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Differential contributions of Mesp1 and Mesp2 to the epithelialization and rostro-caudal patterning of somites

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Author Shuichi Hiraoka was omitted by mistake. The contribution made by Hiraoka was equal to that of the first author during the crucial phase when the main conclusion of the paper was reached.

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The authors apologise to readers for this mistake.

Differential contributions of *Mesp1* and *Mesp2* to the epithelialization and rostro-caudal patterning of somites

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Summary

Mesp1 and *Mesp2* are homologous basic helix-loop-helix (bHLH) transcription factors that are co-expressed in the anterior presomitic mesoderm (PSM) just prior to somite formation. Analysis of possible functional redundancy of *Mesp1* and *Mesp2* has been prevented by the early developmental arrest of *Mesp1/Mesp2* double-null embryos. Here we performed chimera analysis, using either *Mesp2*-null cells or *Mesp1/Mesp2* double-null cells, to clarify (1) possible functional redundancy and the relative contributions of both *Mesp1* and *Mesp2* to somitogenesis and (2) the level of cell autonomy of *Mesp* functions for several aspects of somitogenesis. Both *Mesp2*-null and *Mesp1/Mesp2* double-null cells failed to form initial segment borders or to acquire rostral properties, confirming that the contribution of *Mesp1* is minor during these events. By contrast, *Mesp1/Mesp2* double-null cells contributed to neither epithelial somite nor dermomyotome

formation, whereas *Mesp2*-null cells partially contributed to incomplete somites and the dermomyotome. This indicates that *Mesp1* has a significant role in the epithelialization of somitic mesoderm. We found that the roles of the *Mesp* genes in epithelialization and in the establishment of rostral properties are cell autonomous. However, we also show that epithelial somite formation, with normal rostro-caudal patterning, by wild-type cells was severely disrupted by the presence of *Mesp* mutant cells, demonstrating non-cell autonomous effects and supporting our previous hypothesis that *Mesp2* is responsible for the rostro-caudal patterning process itself in the anterior PSM, via cellular interaction.

Key words: Somitogenesis, Epithelial-mesenchymal conversion, *Mesp2*, Chimera analysis, Mouse

Introduction

Somitogenesis is not only an attractive example of metamerism pattern formation but is also a good model system for the study of morphogenesis, particularly epithelial-mesenchymal interconversion in vertebrate embryos (Gossler and Hrabe de Angelis, 1997; Pourquié, 2001). The primitive streak, or tailbud mesenchyme, supplies the unsegmented paraxial mesoderm, known as presomitic mesoderm (PSM). Mesenchymal cells in the PSM undergo mesenchymal-epithelial conversion to form epithelial somites in a spatially and temporally coordinated manner. Somites then differentiate, in accordance with environmental cues from the surrounding tissues, into dorsal epithelial dermomyotome and ventral mesenchymal sclerotome (Borycki and Emerson, 2000; Fan and Tessier Lavigne, 1994). Hence, the series of events that occur during somitogenesis provide a valuable example of epithelial-mesenchymal conversion. The dermomyotome gives rise to both dermis and skeletal muscle, whereas the sclerotome forms cartilage and bone in both the vertebrae and the ribs. Each somite is subdivided into two compartments, the rostral (anterior) and caudal (posterior) halves. This rostro-caudal polarity appears to be established just prior to somite formation (Saga and Takeda, 2001).

Mesp1 and *Mesp2* are closely related members of the basic helix-loop-helix (bHLH) family of transcription factors but share significant sequence homology only in their bHLH regions (Saga et al., 1996; Saga et al., 1997). During development of the mouse embryo, both *Mesp1* and *Mesp2* are specifically expressed in the early mesoderm just after gastrulation and in the paraxial mesoderm during somitogenesis. *Mesp1/Mesp2* double-null embryos show defects in early mesodermal migration and thus fail to form most of the embryonic mesoderm, leading to developmental arrest (Kitajima et al., 2000). *Mesp1*-null embryos exhibit defects in single heart tube formation, due to a delay in mesodermal migration, but survive to the somitogenesis stage (Saga et al., 1999), suggesting that there is some functional redundancy, i.e. compensatory functions of *Mesp2* in early mesoderm. During somitogenesis, both *Mesp1* and *Mesp2* are expressed in the anterior PSM just prior to somite formation. Although we have shown that *Mesp2*, but not *Mesp1*, is essential for somite formation and the rostro-caudal patterning of somites (Saga et al., 1997), a possible functional redundancy between *Mesp1* and *Mesp2* has not yet been clearly established.

To further clarify the contributions of *Mesp1* and *Mesp2* to somitogenesis, analysis of *Mesp1/Mesp2* double-null embryos

is necessary, but because of the early mesodermal defects already described, these knockout embryos lack a paraxial mesoderm, which prevents any analysis of somitogenesis. We therefore adopted a strategy that utilized chimera analysis. As we have reported previously, the early embryonic lethality of a *Mesp1/Mesp2* double knockout is rescued by the presence of wild-type cells in a chimeric embryo, but the double-null cells cannot contribute to the cardiac mesoderm (Kitajima et al., 2000). This analysis, however, focused only on early heart morphogenesis and did not investigate the behavior of *Mesp1/Mesp2* double-null cells in somitogenesis. In this report, we focus upon somitogenesis and compare two types of chimeras using either *Mesp1/Mesp2* double-null cells or *Mesp2*-null cells to investigate *Mesp1* function during somitogenesis.

Another purpose of our chimera experiments was to elucidate the cell autonomy of *Mesp* functions. In the process of somite formation, mesenchymal cells in the PSM initially undergo epithelialization at the future segment boundary, independently of the already epithelialized dorsal or ventral margin of the PSM (Sato et al., 2002). Epithelial somite formation is disrupted in the *Mesp2*-null embryo, indicating that *Mesp2* is required for epithelialization at the segment boundary. Although *Mesp* products are nuclear transcription factors and their primary functions must therefore be cell autonomous (transcriptional control of target genes), it is possible that the roles of *Mesp2* in epithelialization are mediated by the non-cell autonomous effects of target genes. We therefore asked whether the defects in *Mesp2*-null cells during epithelialization could be rescued by the presence of surrounding wild-type cells. Additionally, we would expect to find that the role of *Mesp2* in establishing rostro-caudal polarity is rescued in a similar way.

Our analysis suggests that *Mesp1* and *Mesp2* have redundant functions and are both cell-autonomously involved in the epithelialization of somitic mesoderm. In addition, our results highlight some non-cell autonomous effect of *Mesp2*-null and *Mesp1/Mesp2*-null cells.

Materials and methods

Generation of chimeric embryos

As described previously (Kitajima et al., 2000), chimeric embryos were generated by aggregating 8-cell embryos of wild-type mice (ICR) with those of mutant mice that were genetically marked with the *ROSA26* transgene (Zambrowicz et al., 1997). *Mesp1/Mesp2* double-null embryos were generated by crossing *wko-del (+/-)* and *Mesp1(+/-)/Mesp2(+/-)* mice as described previously (Kitajima et al., 2000). This strategy enables us to distinguish chimeric embryos derived from homozygous embryos, which have two different mutant alleles, from those derived from heterozygous embryos. Likewise, *Mesp2*-null embryos were generated by crossing *P2v1(+/-)* mice (Saga et al., 1997) and *P2GFP (+/gfp)* mice (Y.S. and S.K., unpublished) that were also labeled with the *ROSA26* locus. The genotype of the chimeric embryos was determined by PCR using yolk sac DNA.

Histology, histochemistry and gene expression analysis

The chimeric embryos were fixed at 11 days postcoitum (dpc) and stained in X-gal solution for the detection of β -galactosidase activity, as described previously (Saga et al., 1999). For histology, samples stained by X-gal were postfixed with 4% paraformaldehyde, dehydrated in an ethanol series, embedded in plastic resin (Technovit

8100, Heraeus Kulzer) and sectioned at 3 μ m. The methods used for gene expression analysis by in-situ hybridization of whole-mount samples and frozen sections and skeletal preparation by Alcian Blue/Alizarin Red staining were described previously (Saga et al., 1997; Takahashi et al., 2000). Probes for in-situ hybridization for *Uncx4.1* (Mansouri et al., 1997; Neidhardt et al., 1997), *Delta-like 1 (Dll1)* (Bettenhausen et al., 1995) and *Paraxis* (Burgess et al., 1995) were kindly provided by Drs Peter Gruss, Achim Gossler and Alan Rawls, respectively. A probe for *EphA4* (Nieto et al., 1992) was cloned by PCR. For detection of actin filaments, frozen sections were stained with AlexaFluor 488-conjugated phalloidin (Molecular Probes) according to the manufacturer's protocol.

Results

Possible functional redundancy and different contributions of *Mesp1* and *Mesp2* in somitogenesis

During somitogenesis, both *Mesp1* and *Mesp2* are expressed in the anterior PSM just prior to somite formation and their expression domains overlap (Fig. 1A). *Mesp1*-null embryos form morphologically normal somites and show normal rostro-caudal patterning within each somite (Fig. 1B,E-H), indicating that *Mesp1* is not essential for somitogenesis. By contrast, *Mesp2* is essential for both the formation and rostro-caudal patterning of somites, as *Mesp2*-null embryos have no epithelial somites and lose rostral half properties, resulting in caudalization of the entire somitic mesoderm (Saga et al., 1997) (Fig. 1C,D).

Although somite formation and rostro-caudal patterning is disrupted in the *Mesp2*-null embryo, histological differentiation into dermomyotome and sclerotome is not affected. It is noteworthy that the *Mesp2*-null embryo still forms disorganized dermomyotomes without forming epithelial somites (Saga et al., 1997). As *Mesp1* is expressed at normal levels in the PSM of *Mesp2*-null embryos (Fig. 1C,D), it is possible that *Mesp1* functions to rescue some aspects of somitogenesis in the *Mesp2*-null embryo. In order to further clarify the contributions of both *Mesp1* and *Mesp2* during somitogenesis, we therefore generated chimeric embryos with either *Mesp2*-null cells or *Mesp1/Mesp2* double-null cells and compared the behavior of mutant cells during somitogenesis (Fig. 2).

Mesp2-null cells tend to be eliminated from the epithelial somite and the dermomyotome, but can partially contribute to both of these structures

We first generated *Mesp2*-null chimeric embryos (*Mesp2*^{-/-} with *Rosa26*: wild) to analyze cell autonomy of *Mesp2* function during somitogenesis. The control chimeric embryo (*Mesp2*^{+/-} with *Rosa26*: wild) showed normal somitogenesis and a random distribution of X-gal stained cells (Fig. 3A). The *Mesp2*-null chimeric embryos formed abnormal somites that exhibited incomplete segmentation (Fig. 3B), but histological differentiation of dermomyotome and sclerotome was observed. Within the incomplete somite, X-gal-stained *Mesp2*-null cells were mainly localized in the rostral and central regions, surrounded by wild-type cells at the dorsal, ventral and caudal sides (Fig. 3B). The surrounding wild-type cells, however, did not form an integrated epithelial sheet, but consisted of several epithelial cell clusters. Such trends were more obviously observed in other sections, where wild-type cells were found to form multiple small epithelial clusters (Fig.

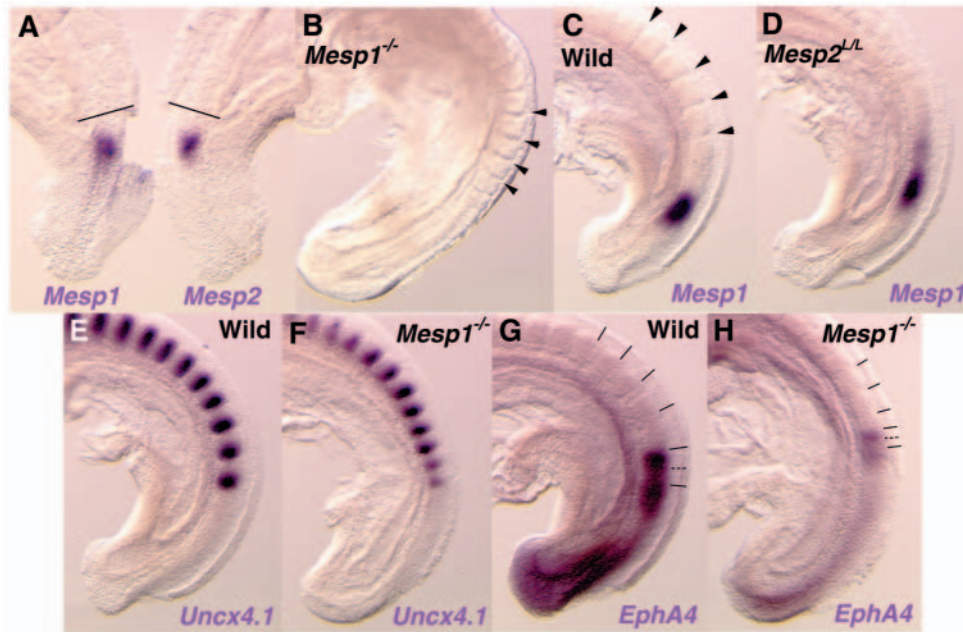


Fig. 1. *Mesp1* and *Mesp2* are co-expressed in the anterior PSM but have differing roles in somitogenesis. (A) Overlapping expression of *Mesp1* and *Mesp2* is revealed by in-situ hybridization using the left and right halves of the same embryo. The lines show most recently formed somite boundaries. (B-C) A *Mesp1*-null embryo (B) shows the same normal somite formation as a wild-type embryo (C). Arrowheads indicate somite boundaries. (D) In *Mesp2*-null embryos, no somite formation is observed but *Mesp1* is expressed at comparable levels to wild type, although its expression is anteriorly extended and blurred. (E-H) *Mesp1*-null embryos show normal rostro-caudal patterning of somites. (E,F) Expression of a caudal half marker, *Uncx4.1*. (G,H) Expression of a rostral half marker, *EphA4*. The lines indicate presumptive or formed somite boundaries and the dotted line indicates approximate position of somite half boundary.

3C,D). *Mesp2*-null cells tended to be eliminated from the epithelial clusters, although they were partially integrated into these structures (blue arrows in Fig. 3C,D). Likewise, small numbers of *Mesp2*-null cells were found to contribute to the dermomyotome (Fig. 3E,F). *Mesp2*-null cells also appeared to form the major part of the sclerotome.

Mesp2 is required for the cell-autonomous acquisition of rostral properties

We have previously demonstrated that suppression by *Mesp2*

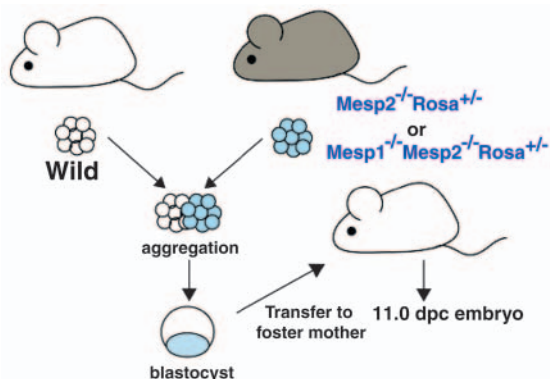


Fig. 2. Schematic representation of chimera analysis method. Either *Mesp2*-null or *Mesp1*/*Mesp2* double-null embryos, genetically labeled with *Rosa* locus, were aggregated with wild-type embryos at the 8-cell stage, and the resulting chimeras were subjected to analysis at 11.0 dpc.

of the caudal genes *Dll1* and *Uncx4.1* in presumptive rostral half somites is a crucial event in the establishment of the rostro-caudal pattern of somites (Saga et al., 1997; Takahashi et al., 2000). As *Mesp2*-null embryos exhibit caudalization of somites, *Mesp2*-null cells are predicted to be unable to express rostral properties. Hence, *Mesp2*-null cells are expected to distribute to the caudal region of each somite where the rostro-caudal patterns are rescued by wild-type cells in a chimeric embryo. In this context, the localization of *Mesp2*-null cells at the rostral side was an unexpected finding. We interpret this to mean that the rostral location of *Mesp2*-null cells is due to a lack of epithelialization functions (see Discussion).

To examine rostro-caudal properties in *Mesp2*-null cells, located in the rostral side, we analyzed the expression of a caudal half marker gene, *Uncx4.1* (Mansouri et al., 1997; Neidhardt et al., 1997). Analysis of adjacent sections revealed that *lacZ*-expressing *Mesp2*-null cells, localized at the rostral and central portion, ectopically expressed *Uncx4.1* (Fig. 4A-D). This strongly suggests that *Mesp2*-null cells cannot acquire rostral properties even if surrounded by wild-type cells, and that *Mesp2* function is cell-autonomously required for the acquisition of rostral properties. We also observed that the small number of *Mesp2*-null cells distributed mostly to the caudal end of the dermomyotome (Fig. 3E,F) and that the expression pattern of *Uncx4.1* was normal in the dermomyotome (Fig. 4E,F). In the sclerotome, *lacZ*-expressing *Mesp2*-null cells often distributed to the rostral side, where expression of *Uncx4.1* was abnormally elevated (Fig. 4G,H). The vertebrae of the *Mesp2*-null chimeric fetus showed a partial fusion of the neural arches, which was reminiscent of

Mesp2-hypomorphic fetuses (Fig. 4I,J) (Nomura-Kitabayashi et al., 2002). Fusion of proximal rib elements was also observed (Fig. 4K,L).

Mesp1/Mesp2 double-null cells cannot contribute to the formation of epithelial somites or to the dermomyotome

To address the question of whether Mesp1, in addition to Mesp2, exhibits any function during somitogenesis, we next generated Mesp1/Mesp2 double-null chimeric embryos and compared them with the Mesp2-null chimeric embryos described in the previous sections. We first performed whole-mount X-gal staining of embryos at 11 dpc. In the control chimeric embryo, the X-gal-stained Mesp1/Mesp2 double-heterozygous cells distributed randomly throughout the embryonic body, including the somite region (Fig. 5A,C). By contrast, the Mesp1/Mesp2

double-null chimeric embryo displayed a strikingly uneven pattern of cellular distribution in the somite region. The X-gal stained Mesp1/Mesp2 double-null cells were localized at the medial part of embryonic tail and were not observed in the lateral part of the somite region (Fig. 5B,D). Histological examination of parasagittal sections further revealed obvious differences in the cellular contribution to somite formation (Fig. 5E,F). In the control chimeric embryo, Mesp1/Mesp2 double-heterozygous cells distributed randomly throughout the different stages of somitogenesis (PSM, somite, dermomyotome and sclerotome: Fig. 5E). In the Mesp1/Mesp2 double-null chimeric embryo, neither the initial segment border nor epithelial somites were formed, but histologically distinguishable dermomyotome-like and sclerotome-like compartments were generated (Fig. 5F). In addition, Mesp1/Mesp2 double-null cells and wild-type cells were randomly mixed in the PSM, whereas the dermomyotome-like epithelium consisted exclusively of wild-type cells and the sclerotome-like compartment consisted mostly of Mesp1/Mesp2 double-null cells. This suggests that either Mesp1 or Mesp2 is cell-autonomously required for the formation of epithelial somite and dermomyotome. These results also indicate that PSM cells with different characteristics are rapidly sorted during somite formation.

Subsequent examination of transverse sections confirmed the elimination of Mesp1/Mesp2 double-null cells from dermomyotome (Fig. 5G,H). In the mature somite region, the wild-type dermomyotome-like epithelium was found to form the myotome (my) (Fig. 5I,J). Furthermore, the ventral part of this dermomyotome-like epithelium became mesenchymal and appeared to contribute to the dorsal sclerotome (dsc), implying that this initial dermomyotome-like epithelium actually corresponds to the epithelial somite exclusively composed of wild-type cells (Fig. 5I,J). Fluorescent phalloidin staining revealed that the apical localization of actin filaments is limited to the dorsal compartments, which are occupied by wild-type cells in the Mesp1/Mesp2 double-null chimeric embryo (Fig. 5K,L), indicating the Mesp1/Mesp2 double-null cells cannot undergo epithelialization.

It is known that the bHLH transcription factor paraxis (Tcf15 – Mouse Genome Informatics), is required for the epithelialization of somite and

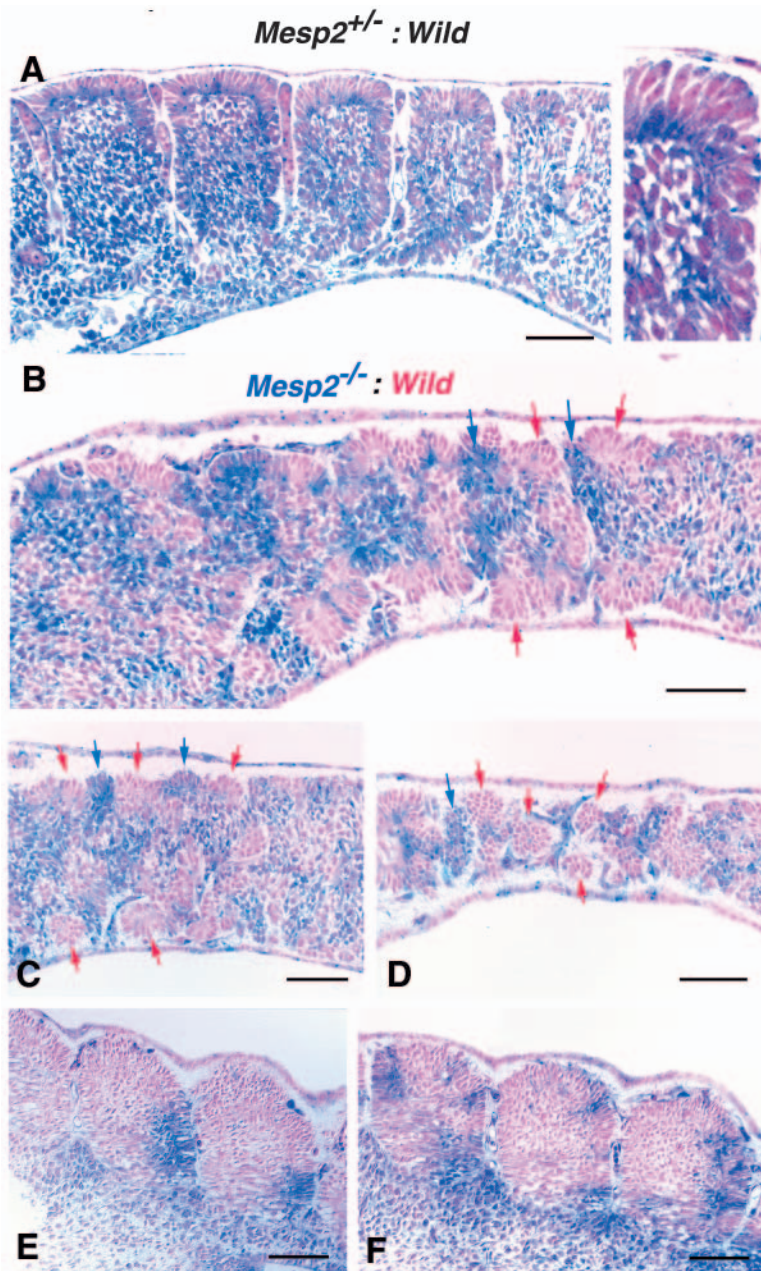


Fig. 3. Mesp2-null cells tend to be excluded from the epithelial region of the somites. (A) The control chimeric embryo undergoes normal somite formation and shows random distribution of labeled cells. The right panel is a high-power view of a somite. (B) In the Mesp2-null chimeric embryo, incompletely segmented somites are formed. Mesp2-null cells tend to be localized at the rostral and central region of these incomplete segments. Red arrows: wild-type cell clusters; blue arrows: Mesp2-null cell clusters. (C,D) Other sections indicating multiple small epithelial cell clusters (arrows). Note that Mesp2-null cells only partially contribute to the epithelial clusters (blue arrows). (E,F) A small number of Mesp2-null cells are distributed in the dermomyotome and are mostly localized at the caudal end. Scale bars: 100 μ m.

dermomyotome (Burgess et al., 1995; Burgess et al., 1996). Although *Paraxis* expression is not affected in *Mesp2*-null embryos (data not shown), it is possible that it is influenced by the loss of both *Mesp1* and *Mesp2*. We therefore examined the expression patterns of *Paraxis* in our *Mesp1/Mesp2* double-null chimeras. In wild-type embryos *Paraxis* is initially expressed throughout the entire somite region (in both the prospective dermomyotomal and sclerotomal regions) in the anteriormost PSM and newly forming somites, and then localizes in the dermomyotomes (Burgess et al., 1995). The dorsal dermomyotomal epithelium, composed of wild-type cells, strongly expressed *Paraxis* in the chimeric embryo (Fig. 6A,B). In addition, adjacent sections revealed that *lacZ*-expressing *Mesp1/Mesp2* double-null cells expressed *Paraxis* in the medial sclerotomal compartment (Fig. 6A,B, brackets). This suggests that *Paraxis* expression in the future sclerotomal region is independent of *Mesp* factors. However, at present we cannot exclude the possibility that the maintenance of *Paraxis* expression in the dermomyotome requires the functions of either *Mesp1* or *Mesp2*.

Mesp1/Mesp2 double-null cells are incapable of acquiring rostral properties

To clarify the rostro-caudal properties of somites in our chimeric embryos, we examined the expression pattern of *Uncx4.1*. Control chimeric embryos exhibited a normal stripe pattern of *Uncx4.1* expression throughout the segmented somite region (Fig. 7A). By contrast, *Mesp1/Mesp2* double-null chimeric embryos exhibited continuous *Uncx4.1* expression in the ventral sclerotomal region (Fig. 7B). This continuity was observed in the entire sclerotome-like compartment of the newly formed somite region and in the ventral sclerotome in the mature somite region. The caudal localization of *Uncx4.1* expression, however, was normal in the dermomyotome and the dorsal sclerotome, which consisted of wild-type cells (Fig. 5), even in *Mesp1/Mesp2* double-null chimeras. This suggests that, like *Mesp2*-null cells, *Mesp1/Mesp2* double-null cells are incapable of acquiring rostral properties. Since the mesoderm of *Mesp1/Mesp2* double-null embryos lacks the expression of the major markers of paraxial mesoderm (Kitajima et al., 2000), and *Mesp1/Mesp2* double-null cells do not exhibit histological features characteristic of epithelial somites in our current study, it is possible that *Mesp1/Mesp2* double-null cells may lack

paraxial mesoderm properties. However, the analysis of adjacent sections suggests that *lacZ*-expressing *Mesp1/Mesp2* double-null cells themselves express *Uncx4.1*, a somite-specific marker (Fig. 7C,D), and they had also been found to have normal expression of *Paraxis* (Fig. 6A,B).

It is believed that the rostro-caudal pattern within somites and dermomyotomes is generated in the PSM and maintained in somites and dermomyotomes. We observed a normal rostro-caudal pattern in the dermomyotome (Fig. 7), although wild-type cells and *Mesp1/Mesp2* double-null cells are mixed in the PSM (Fig. 5), of *Mesp1/Mesp2* double-null chimeric embryos. As *Mesp* products are required for suppression of *Dll1* in the anterior PSM, a normal *Dll1* stripe pattern cannot be formed if *Mesp1/Mesp2* double-null cells are randomly distributed in

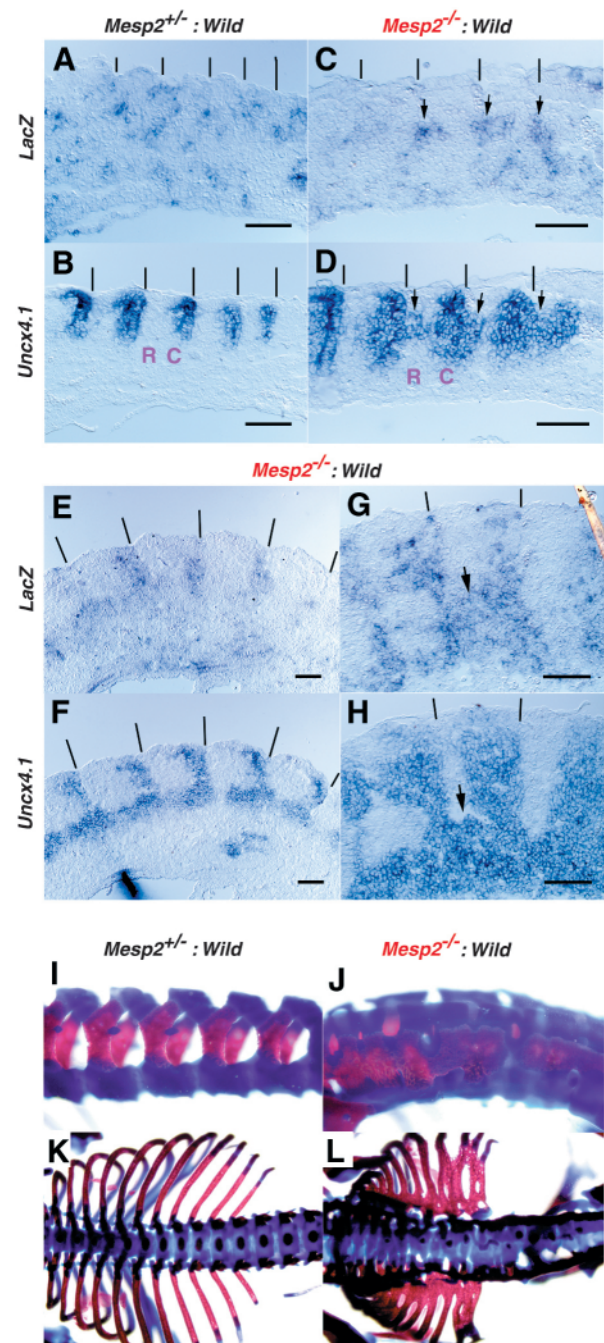


Fig. 4. *Mesp2* function is cell autonomously required for rostral properties. (A-D) Expression of *lacZ* and *Uncx4.1* transcripts at the site of initial somite formation in control (A,B) and *Mesp2*-null (C,D) chimeric embryos. In the control, *lacZ*-expressing cells are randomly distributed and *Uncx4.1* expression is normal. In the *Mesp2*-null chimera, *lacZ*-expressing *Mesp2*-null cells at the rostral part of the incomplete segments (arrows in C) ectopically express *Uncx4.1* (arrows in D). Lines indicate somite boundaries. (E,F) In the dermomyotome, *Mesp2*-null cells are mostly localized at the caudal end, and the *Uncx4.1* expression pattern is normal. (G,H) In the sclerotome, the distribution of *Mesp2*-null cells results in expansion of *Uncx4.1* expression (arrows). (I) The control chimeric fetus shows normal vertebrae. (J) The *Mesp2*-null chimeric fetus exhibits partial fusion of the neural arches. (K) The control chimeric fetus shows normal ribs. (L) The *Mesp2*-null chimeric fetus shows proximal rib fusion. Scale bars: 100 μ m. C, caudal compartment; R, rostral compartment.

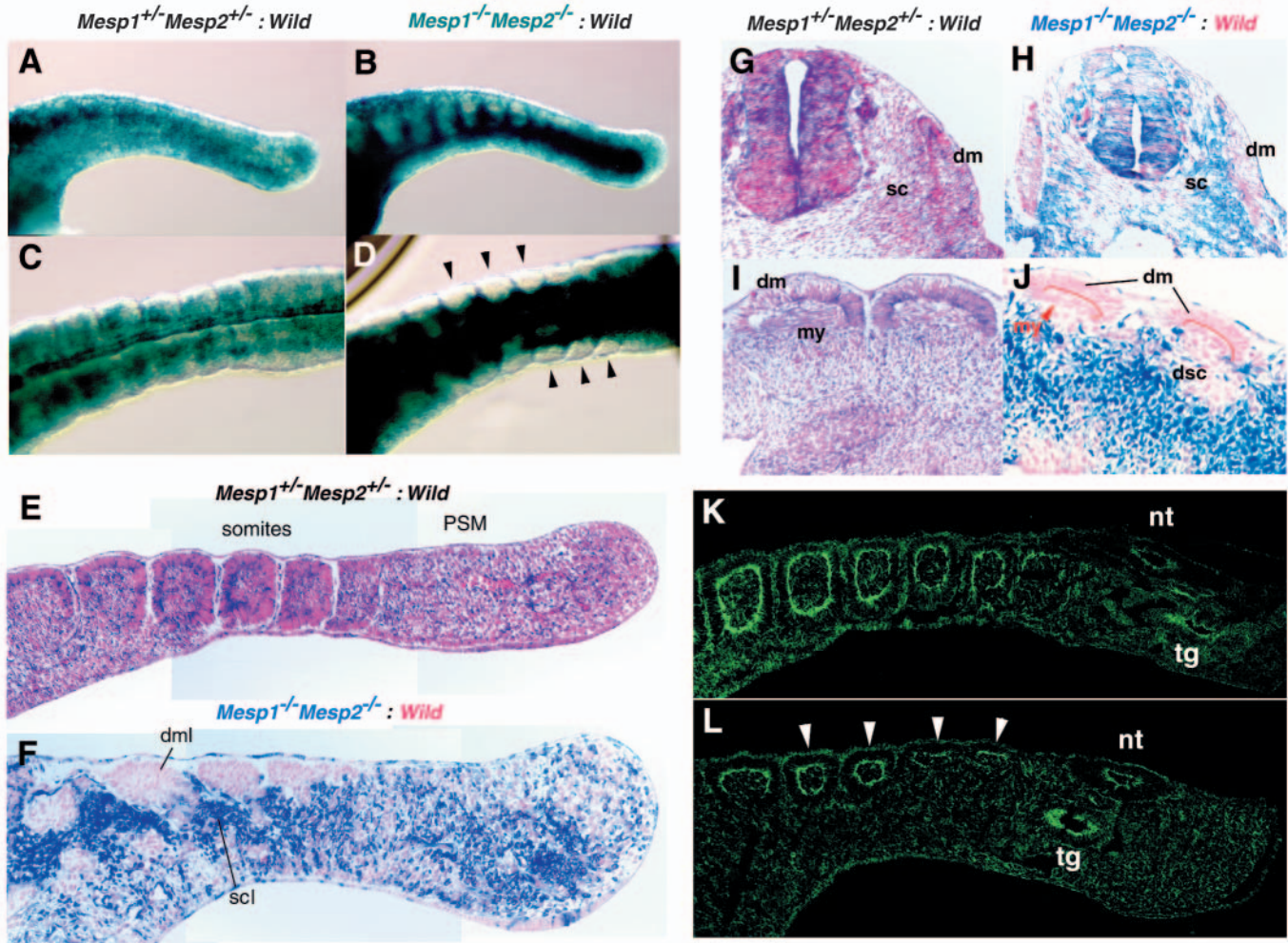


Fig. 5. Mesp1/Mesp2 double-null cells fail to contribute to epithelial somites or to the dermomyotome. (A–D) Tail regions from X-gal-stained whole-mount specimens of control (A,C) and double-null (B,D) chimeric embryos. (A,B) Lateral view. (C,D) Dorsal view. The blue double-heterozygous cells are randomly distributed in the control embryo, whereas the Mesp1/Mesp2 double-null cells are excluded from the lateral region of the somites (arrowheads in D). (E,F) Parasagittal sections of tails from chimeric embryos. (E) The labeled cells are randomly located in the control chimera. (F) The two types of cells are randomly mixed in the PSM, whereas the dermomyotome-like epithelium consisted exclusively of wild-type cells and the sclerotome-like compartment contained mostly Mesp1/Mesp2 double-null cells. Note that normal epithelial somites are not formed in this chimera. (G,H) Transverse sections show elimination of Mesp1/Mesp2 double-null cells from the dermomyotome. (I,J) The dermomyotome-like epithelium in the Mesp1/Mesp2 double-null chimeric embryo gives rise to dermomyotome, myotome (arrowhead in J) and the dorsal part of the sclerotome. Red arches indicate the inner surface of dermomyotome. (K,L) AlexaFluor 488-labeled phalloidin staining shows normal epithelialization of somites in the control chimera (K) and restriction of epithelialization in the dermomyotome-like compartment in the Mesp1/Mesp2 double-null chimera (L). dm, dermomyotome; dml, dermomyotome-like epithelium; dsc, dorsal part of the sclerotome; my, myotome; nt, neural tube; sc, sclerotome; scl, sclerotome-like compartment; tg, tail gut.

the anterior PSM. This is because 50% of cells cannot undergo suppression of *Dll1* even in the future rostral half region. Therefore, our finding of a normal rostro-caudal pattern in the dermomyotome of double-null chimeras is surprising and raises the question of whether wild-type cells can be normally patterned in the presence of surrounding Mesp1/Mesp2 double-null cells. To determine how the rostro-caudal pattern in the dermomyotome is formed in the PSM, we examined the expression pattern of *Dll1* (Bettenhausen et al., 1995), the stripe expression profile of which is established in the anteriormost PSM via the function of Mesp2 (Takahashi et al., 2000). The *lacZ*-expressing Mesp1/Mesp2 double-null cells were subsequently found to be consistently localized in the

sclerotome-like region, where *Dll1* expression was abnormally expanded (Fig. 6C,D). In the dermomyotome-like region, however, *Dll1* expression in the caudal half was normal. Intriguingly, strong *Dll1* expression in the anteriormost PSM was suppressed in a rostrally adjoining cell population, which is mainly occupied by wild-type cells (Fig. 6C,D, arrows). This implies that wild-type cells and Mesp1/Mesp2 double-null cells rapidly segregate at S–1 to S0, after which the rostro-caudal pattern of *Dll1* expression is formed in the partially segregated wild-type cell population but not in the randomly mixed cell population. In other words, the separation from Mesp1/Mesp2 double-null cells enabled normal rostro-caudal patterning of wild-type cells.

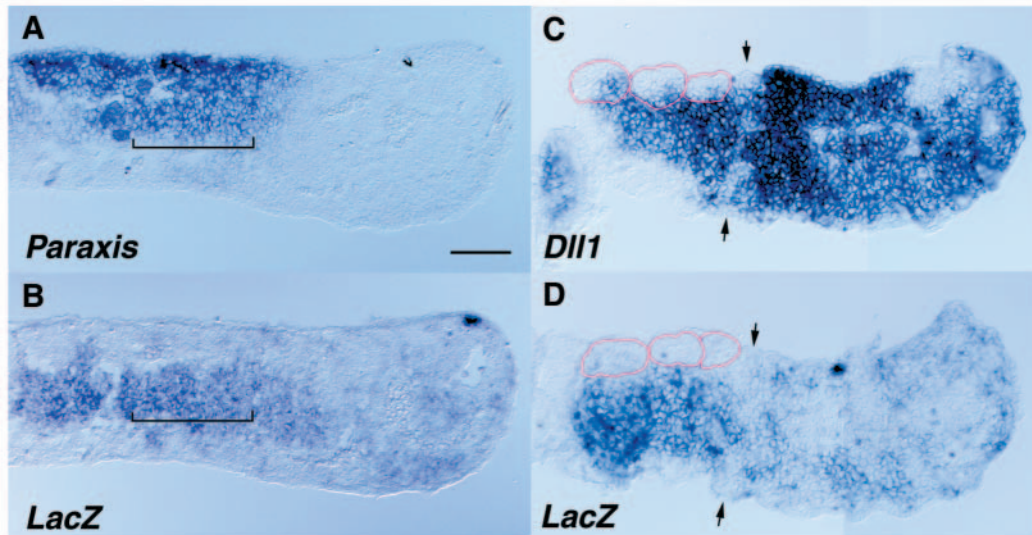
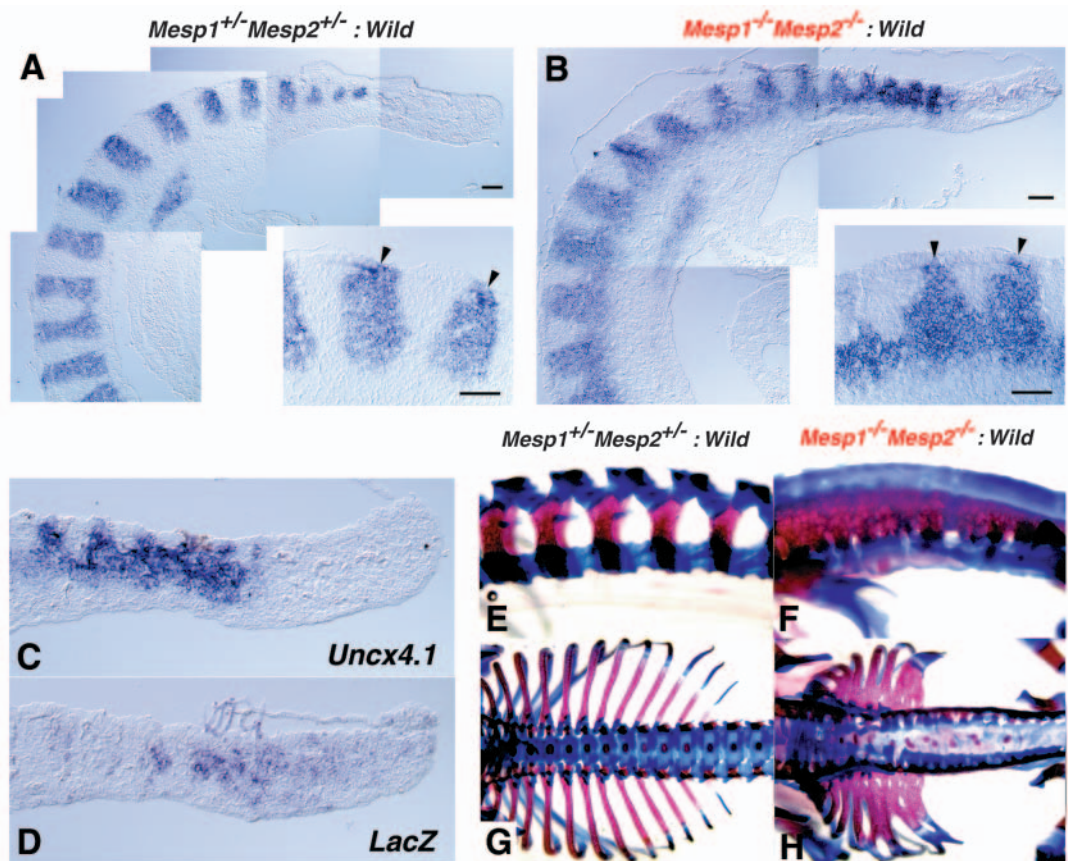


Fig. 6. (A,B) *Mesp1/Mesp2* double-null cells express *Paraxis*. Adjacent parasagittal sections of the *Mesp1/Mesp2* double-null chimeric embryo were stained for either *Paraxis* (A) or *lacZ* (B). Note that the expression domains of the two genes overlap in the medial sclerotomal region (brackets). (C,D) The rostro-caudal pattern in the dermomyotome is formed in a partially segregating wild-type cell population. Adjacent sections of the *Mesp1/Mesp2* double-null chimeric embryos were stained for *Dll1* (C) or *lacZ* (D) mRNA. Red outlines demarcate the dorsal dermomyotome-like compartments. Note that suppression of *Dll1* expression occurs in a region mostly occupied by wild-type cells (arrows). Scale bar: 100 μ m.

Fig. 7. Rostro-caudal patterning of the sclerotome is disrupted in *Mesp1/Mesp2* double-null chimeric embryos. (A) The control chimeric embryos exhibit normal stripe patterns of *Uncx4.1* expression throughout the somite region. (B) The *Mesp1/Mesp2* double-null chimeric embryos exhibit continuous *Uncx4.1* expression in the ventral sclerotomal region. Note that caudal localization of *Uncx4.1* expression is normal in the dermomyotome and dorsal sclerotome. The insets show a higher magnification of lumbar somites. (C,D) Adjacent sections showing that *lacZ*-expressing *Mesp1/Mesp2* double-null cells express *Uncx4.1*. (E-H) The *Mesp1/Mesp2* double-null chimeric fetus exhibits caudalization of the vertebrae and of the proximal ribs. (E) The control chimeric fetus shows normal metameric arrangement of the neural arches. (F) The *Mesp1/Mesp2* double-null chimeric fetus shows severe fusion of the pedicles and the laminae of neural arches. (G) The control chimeric fetus has normal arrangement of ribs. (H) The double-null chimeric fetus shows severe fusion of the proximal elements of the ribs. Scale bars: 100 μ m.



Mesp2-null fetuses display caudalized vertebrae with extensive fusion of the pedicles of neural arches and proximal elements of the ribs (Saga et al., 1997). The *Mesp1/Mesp2* double-null chimeric fetuses also exhibited fusion of the pedicles of neural arches and the proximal ribs (Fig. 7E-H). Furthermore, the vertebrae of severe chimeric fetuses were indistinguishable from those of *Mesp2*-null fetuses. These observations indicate that *Mesp1/Mesp2* double-null cells can differentiate into caudal sclerotome and possibly contribute to chondrogenesis.

Discussion

Mesp1 and *Mesp2* not only exhibit similar expression patterns but also share common bHLH domains as transcription factors. Previous studies using gene replacement experiments (Saga, 1998) (Y.S. and S.K., unpublished) indicate that these genes can compensate for each other. However, the early lethality of double knockout mice hampered any further detailed analysis of somitogenesis. An obvious strategy to further elucidate the functions of *Mesp1* and *Mesp2* was, therefore, the generation of a conditional knockout allele for *Mesp2* in *Mesp1* disrupted cells in which the Cre gene is specifically activated in the paraxial mesoderm, which is now underway. Chimera analysis is also a powerful method as an alternative strategy. Comparisons of chimeras, composed of either *Mesp2*-null or *Mesp1/Mesp2* double-null cells, made it possible to determine the contribution of *Mesp1* to somitogenesis. Our results indicate that *Mesp1* has redundant functions in the epithelialization of somitic mesoderm and additionally, by chimeric analysis, we were able to demonstrate the cell autonomy of *Mesp1* and *Mesp2* function during some critical steps of somitogenesis.

The relative contributions of *Mesp1* and *Mesp2* to somitogenesis

In *Mesp1*-null mice, epithelial somites with normal rostro-caudal polarity are generated, whereas *Mesp2*-null mice exhibit defects in both the generation of epithelial somites and the establishment of rostro-caudal polarity. Thus, it seems likely that *Mesp2* function is both necessary and sufficient for somitogenesis. However, dermomyotome formation was observed, without normal segmentation, even in *Mesp2*-null mice. In view of the apparent redundant functions of *Mesp1* and *Mesp2* in somitogenesis, as demonstrated by our previous gene replacement study, it was possible that the *Mesp1/Mesp2* double-null embryo would exhibit a much more severe phenotype in relation to somitogenesis. In our chimera analyses, both *Mesp2*-null and *Mesp1/Mesp2* double-null cells exhibited complete caudalization of somitic mesoderm, indicating that *Mesp1* function is not sufficient to rescue *Mesp2* deficiency and restore rostro-caudal polarity. Likewise, both *Mesp2*-null and *Mesp1/Mesp2* double-null cells were incapable of forming an initial segment boundary, showing that the contribution of *Mesp1* is also minor during this process. By contrast, whereas *Mesp1/Mesp2* double-null cells lacked any ability to epithelialize, *Mesp2*-null cells were occasionally integrated into epithelial somites and dermomyotome, indicating that the contribution of *Mesp1* to epithelialization is significant and that *Mesp1* can function in the absence of *Mesp2* (Fig. 8). We therefore postulate that the epithelialization

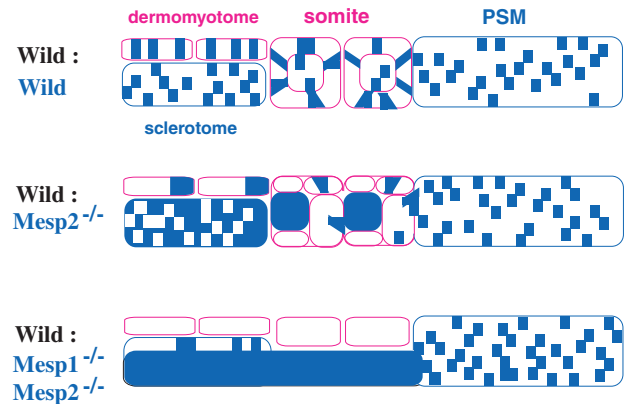


Fig. 8. A schematic summarization of the *Mesp1/Mesp2* chimera experiments. *Mesp1/Mesp2* double-null cells can contribute to neither epithelial somite nor dermomyotome formation, whereas *Mesp2*-null cells can partially contribute to both somites and dermomyotome. Red outlines indicate epithelialized tissues (epithelial somites, dermomyotomes and abnormal small clusters).

of dermomyotome, observed in *Mesp2*-null embryos, is dependent on *Mesp1*.

Mesp factors are cell autonomously required for epithelialization of somitic mesoderm but may also be non-cell autonomously required for morphological boundary formation

Conventional interpretations of the results of chimera analysis are generally based upon the regulative development of the vertebrate embryo and argue cell autonomy of specific gene functions in embryogenesis (Ciruna et al., 1997; Brown et al., 1999; Kitajima et al., 2000; Koizumi et al., 2001). *Mesp1/Mesp2* double-null cells failed to form epithelial somites, even in the presence of surrounding wild-type cells. In addition, they were incapable of contributing to dermomyotome, where cell sorting occurs. This strongly suggests that *Mesp* factors are cell autonomously required for the epithelialization of somitic mesoderm. However, we also found striking non-cell autonomous effects of *Mesp* mutant cells on wild-type cell behaviors. That is, both types of *Mesp* mutant cell not only failed to undergo normal somitogenesis, but also inhibited the normal morphogenesis of wild-type cells. This implies that there are non-cell autonomous roles for *Mesp* factors in the establishment of the future somite boundary, as we will discuss further.

Initial epithelial somite formation is achieved by the mesenchymal-epithelial transition of cells located in the anterior PSM. A future somite boundary is established at a specific position in the PSM, followed by gap formation between the mesenchymal cell populations. Subsequently, cells located anterior to the boundary are epithelialized. This process is known to be mediated by an inductive signal from cells posterior to the boundary (Sato et al., 2002). Therefore, defects in epithelial somite formation can be explained in two principal ways: a lack of cellular ability to epithelialize (cell autonomous) and a lack of an inducing signal, which is produced in the anterior PSM by a mechanism mediated by Notch signaling (thus non-cell autonomous). In the case of chimeras of *Mesp1/Mesp2* double-null cells, no local

boundary formed by locally distributed wild-type cells was observed, i.e. even a gap between wild-type cells was never observed in the mixture of Mesp1/Mesp2 double-null cells and wild-type cells. It is likely, therefore, that the wild-type cell population can form a boundary only after separation from Mesp1/Mesp2 double-null cells (Fig. 8). By contrast, some local boundaries between epithelial wild-type cell clusters were occasionally observed in chimeras with Mesp2-null cells. Considering that there is functional redundancy between these transcription factors, it is possible that either Mesp1 or Mesp2 is necessary for the formation of a signaling center or source of the putative inductive signal. Hence, we cannot exclude the possibility that the lack of Mesp function may affect non-cell autonomous generation of the inductive signal in the anterior PSM.

Formation of epithelial somites requires *paraxis*, which is a transcription factor (Burgess et al., 1996; Nakaya et al., 2004). We observed that Mesp1/Mesp2 double-null cells at the medial sclerotomal region expressed *Paraxis*, indicating that Mesp factors are not absolutely required for *Paraxis* expression. Defects in epithelial somite formation in *paraxis*-null embryos, with normal *Mesp2* expression (Johnson et al., 2001), and in Mesp2-null embryos, with normal *Paraxis* expression, imply that epithelial somite formation independently requires both gene functions.

Mesp2 is cell autonomously required for the acquisition of rostral properties

The distribution of Mesp2-null cells in the Mesp2-null chimeric embryos may appear somewhat paradoxical, as they are localized at the rostral side in the incomplete somites but at the caudal side in the dermomyotome. Initial localization at the rostral and central region, however, is likely to be due to the relative lack of epithelialization functions. In mammalian and avian embryos, mesenchymal-to-epithelial conversion of the PSM commences from the rostral side of the future somite boundary, i.e. the caudal margin of the presumptive somite (Duband et al., 1987). Epithelialization then proceeds anteriorly in the dorsal and ventral faces and in such a process, Mesp2-null cells, which are less able to participate in epithelialization, may therefore be pushed to the central and rostral sides. Thus, the majority of the Mesp2-null cells localize to the central, prospective sclerotomal region and a small number of them are integrated in the future dermomyotomal region. The incomplete somites then undergo reorganization into dermomyotome and sclerotome, and small numbers of Mesp2-null cells in the dermomyotome may be sorted out to the caudal end. Therefore, the apparently complex distribution pattern of Mesp2-null cells is likely to reflect a combination of defects in epithelialization and rostro-caudal patterning. In the incomplete segments of Mesp2-null chimeric embryos, the Mesp2-null cells fail to acquire rostral properties even when localized at the rostral side. Moreover, in the dermomyotome, where rostro-caudal patterning is rescued, Mesp2-null cells are mostly localized in the caudal region. These observations suggested that the requirement of Mesp2 for the acquisition of rostral properties is cell autonomous. Similarly, it has been reported that presenilin 1 (Psen1) is required for acquisition of caudal half properties (Takahashi et al., 2000; Koizumi et al., 2001) and that Psen1-null cells cannot contribute to the caudal half of somites in chimeric embryos,

showing cell autonomous roles for Psen1 (Koizumi et al., 2001).

Mesp mutant cells affect the rostro-caudal patterning of somites due to the lack of cellular interaction with wild-type cells

In a previous study, we have shown that the rostro-caudal patterning of somites is generated by complex cellular interactions involved in positive and negative feedback pathways of Dll1-Notch and Dll3-Notch signaling, and regulation by Mesp2 in the PSM (Takahashi et al., 2003). In chimeras with either Mesp2-null or Mesp1/Mesp2 double-null cells, the mutant cells were distributed evenly and did not show any sorting bias in a rostro-caudal direction in the PSM. Since both Mesp2-null and Mesp1/Mesp2 double-null cells have the ability to form caudal cells, it is likely that if wild-type cells could occupy the rostral part of future somite regions and have the ability to sort in the PSM, a normal rostro-caudal patterning would be generated. We did not observe this, however, and conclude that the presence of mutant cells lacking Mesp factors must have disrupted normal cellular interactions via Notch signaling. Thus these non-cell-autonomous effects of our mutant cells are strongly supportive of our previous contention that rostro-caudal patterning is generated by cellular interactions via Notch signaling.

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References

- Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guénet, J.-L. and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to *Drosophila* Delta. *Development* **121**, 2407-2418.
- Borycki, A. G. and Emerson, C. P., Jr (2000). Multiple tissue interactions and signal transduction pathways control somite myogenesis. *Curr. Top. Dev. Biol.* **48**, 165-224.
- Brown, D., Wagner, D., Li, X., Richardson, D. A. and Olson, E. N. (1999). Dual role of the basic helix-loop-helix transcription factor scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis. *Development* **126**, 4317-4329.
- Burgess, R., Cserjesi, P., Ligon, K. L. and Olson, E. N. (1995). Paraxis: a basic helix-loop-helix protein expressed in paraxial mesoderm and developing somites. *Dev. Biol.* **168**, 296-306.
- Burgess, R., Rawls, A., Brown, D., Bradley, A. and Olson, E. N. (1996). Requirement of the *paraxis* gene for somite formation and musculoskeletal patterning. *Nature* **384**, 570-573.
- Ciruna, B. G., Schwartz, L., Harpal, K., Yamaguchi, T. P. and Rossant, J. (1997). Chimeric analysis of *fibroblast growth factor receptor-1* (*Fgfr1*) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* **124**, 2829-2841.
- Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M. and Thiery, J. P. (1987). Adhesion molecules during somitogenesis in the avian embryo. *J. Cell Biol.* **104**, 1361-1374.
- Fan, C. M. and Tessier Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.
- Gossler, A. and Hrabe de Angelis, M. (1997). Somitogenesis. *Curr. Top. Dev. Biol.* **38**, 225-287.
- Johnson, J., Rhee, J., Parsons, S. M., Brown, D., Olson, E. N. and Rawls, A. (2001). The anterior/posterior polarity of somites is disrupted in Paraxis-deficient mice. *Dev. Biol.* **229**, 176-187.
- Kitajima, S., Takagi, A., Inoue, T. and Saga, Y. (2000). MesP1 and MesP2

- are essential for the development of cardiac mesoderm. *Development* **127**, 3215-3226.
- Koizumi, K., Nakajima, M., Yuasa, S., Saga, Y., Sakai, T., Kuriyama, T., Shirasawa, T. and Koseki, H.** (2001). The role of presenilin 1 during somite segmentation. *Development* **128**, 1391-1402.
- Mansouri, A., Yokota, Y., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P.** (1997). Paired-related murine homeobox gene expressed in the developing sclerotome, kidney, and nervous system. *Dev. Dyn.* **210**, 53-65.
- Nakaya, Y., Kuroda, S., Katagiri, Y. T., Kaibuchi, K. and Takahashi, Y.** (2004). Mesenchymal-epithelial transition during somitic segmentation is regulated by differential roles of Cdc42 and Rac1. *Dev. Cell* **7**, 425-438.
- Neidhardt, L. M., Kispert, A. and Herrmann, B. G.** (1997). A mouse gene of the paired-related homeobox class expressed in the caudal somite compartment and in the developing vertebral column, kidney and nervous system. *Dev. Genes Evol.* **207**, 330-339.
- Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G.** (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-1150.
- Nomura-Kitabayashi, A., Takahashi, Y., Kitajima, S., Inoue, T., Takeda, H. and Saga, Y.** (2002). Hypomorphic *Mesp* allele distinguishes establishment of rostro-caudal polarity and segment border formation in somitogenesis. *Development* **129**, 2473-2481.
- Pourquié, O.** (2001). Vertebrate somitogenesis. *Annu. Rev. Cell. Dev. Biol.* **17**, 311-350.
- Saga, Y.** (1998). Genetic rescue of segmentation defect in *MesP2*-deficient mice by *MesP1* gene replacement. *Mech. Dev.* **75**, 53-66.
- Saga, Y. and Takeda, H.** (2001). The making of the somite: molecular events in vertebrate segmentation. *Nat. Rev. Genet.* **2**, 835-845.
- Saga, Y., Hata, N., Kobayashi, S., Magnuson, T., Seldin, M. and Taketo, M. M.** (1996). *MesP1*: A novel basic helix-loop-helix protein expressed in the nascent mesodermal cells during mouse gastrulation. *Development* **122**, 2769-2778.
- Saga, Y., Hata, N., Koseki, H. and Taketo, M. M.** (1997). *Mesp2*: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. *Genes Dev.* **11**, 1827-1839.
- Saga, Y., Miyagawa-Tomita, S., Takagi, A., Kitajima S., Miyazaki, J. and Inoue, T.** (1999). *MesP1* is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* **126**, 3437-3447.
- Sato, Y., Yasuda, K. and Takahashi, Y.** (2002). Morphological boundary forms by a novel inductive event mediated by Lunatic fringe and Notch during somitic segmentation. *Development* **129**, 3633-3644.
- Takahashi, Y., Koizumi, K., Takagi, A., Kitajima, S., Inoue, T., Koseki, H. and Saga, Y.** (2000). *Mesp2* initiates somite segmentation through the Notch signalling pathway. *Nat. Genet.* **25**, 390-396.
- Takahashi, Y., Inoue, T., Gossler, A. and Saga, Y.** (2003). Feedback loops comprising *Dll1*, *Dll3* and *Mesp2*, and differential involvement of *Psen1* are essential for rostrocaudal patterning of somites. *Development* **130**, 4259-4268.
- Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G. and Soriano, P.** (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **94**, 3789-3794.