

MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube

Yumiko Saga¹, Sachiko Miyagawa-Tomita², Atsuya Takagi¹, Satoshi Kitajima¹, Jun-ichi Miyazaki³ and Tohru Inoue¹

¹Cellular and Molecular Toxicology Division, National Institute of Health Sciences, 1-18-1 Kamiyohga, Setagaya-ku, Tokyo 158, Japan

²Department of Pediatric Cardiology, Heart Institute of Japan, Tokyo Women's Medical College, 8-1, Kawada-cho, Sinjuku-ku, Tokyo 162, Japan

³Department of Nutrition and Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita 565-0871, Japan

*Author for correspondence (e-mail: saga@nihs.go.jp)

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SUMMARY

The *Mesp1* gene encodes the basic HLH protein MesP1 which is expressed in the mesodermal cell lineage during early gastrulation. Disruption of the *Mesp1* gene leads to aberrant heart morphogenesis, resulting in cardia bifida. In order to study the defects in *Mesp1*-expressing cells during gastrulation and in the specification of mesodermal cell lineages, we introduced a β -galactosidase gene (*lacZ*) under the control of the *Mesp1* promoter by homologous recombination. The early expression pattern revealed by β -gal staining in heterozygous embryos was almost identical to that observed by whole mount in situ hybridization. However, the β -gal activity was retained longer than the mRNA signal, which enabled us to follow cell migration during gastrulation. In heterozygous embryos, the *Mesp1*-expressing cells migrated out from the primitive streak and were incorporated into the head mesenchyme and heart field. In contrast, *Mesp1*-expressing cells in the homozygous deficient embryos stayed in the primitive streak for a longer

period of time before departure. The expression of FLK-1, an early marker of endothelial cell precursors including heart precursors, also accumulated abnormally in the posterior region in *Mesp1*-deficient embryos. In addition, using the Cre-loxP site-specific recombination system, we could determine the lineage of the *Mesp1*-expressing cells. The first mesodermal cells that ingressed through the primitive streak were incorporated as the mesodermal component of the amnion, and the next mesodermal population mainly contributed to the myocardium of the heart tube but not to the endocardium. These results strongly suggest that MesP1 is expressed in the heart tube precursor cells and is required for mesodermal cells to depart from the primitive streak and to generate a single heart tube.

Key words: MesP1, Gastrulation, Mesoderm, Heart morphogenesis, Lineage analysis, Mouse

INTRODUCTION

Gastrulation in the mouse embryo involves the active ingression of epiblast cells through the primitive streak and the reassortment of cell populations to the definitive germ layers (Hashimoto and Nakatusji; 1989, Tam and Beddington, 1987; Tam et al., 1993). It is not known how the different populations of presumptive mesodermal and endodermal cells are sorted during germ layer morphogenesis. Fate-mapping studies using either cell-marking or cell transplantation techniques have revealed that both the position of the cells in the proximal-distal axis and their proximity to the primitive streak are major determinants for the patterning of the embryonic mesoderm (Lawson et al., 1991; Parameswaran and Tam, 1995; Tam et al., 1997). According to these results, the first wave of epiblast cells that ingress through the primitive streak are those giving rise to extraembryonic mesoderm, followed by epiblast cells destined for the heart and cranial mesoderm. Thus, cells that

will form the mesoderm of the yolk sac and the amnion make up a major part of the mesodermal layer of the midprimitive-streak-stage embryo. By the late-primitive-streak stage, the mesodermal layer contains only the precursors of the embryonic mesoderm. So far, however, no direct evidence has been presented that proves the results of cell-fate-mapping studies because no suitable genetic markers have been available to trace the cell fate from the beginning of gastrulation to the particular cell lineage.

MesP1, belonging to the bHLH transcription factor family, was found to be expressed in the early mesoderm at the onset of gastrulation (Saga et al., 1996). Since the expression was rapidly down-regulated after 7.0 dpc (days postcoitum), only mesodermal cells that ingressed through the primitive streak in the early stage, between 6.5 and 7.0 dpc, expressed *Mesp1*. Homozygous *Mesp1* null mice exhibited growth retardation after 7.5 dpc and died before 10.5 dpc with many developmental defects (Saga, 1998). The defects included

underdeveloped smaller head folds and abnormal heart morphogenesis; complete or incomplete cardia bifida. Because *Mesp1* was expressed in the nascent mesoderm at the onset of gastrulation, we assumed this abnormality might be due to a precursor cell defect. In order to investigate the behavior of *Mesp1*-expressing cells during gastrulation in the *Mesp1*-null embryo, we introduced the *lacZ* gene under the control of the *Mesp1* promoter so that we could monitor its expression. Because the β -gal activity was retained for a longer period of time than the mRNA signal detected by in situ hybridization, we could trace the cell movement from the onset of gastrulation to the late streak stage. According to lineage studies using cell-marking methods or transplantation experiments, *Mesp1*-expressing cells may include extraembryonic and cranio-cardiac mesoderm (Parameswaran and Tam 1995; Tam et al., 1997). However, *Mesp1*-expressing cells colonized only the embryonic mesoderm proper and were never observed in the extraembryonic region. The anteriormost mesodermal cells expressing *Mesp1* migrated to the region generating the future heart field and this migration was markedly delayed in the *Mesp1*-null embryo. To demonstrate these results and to define the cell lineage of *Mesp1*-expressing mesodermal cells from the beginning of the expression to the final destination, we introduced Cre recombinase under the control of the *Mesp1* promoter-enhancer by homologous recombination and monitored the expression using reporter mice. The results strongly suggest that MesP1 is expressed in the heart precursor cells in addition to the extraembryonic mesoderm and is required for the migratory activity of mesodermal cells in the formation of a single heart tube.

MATERIALS AND METHODS

Vector construction and homologous recombination

Genomic clones for mouse *Mesp1* were isolated by screening a genomic library established in λ FixII from TT2 ES cell DNA with a *Mesp1* cDNA probe (Saga, 1998). To construct the knock-in vector for the β -galactosidase (*lacZ*) or Cre recombinase (*Cre*) genes, an 8 kb DNA fragment spanning the *Hind*III site of the 5' upstream region and the *Bam*HI site of exon 1 was subcloned into Bluescript (SK+) to make *HB-8.0*. A *lacZ* or *Cre* gene and the *pgk-neo* cassette were ligated in frame in the *Nco*I site located at the translational initiation site for the MesP1 protein (to generate *HB-lacZ-neo* or *HB-Cre-neo*). For the short homology arm, a 1 kb *Xba*I fragment starting from exon-2 and extending to the downstream genomic region was ligated with the *pgk-DT-A* cassette to construct *X1.0-DT-A*. The fragment containing *X1.0-DT-A* was ligated with *HB-lacZ-neo* or *HB-Cre-neo* to construct targeting vectors, *Mesp1*- β -gal or *Mesp1*-*Cre* (Fig. 1). These vectors were linearized at the *Not*I site and introduced into TT2 ES cells [C57BL/6(B6) /CBA] by electroporation as described previously (Yagi et al., 1993). After selection with G418, resistant clones were isolated and their DNAs were analyzed by PCR using a neo-specific primer PGK-R (for *Mesp1*- β -gal) or NeoAL (for *Mesp1*-*Cre*), and a *Mesp1* genomic primer GR1. The sequences for these primers were

PGK-R: 5'-CTAAAGCGCATGCTCCAGACT-3',

NeoAL: 5'-GAAAGAACCAGCTGGGGCTCGAG-3' and

MesP1-GR1: 5'-ATATGCCAAGTCATTGAGGTGAGCTTTC-3', respectively.

Generation of chimeras

Embryo manipulations and injection of the ES cell clones into ICR 8 cell embryos were carried out as described previously (Yagi et al.,

1993). Chimeric mice with a high contribution of the TT2 genetic background (monitored by agouti coat color) were bred with ICR mice.

Embryo analysis by β -gal staining, whole-mount in situ hybridization, whole-mount immunohistochemical staining and immunohistochemistry of sections

The fixed embryos at various developmental stages were stained for the detection of β -galactosidase activity as described previously (Saga et al., 1992). For whole-mount in situ hybridization, the basic method was also described previously (Saga et al., 1996), although no glycine treatments were performed after Proteinase K treatment. Whole-mount immunohistochemical staining was performed according to the method of Yoshida et al. (1996) with some modifications. Embryos were fixed by microwave irradiation (600 w) for 30 seconds in 4% paraformaldehyde in PBS and then postfixed in the same solution for 1-2 hours at 4°C. After stepwise dehydration with 25, 50, 75 and 100% methanol in PBS, specimens were soaked in methanol-30% H₂O₂ (98:2) for 1-4 hours at 4°C, washed with 100% methanol and stored at -20°C until use. The method for staining was the same as described by Yoshida et al. (1996). For immunohistochemistry of 6 μ m thick sections, samples were cut and placed on two sets of glass slides for staining with antibodies against muscle-specific protein, i.e., α -sarcomeric muscle actin (HHF35, DAKO A/S) and desmin (intermediate filament in muscle) (Progen BioTec, Heidelberg, Germany). Before incubation with antibodies, the endogenous peroxidase activity was quenched with NaIO₄. Immunohistochemical staining was performed by incubating with the primary antibodies diluted in PBS with 0.1% bovine serum albumin (HHF35 and desmin, 1:1000 for each) for 2 days at 4°C. Sections were then rinsed and immunoreactivity was detected with an ABC kit (Vectastain, Vector, USA). The sections were counterstained with hematoxylin.

RESULTS

Mesp1-null mice show defective heart morphogenesis

As described previously, the *Mesp1*-null embryos exhibited abnormal heart morphogenesis (Saga, 1998). In comparison with the wild-type embryo, the overt morphogenesis of the *Mesp1*-null embryo is delayed after 7.5 dpc and it basically stops growing after reaching the size corresponding to the wild-type 8.5-9.0 dpc embryo. The morphogenetic progression is also delayed in comparison with wild-type littermates. Normally by 8.5 dpc in the wild-type embryos, the lateral wings of the splanchnic mesoderm with the underlying endoderm have migrated ventrally at the level of the heart field to develop into the foregut and the centrally located single primitive heart tube (Fig. 2A,B). However, at the same stage, the *Mesp1*-null embryos still had a horseshoe-shaped field anterior to the buccopharyngeal membrane (Fig. 2C). Although the foregut was closed ventrally and the center of the heart field was fused at the midline, heart anomalies, including two separated heart tubes (Fig. 2), were seen. Wild-type embryos at 9.0 dpc formed a single heart tube with a normal d-loop, showing an atrium, an atrio-ventricular canal, prospective left and right ventricles and an outflow tract (Fig. 2E-G). Development of the myocardium was good and abundant cardiac jelly between the myocardium and endocardium was seen (Fig. 2G). In contrast, homozygous MesP1-null embryos at 9.5 dpc showed various anomalies of heart tube formation and looping (Fig. 2E,F,I-K). The heart tubes ranged from two completely separated tubes (Fig. 2E,F)

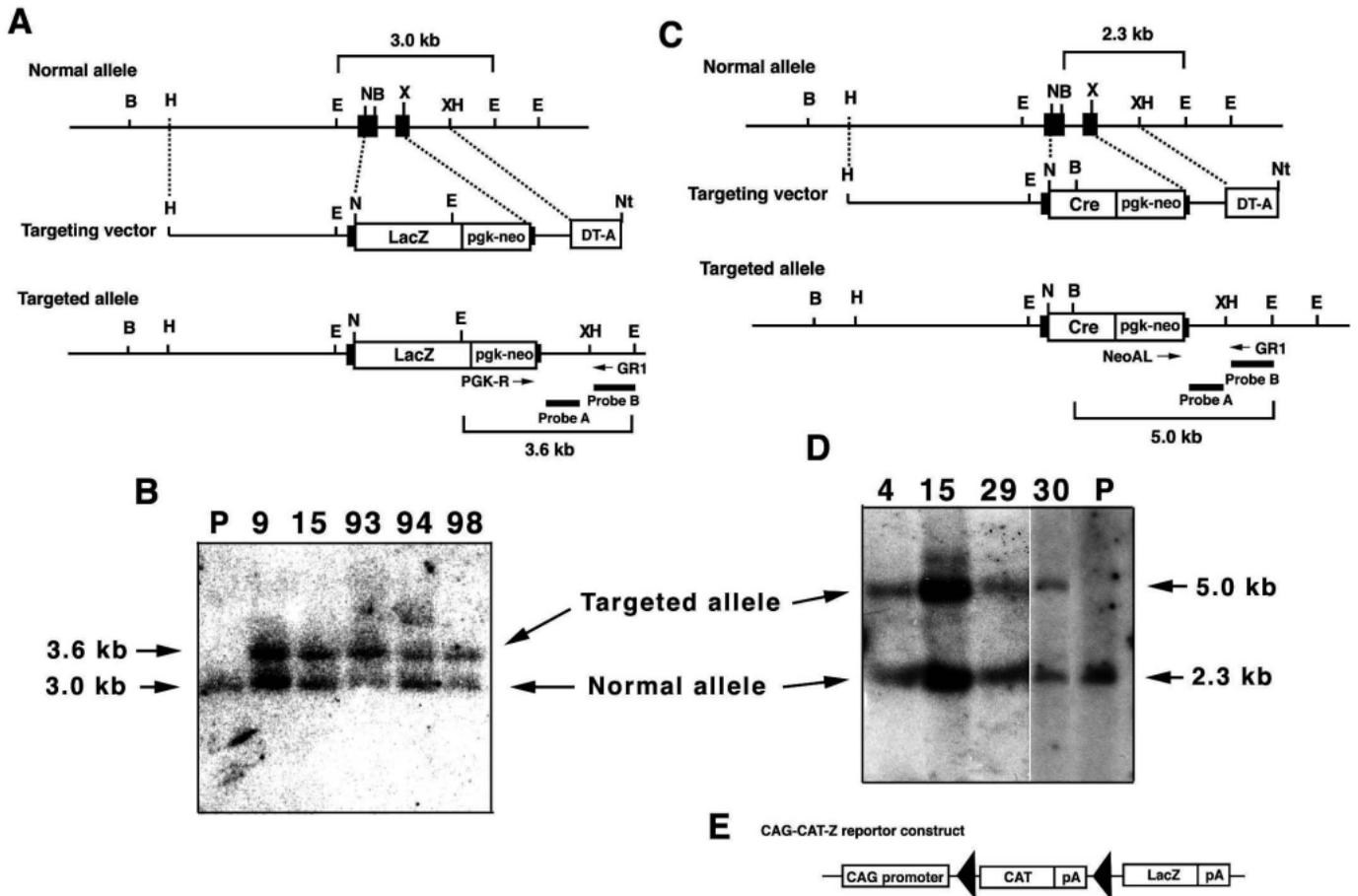


Fig. 1. Introduction of a *lacZ* reporter gene or *Cre recombinase* gene under the control of the *Mesp1* promoter by homologous recombination. (A) Schematic representation of the targeting strategy for *Mesp1*- β -gal. The *Mesp1* coding region was replaced by the *lacZ* and *pgk-neo* cassette. A genomic fragment which extends from the *Hind*III to *Nco*I sites (about 7 kb) and a 1 kb *Xba*I fragment served as the long and short homology arms, respectively. Black boxes represent *Mesp1* exons. Arrows indicate PGK-R and GR1 primers used for PCR. Thick lines represent probes A and B used for the Southern blot analysis. (B) Genomic Southern blot analysis of DNA from G418-resistant ES cell clones. Probe A was used here. P represents parental TT2 ES cells. Arrows indicate the 3.6-kb *Eco*RI fragment of the mutant allele compared with the wild-type 3.0 kb fragment. (C) Schematic representation of the targeting strategy for *Mesp1*-Cre. A similar strategy to that for *Mesp1*- β -gal was used. NeoAL and GR1 primers were used for PCR. Probe A and B represent probes used for Southern analysis. (D) Genomic Southern blot analysis. Probe C was used here. P represents parental TT2 ES cells. Arrows indicate the 5.0 kb *Eco*RI-*Bam*HI fragment of the mutant allele compared with the wild-type 2.3-kb fragment. (E) Schematic representation of a transgene used for the generation of reporter transgenic mouse (Sakai and Miyazaki, 1997).

to single tube (Fig. 2I-K). The separated tubes showed various degrees of cardia bifida, from full (Fig. 2F) to partial bifurcations (data not shown). The two separated small heart tubes had randomized looping (Fig. 2E,F,H). Embryos with a single heart tube also displayed several variations of looping (Fig. 2I-K). Serial sections of these heart tubes revealed that there were middle-line tubes joined to form a single aortic sac cranially and single or double sinus venosus caudally (Fig. 2L,M and data not shown). However, these hearts showed well formed myocardium as indicated by positive reactions for α -sarcomeric muscle actin (HHF35) (Fig. 2L,M) and desmin (Fig. 2N). Trabeculations in the ventricles were well formed and abundant cardiac jelly was seen, as in normal hearts (Fig. 2L-N). Therefore, cellular differentiation accompanied by heart tube formation occurs normally even in *Mesp1*-null embryos. The expression of several marker genes for heart development was also examined. Because *eHand* and *dHand* expression was known to be a regional

cardiac marker in mouse heart (Heikinheimo et al., 1994; Biben and Harvey, 1997), we examined several mutant embryos. The initial bilateral expression of *eHAND* is known to be changed to a left-sided asymmetrical pattern after 9.0 dpc during heart tube looping (Fig. 2E). However, such an asymmetric pattern was not evident in any *Mesp1*-null embryo, although some locally intense regions were occasionally observed in each separated heart tube (Fig. 2E). Because the morphogenesis of *Mesp1*-null embryos is delayed about 1 day compared with wild-type embryos, they may not have reached the stage at which *eHand* expression becomes asymmetrical. Unfortunately, the mutant embryos die before 10.5 dpc, so we cannot follow the change any further. We could not see biased expression of *dHand* even in wild-type embryos, but expression was observed in two small separated heart tubes in *Mesp1*-null embryos (Fig. 2F).

Since *Mesp1* expression occurs in the nascent mesoderm in embryos as early as 6.5 dpc after the onset of gastrulation, this

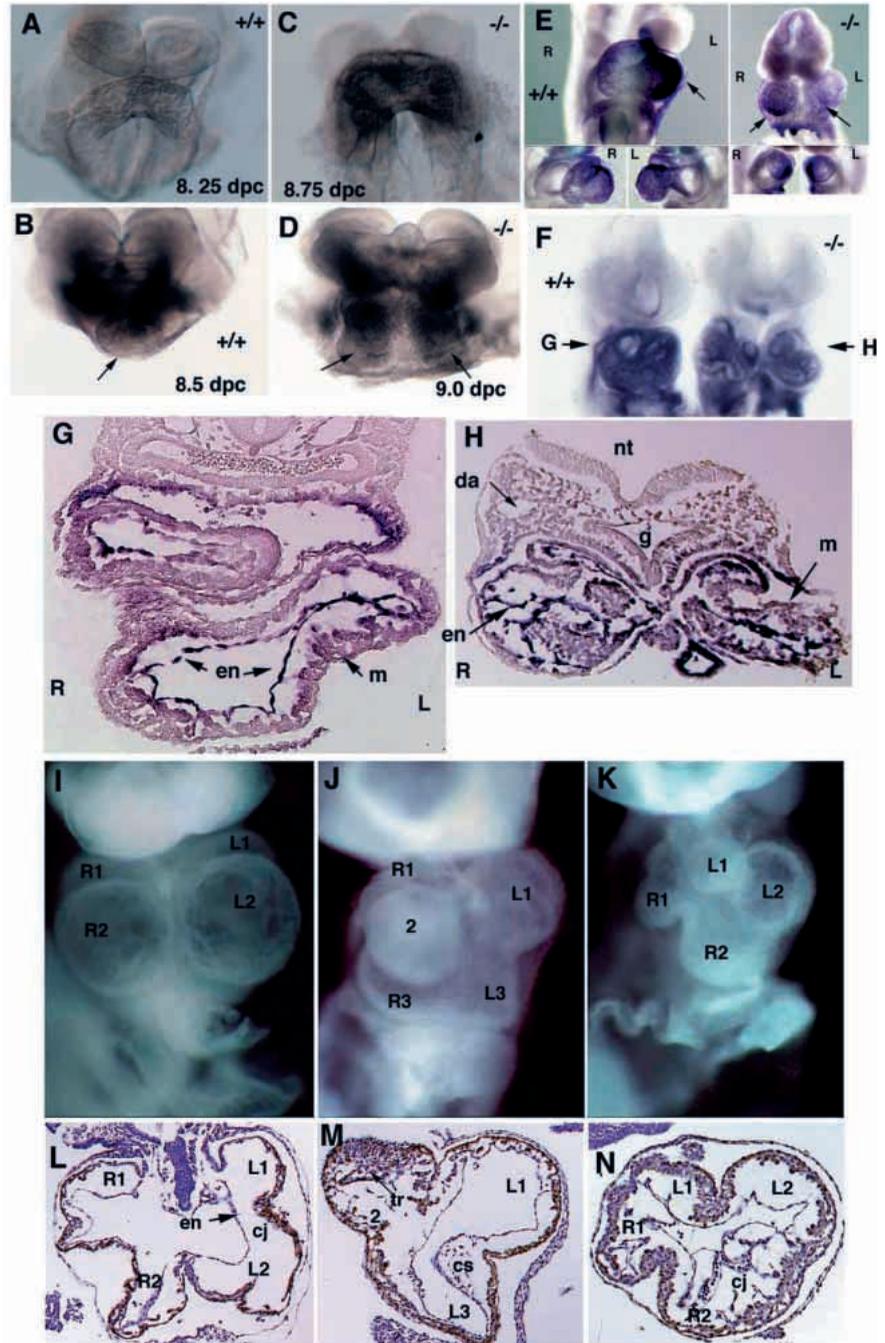


Fig. 2. Abnormal heart morphogenesis in *Mesp1*-null embryos. (A-D) Frontal view showing the comparison of heart development between wild-type embryos, with a single heart tube (A:8.25 dpc and B:8.5 dpc) and the *Mesp1*-null embryo, with two separated heart tubes (C:8.75 dpc and D:9.0 dpc). Embryos were photographed after fixture. Arrows indicate heart tubes. (E) Whole-mount in situ analyses showing *eHand* expression at 9.5 dpc embryos. Right- and left-side views of heart tubes are also shown. Arrows indicate stronger expression sites. (F) *dHand* expression in wild-type and *Mesp1*-null embryos. (G,H) Transverse sections of the embryos shown in F. *dHand* was expressed in the endocardium of the wild-type embryo with a normal d-looping heart tube at 9.0 dpc (G) and in the *Mesp1*-null embryo with two heart tubes showing randomized looping at 9.5 dpc (H). (I-K) Morphological heart anomalies in *Mesp1*-null embryos at 9.5 dpc. Frontal view. Note one heart tube showing randomized looping. Each number (R1-R3, L1-L3) indicates comparable chambers. (L-N) Frontal sections of the hearts, depicted in I-K, stained with HHF35 (L, transverse view; M, front-sagittal view) and desmin (N, frontal view). Development of myocardial trabeculation (tr) was good and abundant cardiac jelly (cj) was seen. One heart showed good development of cushion tissue (M). R; right side, L; left side, cs; cushion tissue, da; dorsal aorta, en; endocardium, g; foregut, m; myocardium, nt; neural tube.

morphological and functional anomaly must be due to the early mesodermal defects. In order to investigate the cause of this defect and to understand the behavior of *Mesp1*-expressing cells in *Mesp1*-null embryos, we placed the *E. coli lacZ* gene under the control of the *Mesp1* promoter, which allowed us to identify *Mesp1*-expressing cells by β -gal staining.

Introduction of a *lacZ* gene under the control of the *Mesp1* promoter

A knock-in vector was constructed to initiate *lacZ* translation at the same initiation site as the *Mesp1*-coding sequence (Fig. 1A). The linearized vector was introduced into TT2 ES cells. Of 69 G418-resistant ES cell clones, five homologous recombinant clones were selected by PCR and verified to have

the correct homologous recombination by Southern blot analysis (Fig. 1B). Clones 9 and 15 were injected into ICR 8 cell embryos and chimeric mice were obtained from both clones. Germline transmission was observed from both chimeric mice. At first, we examined whether β -gal expression reflects endogenous *Mesp1* expression in the targeted locus.

As described previously, the expression of *Mesp1* was observed initially at the onset of gastrulation by whole-mount in situ hybridization (Saga et al., 1996 and Fig. 3G). Strongly β -gal-stained cells were also observed between the extraembryonic and proximal end of the embryonic ectoderm (Fig. 3A). This initial expression pattern monitored by β -gal staining was identical to that observed by in situ hybridization (compare Fig. 3A and G). Transverse sections revealed that

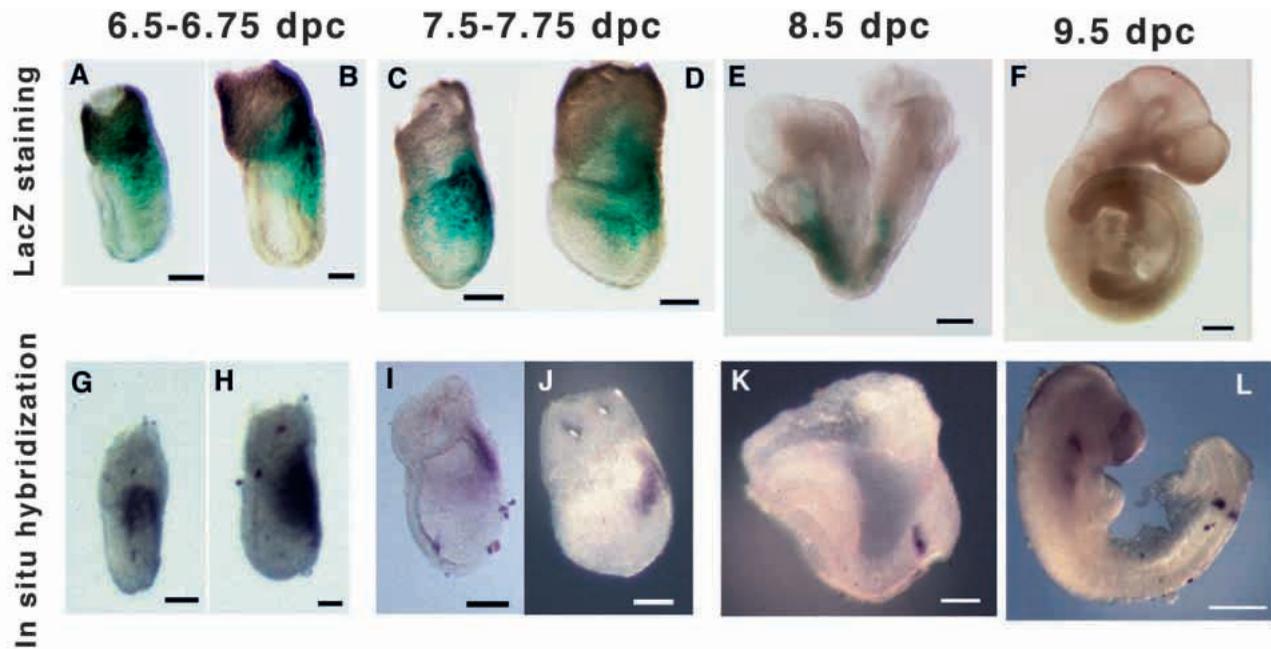


Fig. 3. Comparison of *Mesp1* expression monitored by β -gal staining (A-F) and whole-mount in situ hybridization (G-L). All embryos lateral view. Anterior is to the left. β -gal staining was conducted for 3 hours except for F (18 hours). Developmental stages are indicated at the top of each figure. Bars indicate 100 μ m for A,B,G and H, 200 μ m for C,D,I and J, 300 μ m for E and K, and 500 μ m for F and L.

Mesp1 was expressed in the mesodermal cells that had initially ingressed through the primitive streak (Fig. 4A). Once the initial expression began, most mesodermal cells ingressing through the primitive streak also expressed *Mesp1*. The resulting expression pattern formed a wedge shape, typical of mesodermal cells at the midstreak stage (Fig. 3B,C). This pattern was also observed in the in situ study (Fig. 3H). Transverse sections through these embryos from the distal to the proximal end clearly showed that the stained cells were all mesodermal and included the ingressing primitive-streak cells but not ectodermal or endodermal cells (see Fig. 4B). The initial expression of *Mesp1* is down-regulated as shown by the in situ hybridization study, in which the signal was observed only at the base of the allantois, indicating its expression persisted in this small cell group (Fig. 3I,J). Because β -gal staining detects enzyme activity, however, it persisted even after transcription of *Mesp1* ceased. Therefore, we could follow the cell lineage over a longer period of time by this method than by in situ hybridization. β -gal-stained cells migrated anteriorly and laterally as the gastrulation progressed (Fig. 3C,D). The anteriormost cells, the group that ingressed first, extended to the far anterior region where the presumptive head fold was formed. As revealed by transverse sections, the most distal mesodermal cells destined for the axial notochord and the anteriormost mesodermal cells were not stained (Fig. 4C). By in situ analysis, the second expression domain at the presomitic mesoderm started after the early expression ceased (Fig. 3K,L). However, we detected hardly any β -gal expression in the presomitic mesoderm at 8.5 dpc (Fig. 3E). The staining decreased, and very weak β -gal staining was observed after longer incubation (18 hours) with X-gal substrate at 9.5 dpc (Fig. 3F). Thus, this knock-in strategy may not accurately reproduce the expression in presomitic mesoderm and testis (data not shown, see Discussion). In order to determine that the

β -gal staining observed in the cells located in the anterior region was not due to de novo expression of β -gal and rather reflected cell migration of mesodermal cells previously located in the primitive streak, we conducted in situ hybridization using a *lacZ* probe. The signal was localized only at the base of the allantois and was similar to that detected by the *Mesp1* probe (data not shown). Thus we concluded that the movement of the β -gal staining pattern observed in the developing embryo reflected the migration of mesodermal cells from the primitive streak. However, we did not observe notable migration of β -gal stained cells in the extraembryonic region (Fig. 3C) though *Mesp1* expression was observed in the early ingressed group of mesodermal cells which may contribute to extraembryonic mesoderm (Fig. 3A). To address this question and identify the lineage of *Mesp1*-expressing cells, we used the *Cre-loxP* site-specific recombination system.

Lineage analysis of *Mesp1*-expressing cells

A knock-in vector was constructed so that Cre recombinase is expressed under the control of the *Mesp1* promoter-enhancer similar to the *lacZ* introduction (Fig. 1C). The linearized vector was introduced into TT2 ES cells. Of 105 G418-resistant ES cell clones, four homologous recombinant clones were selected by PCR and verified by Southern blot analysis (Fig. 1D). Clone 30 was injected into ICR 8-cell embryos and a germline chimera mouse was obtained. The mouse was mated with a CAG-CAT-Z reporter mouse (Sakai and Miyazaki, 1997) to know the gene expression and to follow the fate of the cells expressing *Mesp1*. On crossing heterozygous *Mesp1-Cre* and reporter mice, one quarter of the progeny should consist of double transgenics. Only cells that express Cre from the *Mesp1* allele should undergo recombination between the loxP sites of the reporter construct, excising the CAT gene and permitting β -gal expression (Fig. 1E). As the Cre-mediated excision is cell

heritable, the marked cells and all their progeny should express β -gal at later stages even after Cre is no longer expressed. Thus, β -gal staining in the double transgenic embryo should reveal the progeny of all cells that expressed Cre transiently during development. Intercrossed embryos examined at 9.5 dpc showed strong β -gal staining exclusively in the heart tube and the amnion contiguous with closing foregut (Fig. 5D). A few β -gal-positive cells were also observed in the head region. Interestingly, the cross section revealed that the β -gal staining was observed only in the myocardium and not in the endocardium (Fig. 5E), indicating that these cells have different lineages upon gastrulation when the *Mesp1* expression was initiated. The β -gal staining observed in the amnion indicates that extraembryonic cells were also β -gal positive as expected from the initial *Mesp1* expression pattern. To investigate the lineage of cells expressing *Mesp1* in more detail, earlier embryos (7.5 dpc) were examined. Interestingly, extraembryonic mesoderm in addition to lateral embryonic mesoderm was positive for β -gal staining (Fig. 5A), while paraxial and axial mesoderm appeared to be negative when seen from the posterior view (Fig. 5C) as expected from the β -gal pattern of 9.5 dpc embryos (Fig. 5D). The expression pattern contrasts with the β -gal staining pattern of *Mesp1*- β -gal embryos in which no β -gal expression was observed in the extraembryonic region (compare Fig. 5A with 3C). Therefore, we concluded *Mesp1* was activated in the extraembryonic mesoderm first, and then in the heart precursor cells.

Next, using *Mesp1*- β -gal mice, we analyzed the behavior of *Mesp1*-expressing cells in *Mesp1*-null embryos.

Reduced cell migratory activity of *Mesp1*-expressing cells in *Mesp1* deficient embryos

To allow the comparison of the activity from a single copy of the *lacZ* gene in *Mesp1*-expressing cells in homozygous *Mesp1*-deficient embryos (*Mesp1*^{lacZ/-}) with that in heterozygous embryos (*Mesp1*^{lacZ/+}), *Mesp1*^{lacZ/+} mice were crossed with *Mesp1*^{+/-} mice established previously (Saga, 1998). A difference in β -gal staining between *Mesp1*^{lacZ/+} and *Mesp1*^{lacZ/-} embryos was clearly shown to occur at the same time as the morphological difference became obvious at 7.5-7.75 dpc. In *Mesp1*^{lacZ/+} embryos, *Mesp1*-expressing cells ingressed through the primitive streak at an earlier stage, migrated far away from the primitive streak and reached the anterior end where the heart precursor cells are located (Fig. 6A). In contrast, *Mesp1*-expressing cells in *Mesp1*^{lacZ/-} embryos did not actively migrate from the primitive streak but rather remained in the primitive streak (Fig. 6C,D). Furthermore, the level of β -gal

expression clearly differed between the embryos. β -gal staining gradually decreased in *Mesp1*^{lacZ/+} embryos due to the down-regulation of *Mesp1* normally observed after 7.5 dpc (Fig. 6B) and almost disappeared from the cells in the primitive streak at 8.25 dpc (Fig. 6E,G). In *Mesp1*^{lacZ/-} embryos, however, strong β -gal activity was observed even in 8.25 dpc embryos (Fig. 6F). This is not only due to the stability of the β -gal enzyme, but also due to continued *lacZ* transcription, because a *lacZ* RNA probe strongly hybridized giving a similar pattern to that of the β -gal staining (Fig. 6H). A cross section of a *Mesp1*^{lacZ/-} embryo revealed the abnormal accumulation of β -gal-positive mesodermal cells in the primitive streak (Fig. 6;I-

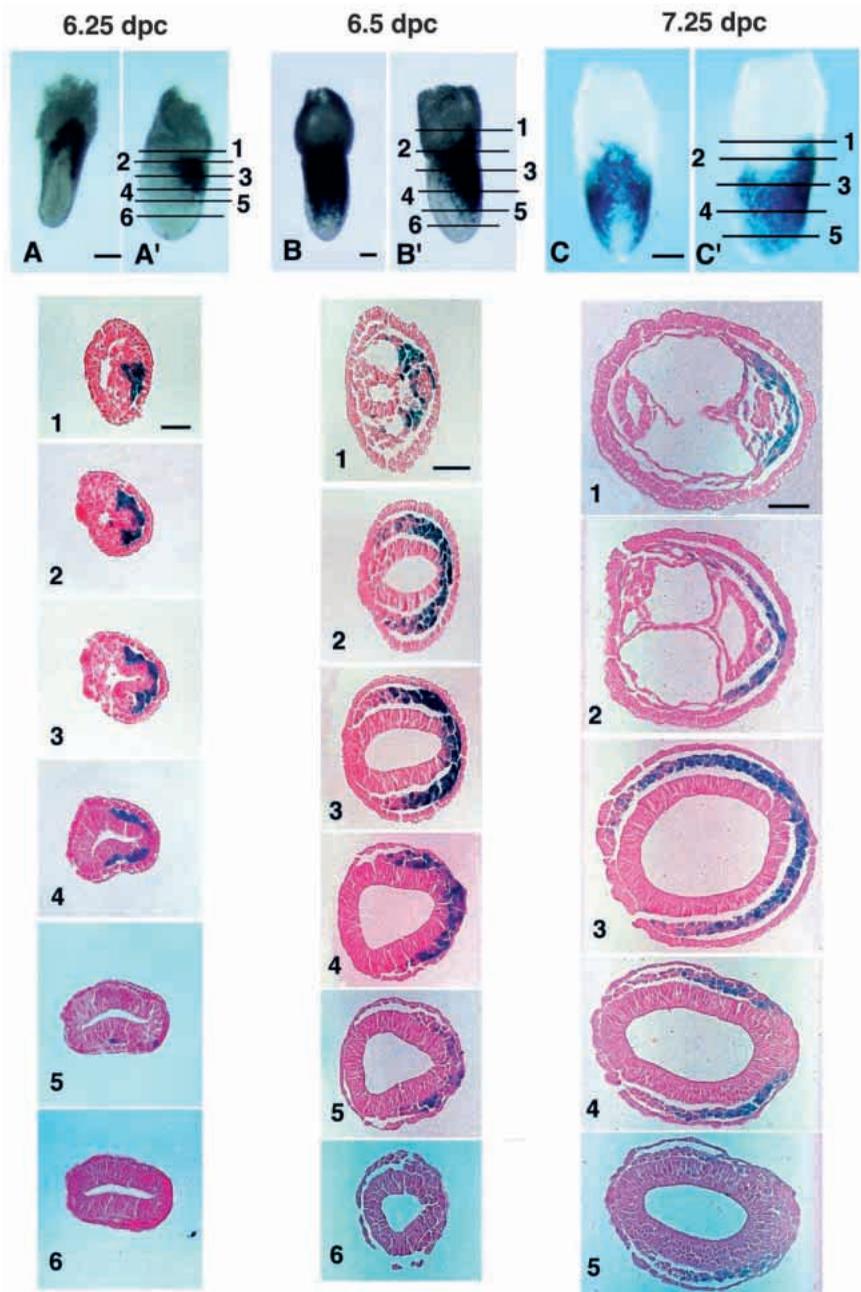


Fig. 4. β -gal stained *Mesp1*^{lacZ/+} embryos at the various developmental stages as indicated. The sectioning planes are indicated on the whole mounts at the top. The transverse sections were counterstained with eosin. Bars represent 100 μ m (A,B) or 200 μ m (C). Anterior is to the left.

5). Therefore, the major defect of the *Mesp1*-null embryo is concluded to be the reduced migratory activity of the mesodermal cells.

Altered genetic activity in gastrulating *Mesp1*^{-/-} embryo

The loss of *Mesp1* expression resulted in defective mesodermal migratory activity, which may accompany defective primitive streak formation and affect other mesendodermal derivatives. To detect other defects in the *Mesp1*^{-/-} embryo in greater detail, several molecular markers were employed to examine the embryos. Because head fold formation was affected in the mutant embryo, we examined the genes involved in the initial head morphogenesis. Mouse *cereberus-like gene-1* (*cer-1*) was expressed in the primitive endoderm, prior to gastrulation, which was later replaced with definitive endoderm generated by gastrulation, and the expression gradually decreased from the anteriormost endoderm and disappeared before somitogenesis (Belo et al., 1997). *Cer-1* was expressed in the *Mesp1*^{-/-} embryo more strongly than in the wild type and no reduction in the level of expression was observed in 7.5 dpc embryos although the prolonged expression may be partly due to the developmental delay (Fig. 7B). Thus initial anterior identity and subsequent head identity were not disturbed in *Mesp1*-null embryos. Next we examined several genes involved in heart morphogenesis in which alterations were anticipated because of the cardiogenetic defects observed. FLK-1 is known as the marker for early endothelial cell precursors fated to become heart cells and is known to be expressed in the proximal-lateral embryonic mesoderm (Yamaguchi et al., 1993). The expression pattern of FLK-1 in *Mesp1*^{lacZ} embryos differs from that in the wild type (Fig. 7A), and is very similar to the β -gal staining pattern observed previously (compare with Fig. 6C). FLK-1-stained cells accumulated in the primitive streak and were not observed in the anterior head fold region, indicating reduced cell migration. However, the early expression patterns of other markers involved in heart morphogenesis, *GATA-4* (Fig. 7D), *eHand* (Fig. 7E) and *dHand* (data not shown) were not affected in the mutant, with strong expression being noted in the extraembryonic endoderm and part of the mesoderm. Because *Mesp1* is also expressed in the paraxial mesoderm from just prior to somitogenesis, we examined the development of the paraxial mesoderm by *Mox-1* (Candia et al., 1992) and *Dll1* (Bettenhausen et al., 1995) staining. As shown in Fig. 7F,G, the expression of both *Mox-1* and *Dll1* was greatly reduced in the *Mesp1*^{-/-} embryo. In contrast, a strong expression domain, an indication of paraxial mesoderm, was observed posterior to the head fold in wild-type embryos. To examine the axial mesoderm, we examined *Brachyury* expression (Herrmann et al., 1990). As expected from the lack of expression of *Mesp1* in the axial mesoderm of the wild-type embryo, *Brachyury* expression was not affected in *Mesp1*^{-/-} embryos (Fig. 7C). Similarly, normal *Brachyury* expression was obtained by staining in the later stages of *Mesp1*^{-/-}

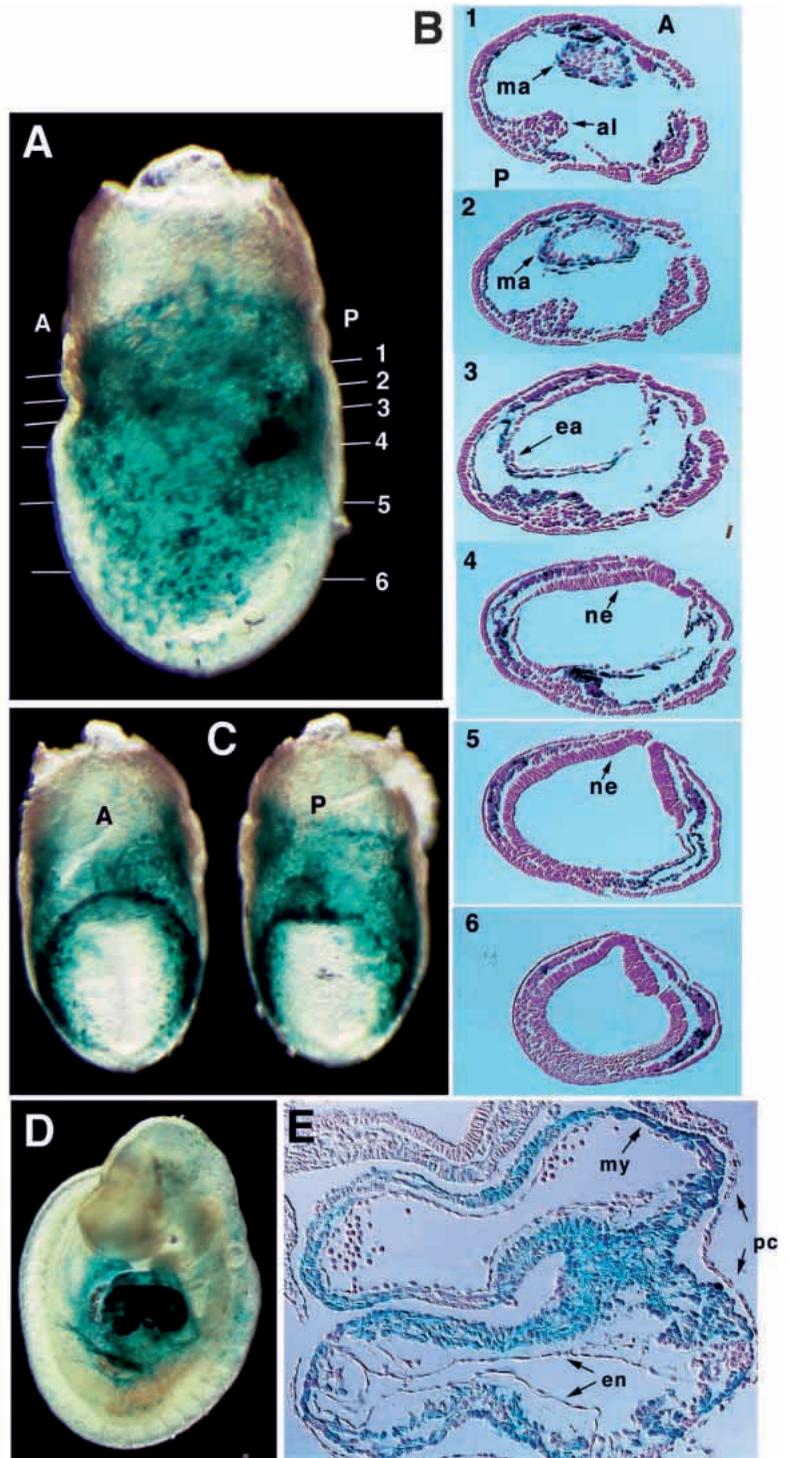


Fig. 5. Lineage analysis revealed by the β -gal expression directed by Cre recombinase introduced into the *Mesp1* locus. (A-C) 7.5 dpc embryo photographed from lateral (A), anterior (C, left) or posterior (C, right) sides. Cross sections are shown in B(1-5) from the proximal to the distal end of the embryo. Note that the extraembryonic mesoderm is positive for β -gal staining. (D,E) 9.5 dpc embryo showing strong β -gal expression in the heart area. The cross section which revealed β -gal expression was confined to the myocardium and does not show the endocardium. A, anterior; P, posterior; al, allantois; ea, ectodermal cells of the amnion; en, endocardium; ma, mesodermal cells of the amnion; my, myocardium; ne, neural ectoderm; pc, pericardium.

embryos (9.5 dpc) as described previously (Saga, 1998). Thus, although the development of the cardiac mesoderm and paraxial mesoderm were strongly affected in embryos lacking *Mesp1*, the axial mesoderm required to generate the notochord was generated normally, implying normal node formation.

DISCUSSION

MesP1, a bHLH transcription factor, is initially expressed in the early ingressing part of the mesoderm which is fated to become extraembryonic and cranial-cardiac mesoderm, and later in paraxial mesoderm to generate somites (Saga et al., 1996). *Mesp1* gene-disruption resulted in early death of the embryo before 10.5 dpc and was associated with several morphogenetic defects in the head and heart regions (Saga, 1998). These defects are attributed to the lack of *Mesp1* expression in the early mesodermal cell lineages. However, the expression was transient and it was difficult to follow the cell lineage using *Mesp1* RNA expression as a marker. In order to follow the cell fate and to identify the *Mesp1*-expressing descendant cells in *Mesp1*-null embryos, we introduced a *lacZ* gene under the control of a *Mesp1* promoter, because β -gal activity was expected to last longer than the *Mesp1* mRNA signal. Although β -gal activity did not last long enough to follow their lineage until the cells were incorporated in the heart tissues, we could observe that the *Mesp1*-expressing mesodermal cells had migrated into the anterior ventral region in which the cardiogenic precursor cells were known to be localized. Furthermore, using the Cre-loxP system, we could determine that the *Mesp1*-expressing mesodermal cells actually contained heart precursor cells. Thus, *Mesp1* is the earliest molecular marker for identifying heart precursor cells. Finally, using β -gal staining as a marker, we found that *Mesp1*-expressing cells accumulated in the primitive streak and that the cell migration activity was greatly reduced in *Mesp1*-null embryos.

Defective heart morphogenesis may be due to the delay in cell migration of mesodermal precursors into the heart field

In *Mesp1*-null mutant embryos, we consistently observed abnormal heart

morphogenesis resulting in complete or incomplete cardia bifida. It is clear from the work in the developing chick that failure of ventral fusion of the pericardial mesoderm results in a variety of cardiac morphogenetic defects including the development of one or two laterally displaced cardiac structures (Osmond et al., 1991; Gannon and Bader, 1995). A similar phenotype has been reported for GATA-4- or Furin-

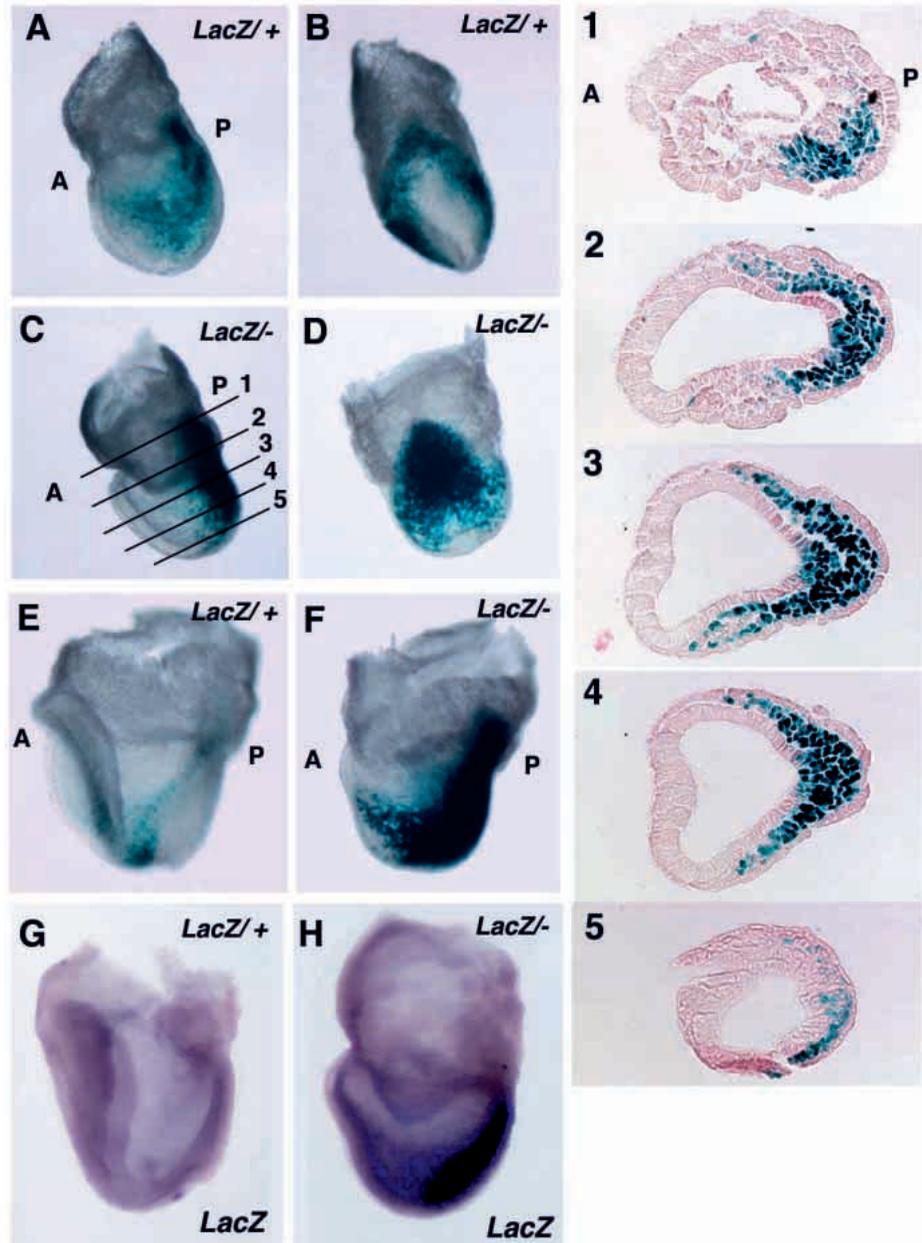


Fig. 6. Comparison of the migration pattern of *Mesp1*-expressing cells in *Mesp1^{lacZ/+}* and *Mesp1^{lacZ/-}* embryos. (A-F) β -gal staining revealed a difference in migratory activity of *Mesp1*-expressing cells. (G-H) Whole-mount in situ hybridization using *lacZ* as a probe. β -gal-stained cells migrated into the anterior region in 7.5 dpc *Mesp1^{lacZ/+}* embryo (A) and *Mesp1* expression was down-regulated in the primitive streak (B). In contrast, β -gal-stained cells accumulated in the primitive streak and cell migration was not apparent in *Mesp1^{lacZ/-}* embryo (C,D). 1-5: Transverse sections of C (sectioning planes are indicated in C). At 8.0 dpc, β -gal staining was greatly reduced (E) and no transcription was apparent in *Mesp1^{lacZ/+}* embryos (G). In contrast, strong β -gal activity (F) and transcription (H) were retained longer in *Mesp1^{lacZ/-}* embryos. A, anterior; P, posterior. (A, C, E-I) lateral view; (B, D) posterior view.

deficient mice, which have a generalized disruption of ventral body patterning (Kuo et al., 1997; Molkentin et al., 1997; Roebroek et al., 1998). In the absence of GATA-4 or Furin, the splanchnic mesoderm failed to migrate to the ventral midline and form the pericardial cavity and heart tube. In contrast, in *Mesp1*-null embryos, the splanchnic mesoderm can generate a horseshoe-shaped field anterior to the buccopharyngeal membrane and result in the generation of two heart tubes by the failure of the primordia to fuse. A major question was to what kind of mesodermal precursor does the *Mesp1*-expressing mesoderm correspond. The answer was obtained by lineage analysis using *Mesp1*-Cre and the reporter system. The cells expressing *Mesp1* at the onset of gastrulation migrate to the heart field and are incorporated into the heart tube. Therefore, *Mesp1* represents the earliest molecular marker of cells destined to generate the heart tube as far as we know. What is the mechanism that generates two heart tubes instead of one? We observed that *Mesp1*-expressing cells showed reduced migratory activity in the *Mesp1*-null mutant and this may be the cause of the heart anomalies. However, after some delay, the heart field was eventually generated even in the *Mesp1*-null embryo and only failed to form a single heart tube. Thus, one possibility is that the mesoderm involved in heart tube formation might be only a portion of the *Mesp1*-expressing mesodermal cells. Actually, only myocardium was stained for β -gal and it was contrasted with no staining in the endocardium. Therefore, there could be some difference in migration speed between the precursors of the myocardium and endocardium. The lack of coordination in migration and possibly in the time of differentiation between these two major components of the heart tube might be responsible for these heart abnormalities. Alternatively, the delay in migration of myocardium might affect on the other cells as well. This is suggested by the fact that the altered FLK-1 expression, which is known as an early marker of endothelial cell precursors including endocardium. Taken together, although MesP1 is not expressed in the differentiated heart cells, MesP1 is expressed in the myocardium precursors and is required for single heart tube formation.

Mesp1 may be involved in the lineage restriction of the heart precursors

Subsequent fusion of the bilateral heart primordia gives rise to a single tubular heart consisting of two epithelial layers: an outer myocardium and an inner endocardium. To date, it was uncertain whether these two distinct cell types of the heart arise from common or separate progenitor populations of mesodermal cells within the heart field. By retroviral single-cell-marking and tracking studies in chicken, it was suggested that the heart field mesoderm at stage 4 consisted of at least two distinct subpopulations, containing more premyocardial cells than preendocardial cells, indicating that the lineage diversification must

occur at or prior to the arrival of mesodermal cells to the heart field (Cohen-Gould and Mikawa, 1996). Regarding this problem, our data in which only myocardium was marked by β -gal directed by *Mesp1*-Cre is extremely interesting. Because *Mesp1* expression is initiated in very early gastrulation stage embryos no endocardium cells were marked by β -gal, and therefore *Mesp1* is expressed only in the precursor cells of the myocardium indicating that the lineage might be separated from the beginning and may not have a common mesodermal precursor. Alternatively, premyocardial cells were derived from common or endocardium precursors before *Mesp1* expression. The sections of 7.5 dpc double-transgenic embryos shown in Fig. 5 revealed some β -gal-negative mesodermal cells

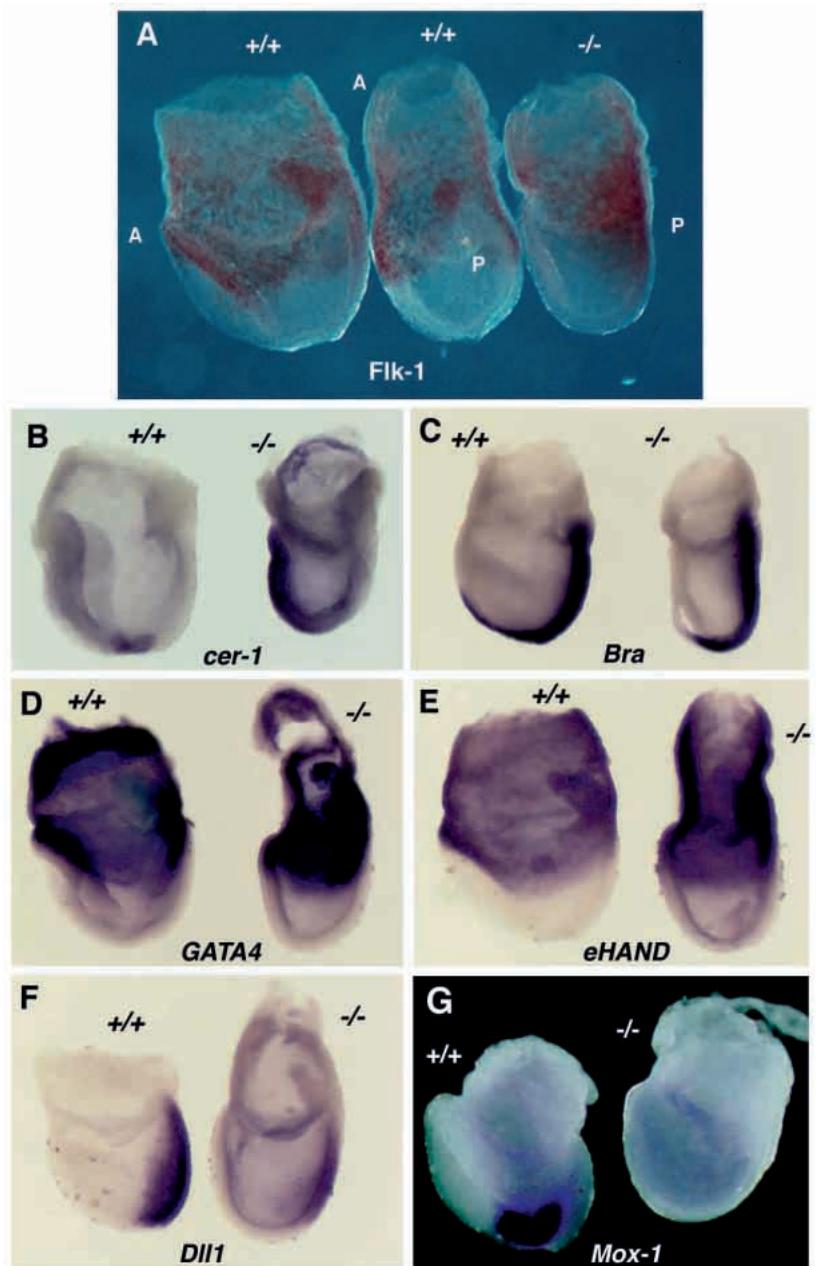


Fig. 7. Comparison of gene expression during gastrulation by whole-mount immunohistochemical analysis (A) or in situ hybridization (B-G) of 7.5 dpc embryos. Embryos were produced by intercrosses between *Mesp1* (+/-) heterozygous mice. Probes used and genotype of each sample are indicated.

among the β -gal-positive cells. The lineage of these β -gal-negative cells, and when and where these cells are derived, cannot be assessed because no good early marker to distinguish endocardial precursors in gastrulation stage embryos is available. However, the detailed analysis of this transgenic embryo may help to determine when these heart precursor cells are discriminated in the early gastrulation stage embryo and how these cells are incorporated in the heart tube upon their differentiation during heart morphogenesis.

MesP1 function in other mesodermal lineages

In addition to the defective migratory activity of mesodermal cells, the generation of paraxial mesoderm was delayed in *Mesp1*-null embryos. However this defect appears to be rescued later in development because *Mesp1*-null embryos eventually generated segmented somites as described previously (Saga, 1998). This rescue event may be due to the expression of the *Mesp2* gene, belonging to the same family as *Mesp1*, which starts just before somitogenesis in the paraxial mesoderm (Saga et al., 1997) and the function of these two genes has been demonstrated to be redundant during somitogenesis (Saga, 1998).

An interesting aspect of the *Mesp1*- β -gal expression is the apparent lack of contribution of *Mesp1*-expressing cells in the extraembryonic cell lineage. The earliest expression of *Mesp1* was observed in the newly formed mesoderm at the proximalmost end of the early-streak-stage embryo (Fig. 3G) and matched perfectly the prospective extraembryonic mesodermal lineage. According to the fate-mapping study, the precursors of the extraembryonic mesoderm were localized in the proximal region of the lateral and posterior epiblast, and there may be a progressive displacement of the mesodermal layers proximally and anteriorly during gastrulation (Lawson et al., 1992; Parameswaran and Tam, 1995). However, most *Mesp1*- β -gal-stained cells were distributed within the embryo as development progressed, and only a few were found in the extraembryonic tissues. This discrepancy could be resolved if the *Mesp1*-positive cells constituted only a subgroup of the cells within the proximal mesoderm that ingressed at the beginning of gastrulation. However, transverse sections of the early-streak-stage embryo revealed that the majority of mesodermal cells were stained (Fig. 4A). Another possible explanation is a difference in the β -gal stability between mesodermal cells destined for extraembryonic and embryonic lineages. Mesodermal cells in the embryonic lineage may retain β -gal activity for periods of time longer than those of the extraembryonic mesoderm. The answer was obtained by the lineage analysis using embryos intercrossed between *Mesp1*-Cre and CAG-CAT-Z, in which β -gal staining driven by *Mesp1*-Cre was observed both in extraembryonic and embryonic mesoderm. Therefore, *Mesp1* is really expressed in the initial mesoderm destined to contribute to the extraembryonic mesoderm and then the expression in embryonic mesoderm follows. The time difference must result in the apparent difference in the β -gal stability. The function of MesP1 in the extraembryonic component was not clearly shown in this study. The allantois was generated, but it was small and the truncation occasionally resulted in failure to connect with the chorion (Saga, 1998). Therefore, this disfunction might contribute to the early death of the MesP1-null embryo.

Regulation of transcription or translation may attenuate MesP1 function in later development

We previously described that *Mesp1* was also expressed at the rostral part of the presomitic mesoderm like the family gene *Mesp2* (Saga et al., 1997). In fact, MesP1 introduced into the *Mesp2* locus could rescue the *Mesp2* deficiency in a dosage-dependent manner, indicating the similarity of the functions of MesP1 and MesP2 during somitogenesis (Saga, 1998). However, *Mesp1*- β -gal and likewise *Mesp1*-Cre, did not induce β -gal expression in the presomitic mesoderm. This is very likely due to the variation in transcriptional initiation and subsequent translational initiation because, although *Mesp1* is strongly expressed in the adult mature testis, the transcription starts from far downstream sites compared with transcripts prepared from 7.0-7.5 dpc embryos and therefore may not generate a functional protein (Saga et al., 1996). We have not determined the transcriptional initiation site for the *Mesp1* gene in the presomitic mesoderm; however, embryos prepared from either *Mesp1*- β -gal or *Mesp1*-Cre did not show significant staining either in the presomitic mesoderm or in adult testis (data not shown), indicating that transcription in the presomitic mesoderm may occur in a similar way to that in testis. In contrast, in the other *Mesp1*- β -gal strain we generated by homologous recombination with targeting vectors in which the *lacZ* gene was fused with *Mesp1*-coding sequence after bHLH motif, we could see strong expression both in the presomitic mesoderm and the adult testis (data not shown), supporting the above idea. Therefore, functional full-length MesP1 may only exist in the early embryo during gastrulation. This could be part of reason why we did not see any segmentation defects during somitogenesis in *Mesp1*-null embryos (Saga, 1998).

MesP1 function in the primitive streak

Initial expression of *Mesp1* is observed in the most proximal region of the posterior side of the epiblast where the initial primitive streak activity appears to be localized. Thus, *Mesp1* must be one of the earliest genes expressed in response to a signal derived from the primitive streak. Upon the subsequent lengthening of the primitive streak in the midstreak embryo, newly ingressed mesodermal cells along with the primitive streak also express *Mesp1*. These mesodermal cells, however, stop expressing *Mesp1* once they depart from the primitive streak. In the later streak stage embryo, the mesodermal cells ingressing along with the more distal part of the primitive streak do not express *Mesp1*. Thus, the signal-inducing *Mesp1* expression must be down-regulated after *Mesp1*-expressing cells depart from the primitive streak. In *Mesp1*-null mice, however, *Mesp1*-expressing cells failed to depart from the primitive streak and continued to activate *Mesp1* transcription, resulting in the abnormal accumulation of *Mesp1*-expressing cells in the primitive streak. This phenomenon may reflect the persistence of the signal in the primitive streak in the *Mesp1*-null embryo, which is ordinarily inhibited by a feedback mechanism involving MesP1. The migration of prospective mesodermal cells through the primitive streak appears to be regulated by the activity of growth factors. The TGF- β and FGF families have been implicated in the induction of mesoderm migration in *Xenopus* (Cunliffe and Smith, 1994; Slack, 1994; Smith, 1995). In mice, blocking FGF activity by a mutation in FGFR1 resulted in the retention of the ingressed mesodermal cells at the primitive streak (Deng et al., 1994;

Yamaguchi et al., 1994; Ciruna et al., 1997). Mesodermal migration abnormalities are also associated with the *Brachyury* mutation in mice (Wilson et al., 1995), which may be mediated through alterations in FGF signaling. However, the factors that are essential for mesoderm induction in the mouse embryo remain essentially unknown. We analyzed the expression of several FGFs (*FGF-3*, *FGF-4* and *FGF-8*) in *Mesp1*-null embryos by whole-mount in situ hybridization, but no significant alteration in the expression pattern was observed for any FGF. Because *Mesp1* is expressed in the extraembryonic mesoderm ingressing first through the primitive streak, the mechanism inducing *Mesp1* expression is critically important for determining the signal involved in the initial mesoderm induction in mouse. Therefore, the detection of an enhancer of the *Mesp1* gene and the subsequent identification of and/or discrimination between molecules inducing the primitive streak, the mesoderm of different lineages or *Mesp1* will contribute to a better understanding of the mechanism of mouse gastrulation.

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