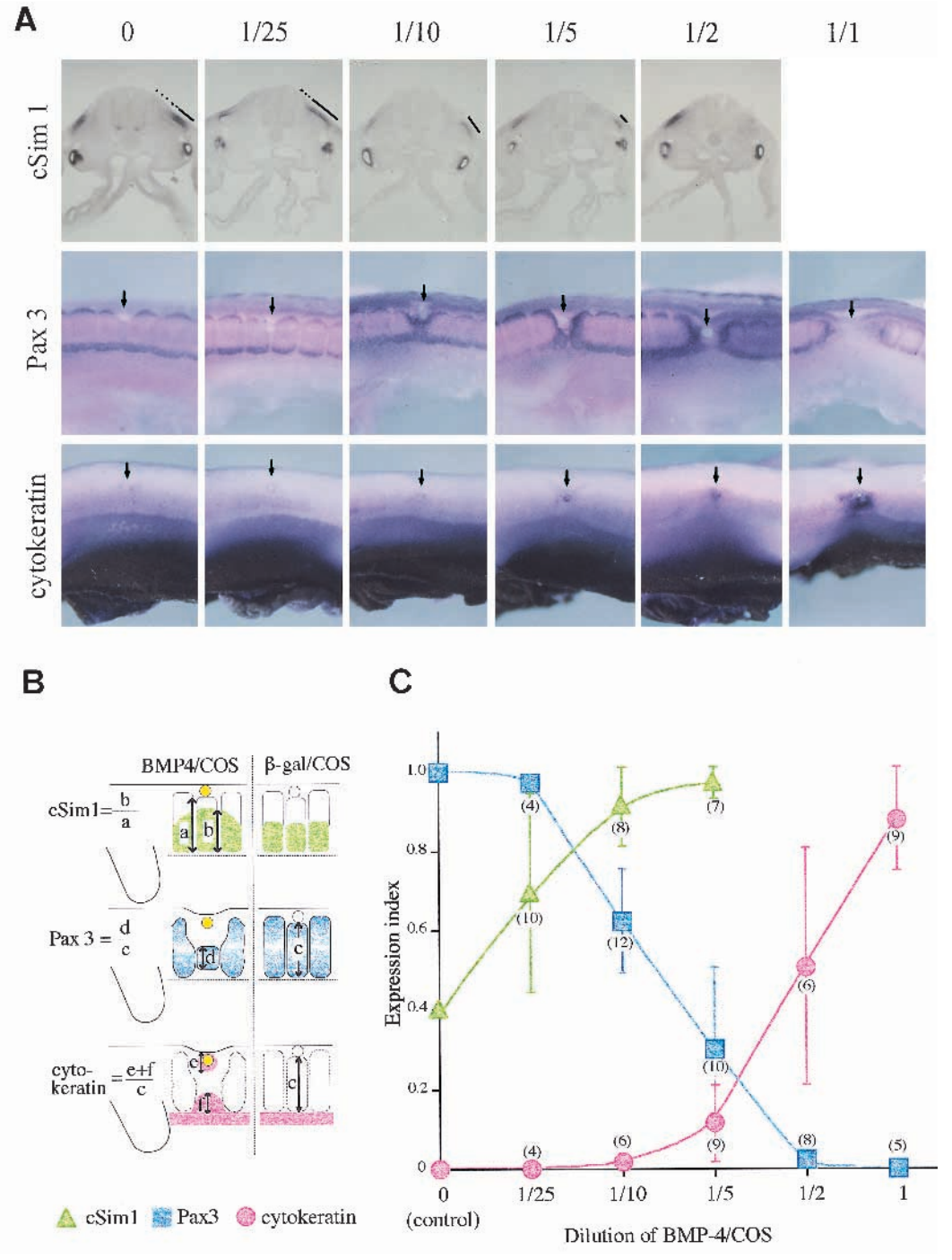


**Fig. 5.** Concentration-dependent lateralizing effect of BMP-4 on the somitic mesoderm. The embryos were assayed 24 hours after the graft of COS cells for expression of *cSim 1*, *Pax 3* and *cytokeratin* mRNAs. (A) BMP-4/COS was diluted as indicated above. 0 and 1/1 represent a graft with control COS cells and non-diluted BMP-4/COS, respectively (see text for details). *cSim 1* is normally expressed in the lateral 40% of the dermomyotome (also see Fig. 2, right diagram). The solid line indicates the *cSim 1*-positive region and the dotted line is the rest of the dermomyotome, which is negative for *cSim 1*. As the relative concentration of BMP-4 increased, the proportion of *cSim 1* became greater, whereas the size of the entire dermomyotome diminished. At the levels 1/10 and 1/5, all the remaining dermomyotome was positive for *cSim 1*. *Pax 3*-positive fragment of the dermomyotome along the M-L axis at the grafted level (arrow) declined as the relative level of BMP-4 increased. Induction of *cytokeratin* mRNA was obvious with 1/5 and became more intense with a higher concentration of BMP-4. (B) Definition of expression indices of *cSim 1*, *Pax 3* and *cytokeratin* mRNAs. The diagram is a lateral view of embryos after transplantation of either BMP-4/COS or β-gal/COS. *cSim 1* expression index: [*cSim1*-positive region/ remaining dermomyotome] (b/a); *Pax 3* expression index: [Pax 3-positive region along the M-L line with BMP-4/COS/ Pax 3-positive region along the M-L line with β-gal/COS] (d/c); *cytokeratin* expression index: [mediolateral length of induced *cytokeratin* mRNA in the side with BMP-4/COS/ mediolateral length of the dermomyotome in the side with β-gal/COS] (e+f/c). (C) Expression index of either *cSim 1*(green triangle) or *Pax 3* (blue square) or *cytokeratin* (pink circle). An average of each index at each dilution level of BMP-4/COS is plotted. Number of specimen analyzed for each point is shown in parenthesis.

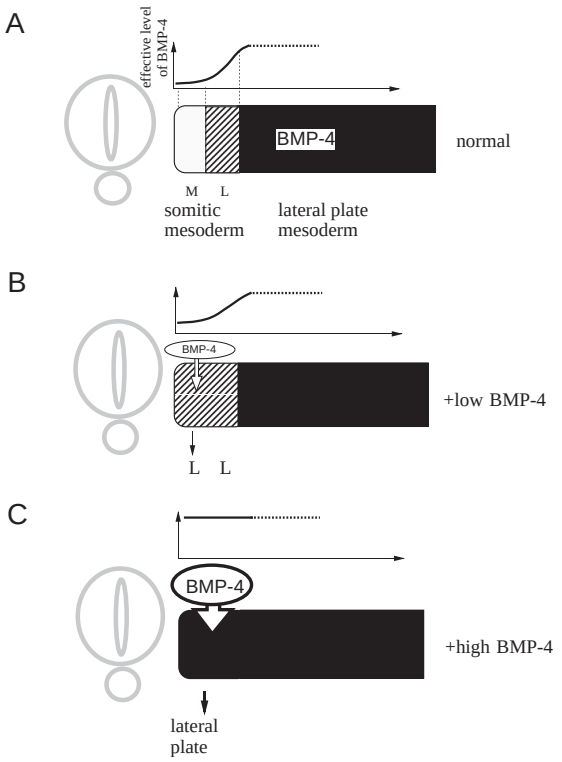


**Fig. 6.** A summary of the present study and a model for the mesodermal subdivision along the mediolateral axis controlled by BMP-4. (A) During normal embryogenesis, the mesoderm may be exposed to a different level of effective BMP-4 according to the position along the M-L axis and respond to produce distinct subtypes of the mesoderm, medial (M) and lateral (L) components of the somite, and the lateral plate mesoderm in the increasing order of an effective level of BMP-4. In this model, BMP-4 emanates from the lateral plate. It is unknown whether there is a gradient of BMP-4 within the lateral plate. This model is supported by the present results, which are shown in B and C. When a low concentration of BMP-4 (1/25 to 1/10) was administered to the somitic mesoderm, the medial portion of the somite was transformed to the lateral somite (*cSim-1* positive) (B). In contrast, when a high concentration of BMP-4 (1/5 to 1) was administered, the somite was converted to the lateral plate mesoderm (*cytokeratin*-positive) (C).

value of 0.4, observed with β-gal/COS, indicating that a greater fraction of the somite displayed characteristics of lateral somite (Fig. 5A,C). By the dilution level 1/10, the dermomyotome was shortened and became fully positive for *cSim1*, and, at dilution levels higher than 1/5, the dermomyotome formation was lost. Consistent with this mediolateral conversion within the somite by low BMP-4 revealed by *cSim 1* expression, *Pax 3*, which is normally more intensely expressed in the lateral somite, was also upregulated in regions some distance away along the A-P axis from the implanted site even with the undiluted BMP-4/COS, showing that declining concentration of BMP-4 conferred lateral somite characteristics.

These results demonstrated that there are multiple thresholds in the responsiveness to BMP-4 of the somitic mesoderm to be lateralized: a low concentration of BMP-4 results in somitic mesoderm with some lateral-somite characteristics and a high BMP-4 concentration induces a complete transformation of the somite to the lateral plate.

## DISCUSSION

The somitic mesoderm, when it is formed, is morphologically distinct from more lateral mesodermal tissues. It has not heretofore been known to what extent the morphological segregation of the somitic mesoderm reflects the cellular specification and commitment. It has been reported, using a cell labeling technique (Stern et al., 1988), that the lineage of the cells located in the rostral portion of the segmental plate is restricted to the somite whereas those in the caudal portion contribute to the somites and also to the more lateral mesoderm. In this study, we have demonstrated that the presomitic mesoderm is not fully committed to the somite, since. when positioned ectopically in the lateral plate, it was respecified to the lateral plate. We have provided evidence that BMP-4, which is expressed in the lateral plate, is one of the signals involved in this mesodermal specification (Fig. 6A), and have also demonstrated that different concentrations of BMP-4 elicit distinct outputs in mesodermal subdivisions along the M-L axis.

### Regional specification of the mesoderm along the M-L axis

The ventralizing activity of BMP-4 has also been reported in *Xenopus* embryos: the 'fate' of the dorsal mesoderm is changed to the ventral one by BMP-4 (Jones et al., 1992; Fainsod et al., 1994; Harland, 1994; Schmidt et al., 1995). These findings are largely consistent with our observations described in this study. However, most studies in *Xenopus* embryos are performed by injecting *BMP* mRNAs at earlier stages where cells are not yet specified. Further, manipulations of embryos at such early stages often interfere with the formation of both the D-V and A-P axes at the same time.

Here, the effect of BMP-4 was studied in a specific tissue and at a specific developmental stage. Transplantation of BMP-4-expressing cells induced a local conversion of the presomite to the lateral plate as shown by expression of several markers for the lateral plate, and also by labeling the presomite at the graft site. Lack of rib cartilages in a region corresponding to the graft site at later stages also supports this mediolateral conversion of the mesoderm. Our observation on cartilagenous formation affected by BMP-4 is also largely consistent with that reported recently by LeDouarin's group (Monsoro-Burq et al., 1996). The results presented in this study suggest that, during normal development, BMP-4 expressed in the lateral plate is a cue that produces the lateral characteristics in the mesodermal tissues. Our results are also consistent with the reports using younger materials: when the prospective somitic mesoderm taken from the primitive streak at stage 4 (gastrulation stage) was heterotopically grafted into the prospective lateral plate region of the primitive streak, the graft contributed to the lateral plate (Garcia-Martinez and Schoenwolf, 1992). The axial tissues, Hensen's node or notochord, are thought to provide the dorsalizing factors that are important for the formation of the axial and paraxial structures (Spemann, 1938; van Straaten and Hekking, 1991). BMP-4 administered in the somitic region in this study appears to override activities of these dorsalizing factors.

BMP-4 expression in the lateral plate mesoderm is readily detected after stage 10 (10-somite) (this study, and Watanabe and LeDouarin, 1996). Prior to this stage, *BMP-4* mRNA is instead observed in the ectoderm where the prospective lateral plate mesoderm (BMP-4 negative) underlies it (Watanabe and

LeDouarin, 1996). It is conceivable that the lateral ectoderm first signals the mesoderm to be specified to the lateral plate by the action of BMP-4 and that this action, in turn, produces a high concentration of BMP-4 in the lateral plate complex including the mesoderm. It has been reported, in *Drosophila*, that signals from the ectoderm specify the underlying mesodermal subtypes and also that decapentaplegic (dpp), a *Drosophila* homolog of BMP-4, mediates these interactions (Frasch, 1995).

The intermediate mesoderm, located between the somite and the lateral plate, differentiated into the mesonephric tissues including the Wolffian duct and mesonephric tubules despite BMP-4 administration. It is conceivable that the presomitic cells are responsive to BMP-4 signal by expressing BMP-4 receptors while the intermediate mesoderm is not responsive due to the lack of the receptors. The distribution pattern of BMP-4 type I and type II receptors remains to be investigated.

When BMP-4/COS was transplanted into the somite of somite-stage V, the responsiveness of the somite to BMP-4 declined. This suggests that determination of the somitic mesoderm along the M-L axis takes place around somitic-stage V, where dermomyotomal and sclerotomal differentiation becomes distinct. It has been reported that axial signals such as Sonic hedgehog emanating from the notochord (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) and signals from the neural tube (Munsterberg and Lassar, 1995; Pourquie et al., 1996) are important for these differentiations. It is conceivable that an initial lateralizing signal conveyed by BMP-4 produces a plastic polarity in the somite, and that axial signals, coming later, fix and elaborate the polarity.

### Concentration-dependent effect of BMP-4 in the mesodermal subdivision along the M-L axis

We have found that different concentrations of BMP-4, locally administered in ovo, result in different mesodermal subtypes. At a low concentration, the medial component of the somite was taken over by the lateral somite (Fig. 6B), whereas a higher level converted the entire somite to the lateral plate (Fig. 6C). This suggests that, during normal embryogenesis, BMP-4 acts at different levels of concentration for the mesodermal specification along the M-L axis. A simple model is that the mesoderm positioned laterally, which is exposed to the highest level of BMP-4, is specified to the lateral plate, whereas the medial-most mesoderm is exposed to the lowest level and acquires characteristics of the medial somite (Fig. 6A). It seems that, at the relative level of BMP-4 in our experimental system above the threshold 1/5, the characteristics of the lateral plate were established, whereas the level above 1/25 conferred the lateral somite characteristics. Since *BMP-4* mRNA is not detected in the somite mesoderm, in order for BMP-4 to be effective in the somite, its signal must be transmitted from the lateral plate to the somite either directly or indirectly. It has been reported that dpp acts as a gradient morphogen (Lecuit et al., 1996; Nellen et al., 1996). Distribution of the effective BMP-4 molecule in vivo remains to be investigated. It is unknown whether the lateromedial decline of the effective level of BMP-4 is simply by diffusion or is negatively controlled by unidentified inhibitors. This model is obviously oversimplified because it excludes possible effects emanating from the axial tissues such as chordin or noggin (Harland, 1994; Sasai et al., 1994), or those from the dorsal neural tube (Munsterberg and Lassar, 1995; Pourquie et al., 1996). Noggin and chordin, expressed in the Spemann's organizer in *Xenopus*, have been reported to counteract BMP-4 by a direct binding at the protein level (Piccolo et al., 1996; Zimmerman et al., 1996). Our preliminary observations also support that these molecules may act along the axial tissues (A. T. and Y. T., unpublished). Therefore, it is most likely that the subdivision of the mesoderm along the M-L axis is established by possible cooperation and counteraction between the axial-derived factors and different concentrations of BMP-4. A similar model for the mesodermal subdivision has been proposed by Pourquie et al. (1996) by showing that the M-L polarity *within* the somite was perturbed by retrovirus-encoded BMP-4. The present study has revealed that the BMP-4 concentration that Pourquie et al. (1996) achieved roughly corresponds to the 1/25 dilution level of our experimental system, which causes the somite to be lateralized. It has also been reported that different concentrations of BMP-4 and activin, another TGFβ-family member, induced different subtypes of ectoderm (Wilson and Hemmati-Brivanlou, 1995) and mesoderm (Green et al., 1992; Gurdon et al., 1995), respectively, during gastrulation, using the animal cap assay in *Xenopus* embryos.

In a previous study, we observed that, when the roof plate, which is positive for *BMP-4* mRNA, was transplanted in the vicinity of the host neural tube, it induced ectopic expression of *Msx 2* in its overlying cells (Takahashi et al., 1992). In this study, we did not detect induction of *cytokeratin* mRNA expression upon the roof plate transplantation ($n$=8, data not shown). It remains to be studied whether this is because the roof plate secretes some unknown inhibitory factor(s) for lateral plate formation although it produces a high level of BMP-4, or whether it secretes a low concentration of BMP-4 protein, which is effective only for induction of *Msx 2* and not for *cytokeratin* expression.

Lastly, importance of BMP-4 during early developmental processes was also demonstrated by a loss-of-function analysis in mice (Winnier et al., 1995). Although majority of homozygous null *BMP-4* embryos died around gastrulation, some surviving embryos exhibited defects in the formation of the ventral-most mesoderm. A gain-of-function analysis of BMP-4, which is administered at a specific site and at a specific stage as presented in this report, is particularly useful to study the effects of the molecule on mesodermal specification and determination at relatively late stages, and also to study morphological consequences of ectopic action of BMP-4.

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