BMP signaling in the epiblast is required for proper recruitment of the prospective paraxial mesoderm and development of the somites

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Bmp1a encodes the BMP type IA receptor for bone morphogenetic proteins (BMPs), including 2 and 4. Here, we use mosaic inactivation of Bmpr1a in the epiblast of the mouse embryo (Bmpr-MORE embryos) to assess functions of this gene in mesoderm development. Unlike Bmpr1a-null embryos, which fail to gastrulate, Bmpr-MORE embryos initiate gastrulation, but the recruitment of prospective paraxial mesoderm cells to the primitive streak is delayed. This delay causes a more proximal distribution of cells with paraxial mesoderm character within the primitive streak, resulting in a lateral expansion of somitic mesoderm to form multiple columns. Inhibition of FGF signaling restores the normal timing of recruitment of prospective paraxial mesoderm and partially rescues the development of somites. This suggests that BMP and FGF signaling function antagonistically during paraxial mesoderm development.

KEY WORDS: Epiblast, Primitive streak, Paraxial mesoderm, Somites, BMP, FGF, Mouse

INTRODUCTION

Gastrulation begins around embryonic day 6.5 (E6.5), when the primitive streak, a transient structure, forms in the posterior epiblast at its junction with the extra-embryonic ectoderm. As gastrulation progresses, cells are recruited to intercalate between the proximal and the distal ends of the primitive streak, and ultimately the primitive streak extends to the distal tip of the egg cylinder (Tam and Behringer, 1997). In this case, ‘proximal’ refers to the end of the primitive streak closest to the extra-embryonic tissues. At the early to mid streak stage, primarily future extra-embryonic mesodermal cells are recruited to the proximal primitive streak and they are committed to yolk sac mesoderm (Kinder et al., 1999; Lawson et al., 1991; Parameswaran and Tam, 1995). By the late streak stage, prospective lateral plate mesoderm (LPM) and paraxial mesoderm (PXM) cells are recruited to the middle and anterior primitive streak, respectively, and become committed to trunk mesoderm (Kinder et al., 1999; Parameswaran and Tam, 1995; Tam and Quinlan, 1996). However, the molecular mechanisms involved in recruitment of the epiblast cells are largely unknown (Tam and Behringer, 1997).

It is now well established that BMP signaling regulates the development of many embryonic cell types, including the heart and hematopoietic tissues (Kishigami and Mishina, 2005; Mishina, 2003). The development of the lateral plate is also regulated by BMP signaling. Hensen’s node or the BMP antagonist Noggin can generate somites from lateral plate cells (Hornbruch et al., 1979; Tonegawa and Takahashi, 1998). Thus, BMP signaling plays a role in patterning the LPM. In addition to BMPs, other growth factors are crucial for somitic development. For example, fibroblast growth factor receptor 1 (Fgfr1) deficient embryos do not form somites, and FGF signaling is involved in the development of the PXM lineage (Ciruna and Rossant, 2001; Yamaguchi et al., 1994). Thus, one can speculate that there is a high potential for interaction between BMP and FGF signaling during mouse mesoderm development. However, little is known about how they might interact to regulate paraxial versus other mesodermal tissues.

Bmpr1a encodes the type 1 BMP receptor BMPR1A, which is known to be a receptor for BMP2 and BMP4 (Mishina, 2003). These molecules constitute a major BMP signaling component in early mouse development. However, because Bmpr1a-null mutant embryos do not initiate gastrulation (Mishina et al., 1995), the function of Bmpr1a during gastrulation in mice is largely unknown. To elucidate functions of BMP signaling through BMPR1A during and after gastrulation, we have generated a conditional allele of Bmpr1a (Mishina et al., 2002). The Mox2-Cre (MORE) strain drives mosaic Cre-mediated recombination in the epiblast, providing a useful tool for reducing but not completely abolishing gene expression in the epiblast (Hayashi et al., 2002; Talilquist and Soriano, 2000). For simplicity, we refer to embryos in which Bmpr1a is ablated as a result of MORE activity as Bmpr-MORE embryos. Our previous studies using Bmpr-MORE embryos showed that the anterior neural ectoderm was enlarged at the expense of surface ectoderm, and that endodermal morphogenesis was defective (Davis et al., 2004). Here, the development of mesodermal tissues is studied in Bmpr-MORE embryos. The results indicate that Bmpr1a in the epiblast is required to regulate the recruitment of prospective PXM cells correctly and consequently to direct normal somite development. Our results also indicate that BMP and FGF signaling interact for proper development of the mesoderm during gastrulation.

MATERIALS AND METHODS

Mouse

The strain information and genotyping procedures were described previously (Davis et al., 2004). All mouse experiments were performed in accordance with NIH and institutional guidelines covering the humane care and use of animals in research.
Whole-mount in situ analysis

Whole-mount in situ was performed as described (Belo et al., 1997). Probes used were brachury (Wilkinson et al., 1990), Lefty2 (Menon et al., 1999), Foxf1 (Mahlapuu et al., 2001), Bmp4 (Winnerin et al., 1995), Lim1 (Tsang et al., 2000), Mox1 (Candia et al., 1992), Uncx4.1 (Leite et al., 2000), Pax1 (Wallin et al., 1996), Foxa2 (Sasaki and Hogan, 1994), Tbx6 (Chapman et al., 1996), Pdcdh8 (Yamamoto et al., 2000), Epha4 (Durbin et al., 1998) and noggin (McMahon et al., 1998).

Cell lineage analysis

Embryos were harvested in dissecting medium (Miura and Mishina, 2003). Cell lineage analysis was carried out as described previously (Wilson and Beddington, 1996). Briefly, a 0.5% stock solution of 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) (Molecular Probes) in 100% ethanol was diluted tenfold with 0.3 M sucrose before use. DiI solution (0.05%) was sucked into a glass needle of 25 μm diameter (HUMAGEN). The tip of the needle softly touched the posterior region of the embryo, tore through the visceral endoderm and then DiI was released to label the mesoderm. These embryos were cultured with DR50 in a rotating glass bottle (BTC engineering) supplied with 5% O2 and CO2 balanced with N2 at 37°C. After culture, embryos were placed in phosphate-buffered saline (PBS) and observed with a stereo-dissection microscope (Leica) under visible or ultra violet light, and imaged.

Inhibition of FGF signaling

SU5402 (CALBIOCHEM) was suspended in dimethyl sulfoxide (DMSO) (Sigma) as a 10 mM solution and kept at –20°C until use. E6.5-6.75 embryos were cultured with DR50 containing either SU5402 or DMSO for indicated lengths of time in rotating glass bottles.

Immunohistochemistry

Cultured embryos were washed in cold PBS and fixed with 4% paraformaldehyde overnight, dehydrated and embedded in paraffin. Sections (7 μm) were deparaffinized and stained with anti-phosphoErk1/2 antibody (Cell Signaling Technology) using an ABC kit (Vector Laboratories) according to manufacturers’ protocols.

RESULTS

Bmp-MORE embryos show multiple defects in mesodermal development

Bmp-MORE embryos initiated gastrulation and were morphologically indistinguishable from control embryos at E6.5-6.75 (Davis et al., 2004). However, morphological defects were apparent in Bmp-MORE embryos starting at E7.5. At the allantoic bud stage, control embryos had a distinctly formed amnion and chorion (Fig. 1A), while mutant embryos were characterized by a poorly formed amniotic fold and acute posterior curvature (Fig. 1A’). Amnion and chorion were not formed yet at the head-fold stage in Bmp-MORE embryos (Fig. 1B’). In addition, a typical cardiac crescent did not develop in Bmp-MORE embryos (Fig. 1C,C’ arrowheads). At E8.5, multiple defects were observed, including a striking lateral expansion of somites (Fig. 1D,F’).

Histological sectioning at E8.5 revealed important aspects of these and other mesodermal phenotypes. In control embryos, somites developed as a single column on each side of the neural tube (Fig. 1G,F’). In Bmp-MORE embryos, ectopic mutant somites developed and extended laterally to form multiple irregular columns (Fig. 1G,F’). The mutant somites formed typical ball-like epithelial-mesenchymal structures (Fig. 1G,F’). In control embryos, LPM formed lateral to somites and medial to visceral yolk sac (Fig. 1E,G’). In Bmp-MORE embryos, lateral plate was apparently lacking, but cell masses were observed anterior and lateral to the paraxial somites and medial to the yolk sac, suggesting abnormal development of LPM (Fig. 1F,G’). The heart formed at the most anterior end of normal embryos, but no analogous structure formed in Bmp-MORE embryos (Fig. 1G,G’). Notochord was formed and expressed markers such as sonic hedgehog (Fig. 1G’) (Davis et al., 2004). The amnion was formed at this stage, but the allantois was notably small in the mutant embryos (Fig. 1H’).

Bmp-MORE embryos were mosaic for recombinated Bmpr1a−/− (mutant) cells and heterozygous cells (Davis et al., 2004). It is therefore important to know if both cell types distribute evenly or unevenly during development among all three germ layers. If there were a bias in their distribution, it might affect the mutant phenotype. We used a mouse line R26R to visualize mutant and heterozygous cells (Soriano, 1999). This is a reporter line for Cre recombinase, which marks those cells that have undergone recombination by expression of lacZ. As observed previously, both cell types were distributed evenly among tissues in control and mutant embryos (Fig. 1H-L) (Davis et al., 2004). These results indicate that Bmpr1a signaling in the epiblast did not restrict cells to or from any germ layer or major tissue in the early embryo. Thus, both mutant and heterozygous cells simultaneously traversed the primitive streak and ingressed into mesodermal tissues, resulting in a loss of BMP1A signaling in most, but not all, epiblast-derived cells.

By contrast, Sox2-Cre mice catalyze complete recombination of loxp sequence in the epiblast (Fig. 1M,O) (Hayashi et al., 2002), allowing us to obtain embryos with total loss of BMP1A signaling in the epiblast-derived tissues (Fig. 1N,P). Many of such mutant embryos showed a remarkably similar phenotype to Bmp-MORE embryos, namely lateral expansion of somites (Fig. 1N,P). Having the similar phenotype both in Bmp-MORE and Sox2Cre; Bmpr1afllox/null embryos implies that downstream targets of BMP1A signaling act in a non-cell-autonomous manner to regulate early mouse epiblast development.

Correct patterning of ectopic somites and improper patterning of lateral plate mesoderm

To further explore how mutant somites were patterned, the expression for several markers for PXM development was examined. Normally, Uncx4.1 (a marker for the caudal region of each somite) (Leite et al., 2000) or Dll1 (Bettenhausen et al., 1995) (a marker for caudal somite and presomitic mesoderm) was expressed as a column on each side of the embryo (Fig. 2A; data not shown). In mutant embryos, Uncx4.1 or Dll1 was expressed in multiple irregular columns on each side of the embryo, forming short mediolateral rows of expression domains (Fig. 2A’; data not shown). Uncx4.1 was correctly expressed in caudal region of each somite in both control and mutant embryos (Fig. 2B,B’). The expression domain of Epha4 (Durbin et al., 2000) or Mesp2 (Saga et al., 1997) (markers of rostral presomitic mesoderm) was laterally broadened, indicating the expansion of presomitic mesoderm (Fig. 2C’; data not shown). Mox1 (a paraxial mesoderm marker) (Candia et al., 1992), which is normally expressed in the somites (Fig. 2D,E), was not only expressed in expanded somites but also in cells of the LPM domain (Fig. 2D,E’), suggesting the LPM had acquired PXM fate. The expression domain of Pax1 (a marker for sclerotome) (Wallin et al., 1996) was also expanded in mutant embryos (Fig. 2F’). These data show that somites extended abnormally to the lateral edges of the embryo but individual somites were largely patterned correctly in Bmp-MORE embryos.

By contrast, mutant LPM was very poorly patterned. Foxf1 was normally expressed in LPM starting at E8.5 (Fig. 2G) (Mahlapuu et al., 2001). In Bmp-MORE embryos, expression of Foxf1 was patchy and often weak (Fig. 2G’). Some mutants did not express Foxf1 at all, suggesting some variability of phenotype; this is possibly a result of the mosaic nature of the mutant embryos. Bmp4 was expressed in allantois, the posterior primitive streak and LPM at E8.5 (Fig. 2H).
In *Bmpr-MORE* embryos, *Bmp4* was expressed in allantois and the primitive streak but not in LPM (Fig. 2H). *Lim1* expression was normally observed in LPM at E7.75 (Fig. 2I) (Tsang et al., 2000). In *Bmpr-MORE* embryos, *Lim1* expression was either not expressed or lower than normal (Fig. 2I; data not shown). These data indicate that *Bmpr1a* is required for proper patterning of LPM in normal development.

**Delay in recruitment of prospective PXM**

In *Bmpr-MORE* embryos, gastrulation initiated normally as described above. However, the primitive streak of mutant embryos was consistently shorter than that of control embryos during late stages of gastrulation. At the no allantoic bud stage (just prior to formation of the allantoic bud), brachyury was expressed in the entire primitive streak, the node and notochord in control embryos (Fig. 3A) (Wilkinson et al., 1990). In the notochord, the expression reached the anterior end of embryos (Fig. 3A). However, in *Bmpr-MORE* embryos, the most anterior domain of brachyury expression was in the distal region of the embryo (Fig. 3A'). In a control embryo at the allantoic bud stage, *Foxa2* was expressed in the node, notochord and anterior definitive endoderm (Fig. 3B) (Sasaki and Hogan, 1994). The node is formed at the most distal region of the entire primitive streak, the node and notochord in control embryos (Fig. 3A) (Wilkinson et al., 1990). In the notochord, the expression reached the anterior end of embryos (Fig. 3A). However, in *Bmpr-MORE* embryos, the most anterior domain of brachyury expression was in the distal region of the embryo (Fig. 3A'). In a control embryo at the allantoic bud stage, *Foxa2* was expressed in the node, notochord and anterior definitive endoderm (Fig. 3B) (Sasaki and Hogan, 1994). The node is formed at the most distal region of the primitive streak in *Bmpr-MORE* embryos, the node was formed more proximally than in control embryos (Fig. 3B'). Similar observations were made using probes for noggin (Fig. 3C') and *Lim1* (see Fig. S1A' in the supplementary material) (McMahon et al., 1998; Tsang et al., 2000). These results indicate that the distoanterior extension of the primitive streak was defective in
Bmpr-MORE embryos. However, by the head-fold stage, the primitive streak of Bmpr-MORE appeared to be fully elongated (Fig. 3D', see Fig. S1B' in the supplementary material). Thus, primitive streak extension was temporally delayed in Bmpr-MORE embryos during gastrulation.

Because the delay of primitive streak extension became apparent around the late stage streak stage, we examined if the production of LPM and/or PXM, which is normally induced by the late streak stage, was altered in Bmpr-MORE embryos. In control embryos, Lefty2 was expressed in LPM and PXM at this stage (Fig. 3E) (Iratni et al., 2002; Meno et al., 1999). In Bmpr-MORE embryos, the expression domain for Lefty2 was decreased and observed only in the proximal embryonic region (Fig. 3E'). Tbx6 was also expressed in LPM and PXM at the late stage streak (see Fig. S1C in the supplementary material) (Chapman et al., 1996), but its expression was also decreased and observed in only the proximal embryonic region in Bmpr-MORE embryos (see Fig. S1C' in the supplementary material). Thus, a reduced amount of LPM and/or PXM was induced in Bmpr-MORE embryos by the late-stage stage. These results suggest that the recruitment of prospective LPM and/or PXM to the primitive streak was delayed. This, in turn, caused a delay in the extension of the primitive streak in Bmpr-MORE embryos.

To further probe the temporal nature of mesoderm formation, we performed a cell lineage experiment using Dil, a tracer of living cells; this allowed us to label mesodermal cells with Dil, then to analyze their fate after culturing them for a defined period. Dil was injected into the proximal region of the primitive streak to label mesoderm cells of control or Bmpr-MORE embryos at the late stage streak (Fig. 4A,B). The embryos were then cultured for approximately 30 hours until the organogenesis stage (Fig. 4A',B'). As expected, in control embryos, labeled cells contributed to lateral plate (3/3) and yolk sac (3/3) (Fig. 4A', A'). Similarly, in all mutant embryos (5/5), labeled cells were distributed in regions of the embryo anterior and lateral to the paraxial region, but not to the somites (Fig. 4B',B'). Labeled cells were also distributed in the yolk sac (3/3) (Fig. 4B',B'). Dil was also injected into the anterior and distal region of the primitive streak of control or Bmpr-MORE embryos at the late stage streak (Fig. 4C,D). In control embryos, labeled cells contributed to somites and an axial tissue, which was probably axial mesoderm (3/3) (Fig. 4C',C'). However, in the mutant embryos, labeled cells did not contribute to the paraxial region but rather to the anterior region and an axial tissue of the embryo (4/4) (Fig. 4D',D'). These results indicate that the recruitment of prospective PXM had not occurred yet at the late stage streak in Bmpr-MORE embryos.

At the allantoic bud stage, Lefty2 was normally expressed in migrating LPM and PXM cells (Fig. 3F). The two domains of expression were basically contiguous, suggesting that recruitment of prospective LPM and PXM occurs continuously. In Bmpr-MORE embryos, we found two domains of expression for Lefty2 that were...
Fig. 3. Recruitment of prospective PXM is delayed during gastrulation in Bmpr-MORE embryos. (A,A') No allantoic bud stage. Brachyury was expressed in the primitive streak (A, arrow), node and notochord of control embryos. The rostral end of the notochord was at the anterior of the embryo (A, arrowhead). The expression of Brachyury did not extend to the anterior in Bmpr-MORE embryos (A', arrow and arrowhead). (B-C) The early to late allantoic bud stage. Foxa2 was expressed in the node (B, lower arrowhead) and notochord of control embryos. Upper arrowheads indicate embryonic/extra-embryonic border. As the node is formed at the distal region of the primitive streak, the length of the primitive streak corresponds to the length between two arrowheads. The length of the primitive streak of Bmpr-MORE embryos was shorter compared with that of control embryos (B', arrowheads). Definitive endoderm develops in the mutant embryo (B', arrow). (C,C') Noggin was expressed in the node (lower arrowheads). The length of the primitive streak was shorter in mutant embryos (distance between arrowheads). (D,D') The head-fold stage. (D) Tbx6 was expressed in the mesoderm except axial mesoderm (between arrowheads). (D') The length of the primitive streak appeared to be similar between control and Bmpr-MORE embryos (distance between arrowheads). (E,E') The late stage streak. Lefty2 was expressed in LPM and PXM (between arrowheads). (E) In Bmpr-MORE, Lefty2 expression was decreased (E', between arrowheads). (F-G') The early to late allantoic bud stage. Lefty2 was expressed in migrating LPM and PXM (F, black and red arrowheads, respectively). In Bmpr-MORE, the migrating LPM expressed Lefty2 (F', black arrowhead). More recently recruited mesoderm expressing Lefty2 was observed at the middle and distal regions of the primitive streak (F', red arrow and arrowhead, respectively). Pcdh8 (PAPC on figure) was also expressed in LPM and PXM (G, black and red arrowheads, respectively). In Bmpr-MORE, the migrating LPM expressed Pcdh8 (G', black arrowhead). More recently recruited mesoderm expressing Pcdh8 was observed at middle and distal regions of the primitive streak (G', red arrow and arrowhead, respectively). Scale bars: 100 μm for A,A',E,E'; 250 μm for B-D', 200 μm for F-H'.

These results indicate that mesodermal cells formed relatively late in the middle region of the primitive streak were mostly committed to PXM. Collectively, our results suggest that the delay in the recruitment of prospective PXM results in a more proximal distribution of mesoderm cells with PXM character within the primitive streak, which probably leads to the lateral extension of somites in Bmpr-MORE embryos.

Inhibition of FGF signaling restores proper recruitment of prospective PXM and partially rescues the abnormal expansion of somites in Bmpr-MORE embryos

Embryos deficient for fibroblast growth factor receptor type 1 (FGFR1) do not form somites, indicating that FGF signaling is involved in the development of the PXM lineage (Ciruna and Rossant, 2001; Yamaguchi et al., 1994). In Bmpr-MORE embryos, LPM acquired PXM character (Fig. 2D',E'). These observation led us to hypothesize that BMP signaling protects LPM from FGF signaling, which otherwise might cause the LPM to acquire PXM character. This hypothesis was tested by culturing E6.5-6.75 embryos in the presence of the FGFR1 antagonist SU5402 (Mohammadi et al., 1997). We reasoned that if the essential function
of Bmpr1a in terms of LPM development is to antagonize FGF signaling, SU5402 should be able to mitigate the abnormal patterning of LPM in Bmpr-MORE mutants.

Mutant and control embryos at E6.5-6.75 were cultured in DMEM with 50% rat serum (DR50) supplied with either dimethyl sulfoxide (DMSO) (vehicle for SU5402) or various concentration of SU5402. When cultured with DMSO or 10 μM SU5402 for around 20-24 hours, most control embryos reached a stage corresponding to the allantoic bud to head-fold stage (Fig. 5A; data not shown). Mutant embryos cultured with DMSO or 10 μM SU5402 showed poorly developed amniotic folds and a shorter primitive streak (Fig. 5A; data not shown). When cultured with 40 μM SU5402, all embryos showed abnormal development (see Fig. S2 in the supplementary material), as expected, with a strong reduction in FGFR1 activity. As a 50% inhibition of FGFR1 function is achieved at 10-20 μM (Mohammadi et al., 1997), we also tested the effect of 20 μM of SU5402. Control embryos developed normally at this concentration. By contrast, mutant embryos developed an amnion, chorion and allantois (12/12) (Fig. 5B,F). Moreover, the primitive streak appeared to extend normally in such embryos (Fig. 5B), suggesting that the recruitment of prospective PXM had occurred normally in Bmpr-MORE embryos in culture. Next, cultured embryos were tested for their expression of phosphorylated Erk1/2 to evaluate the level of signaling transduced by FGFR1 (Corson et al., 2003). The expression was observed most strongly in extraembryonic ectoderm derived tissues and ectoplacental cone (Corson et al., 2003). In embryos cultured with DMSO, strong expression was observed in the chorion or amniotic fold and ectoplacental cone (Fig. 5C,D). When cultured with 20 μM SU5402, expression was still observed strongly in the ectoplacental cone. However, the expression in the chorion was significantly reduced, indicating that signaling from FGFR1 was inhibited in both control and Bmpr-MORE embryos (Fig. 5E,F). We then examined the expression of Lefty2 in cultured embryos. As observed in vivo, the expression of Lefty2 in mutant embryos cultured with DMSO was abnormal; the expression was shifted proximally and decreased (3/3) (Fig. 6B). However, when cultured with 20 μM SU5402, Lefty2 was expressed.
at a normal level in mutant embryos (2/2) (Fig. 6D). These results indicate that inhibition of FGF signaling via reduction of FGFR1 activity rescued the delayed recruitment of prospective PXM in Bmpr-MORE embryos.

To further explore the impact of FGF signaling inhibition on somite development, we cultured embryos with 20 μM SU5402 for 48 hours. However, somite development was inhibited in such many embryos (see Fig. S2 in the supplementary material). Therefore, we cultured embryos with 20 μM SU5402 for 12-15 hours, removed the SU5402 by extensive washing, and continued to culture the embryos with DMSO for 48 hours in total. Under these conditions, somites developed normally (12/12) (Fig. 6G). Although somites were significantly expanded laterally in all mutant embryos cultured with DMSO (9/9) (Fig. 6F, see Fig. S3 in the supplementary material), treatment with SU5402 resulted in a variable level of rescue for somite development in six out of ten mutant embryos tested (Fig. 6H, see Fig. S3 in the supplementary material). Three mutant embryos developed only one or two rows of somites on both sides (Fig. 6H). In three other mutant embryos, the rescue in somite development was observed on one side of each embryo (see Fig. S3).

Fig. 5. Inhibition of FGF signaling by a specific antagonist for FGFR1 in Bmpr-MORE embryos. (A) Control embryos developed to the late streak to allantoic bud stage. Bmpr-MORE embryos showed a poorly developed amniotic fold (red arrowheads) and less extended primitive streak (yellow arrows) in culture, as was observed in vivo. Inset shows genotyping results for these embryos. Embryos positive for Cre and heterozygous for Bmp41a-null allele are Bmpr-MORE embryos (inset, pink stars). (B) Control embryos developed to the allantoic bud stage. Bmpr-MORE embryos developed amnion, chorion and allantois (red arrowheads), and a well-extended primitive streak (yellow arrow). Embryos positive for Cre and heterozygous for Bmp41a-null allele are Bmpr-MORE embryos (inset, pink star). (C-F) Cultured embryos stained with phospho-Erk1/2 antibody. (C) Control embryos cultured with DMSO. Ectoplacental cone (EPC) (arrow) and chorion (arrowheads) were strongly stained. (D) Mutant embryos cultured with DMSO. Ectoplacental cone (arrow) and the amniotic fold (orange arrowhead) showed strong expression. The extra-embryonic ectoderm in the ectoplacental cavity was weakly stained (black arrowheads). (E) Control embryos cultured with 20 μM SU5402. Expression in the ectoplacental cone was not affected (arrow), but that of the chorion was significantly decreased (arrowheads). (F) Mutant embryos cultured with 20 μM SU5402. Expression in the ectoplacental cone was not affected (arrow) but that of the chorion was significantly decreased (arrowheads). Scale bars: 500 μm for A,B; 100 μm for C-F.

Fig. 6. Inhibition of FGF signaling partially rescues the development of somites in Bmpr-MORE embryos. (A-D) Control and Bmpr-MORE embryos at E6.5-6.75 grown in culture for 20-24 hours were examined for Lefty2 expression. Control embryos cultured with DMSO normally expressed Lefty2 (A, arrowheads). Mutant embryos cultured with DMSO showed a proximal shift and decreased expression of Lefty2 (3/3) (B, arrowheads). Control embryos cultured with 20 μM SU5402 showed normal expression of Lefty2 (C, arrowheads). Exposure to SU5402 normalized the expression of Lefty2 in Bmpr-MORE embryos (2/2) (D, arrowheads). (E-H) Control and Bmpr-MORE embryos at E6.5-6.75 were cultured for around 48 hours and examined for the expression of Uncx4.1. (E) Control embryos cultured with DMSO normally expressed Uncx4.1 (arrowheads). (F) Mutant embryos cultured with DMSO showed a significant expansion of somites to the lateral edge of the embryos (arrows) (n=9). (G) Control embryos were cultured with 20 μM SU5402 for 12-15 hours, extensively washed and then cultured with DMSO for around 48 hours in total. In this condition, somites developed normally (arrowheads). (H) Mutant embryos were cultured with 20 μM SU5402 for 12-15 hours, extensively washed and then with DMSO for around 48 hours in total. In six mutant embryos out of 10 tested, partial rescue of somite development was observed (arrowheads). Scale bars: 85 μm for A,B; 100 μm for C,D; 600 μm for E-H.
in the supplementary material). These data indicate that inhibition of FGF signaling during gastrulation partially rescued abnormal development of somites in Bmpr-MORE embryos.

It was possible that the observed rescue of somite development was due to normal development of the LPM in Bmpr-MORE embryos. Therefore, we also examined the expression of Foxf1 in embryos cultured as described above. Foxf1 was not expressed in these mutant embryos, indicating that inhibition of FGF signaling did not rescue the abnormal development of the LPM (data not shown).

**DISCUSSION**

In this paper, we have shown that BMPRIA in the epiblast is essential for proper recruitment of epiblast cells into the primitive streak. This, in turn, has important consequences for development of the mesodermal region of the embryo, such as the somites.

Bmpr-MORE embryos at the allantoic bud stage showed a characteristic morphology: an acute curvature in the posterior side, which was due to a delay of primitive streak extension. Our results indicate that recruitment of prospective PXM that normally occurs during the mid to late streak stage was delayed and took place during the allantoic bud to head fold stage in Bmpr-MORE embryos. Normally, the prospective PXM population arises mainly at the anterior primitive streak. Our results indicate mesoderm cells with PXM character arise in the middle in addition to the anterior part of the primitive streak. The distoproximal order of the prospective mesoderm cells within the primitive streak is maintained during progression of cells from the primitive streak into the mesoderm (Smith et al., 1994). Presumably, PXM cells in the middle part of the primitive streak migrate more laterally and PXM cells at the distal part of the primitive streak migrate more medially, thus causing an altered morphogenesis of somites in Bmpr-MORE embryos.

We propose two possible models to explain the mechanism of how ectopic somites develop in Bmpr-MORE mutant embryos. The first model proposes that loss of BMP signaling causes a fate change of epiblast cells passing through the middle primitive streak, which would normally form the LPM, and that these cells consequently form ectopic somites (Fig. 7B, yellow arrow). The second model posits that the delay of recruitment in Bmpr-MORE embryos causes abnormal morphogenetic movement of prospective PXM cells upon recruitment to the primitive streak (Fig. 7C). Normally, these cells are recruited to the anterior primitive streak and exit from the anterior primitive streak (Fig. 7A, red arrow). In the mutant embryos, these cells are abnormally recruited to the middle primitive streak to form ectopic somites (Fig. 7C, yellow arrow).

FGF signaling induces PXM patterning via expression of brachyury and Tbx6 (Ciruna and Rossant, 2001). However, inhibition of FGF signaling in Bmpr-MORE embryos restored normal timing of prospective PXM recruitment. Together, these data indicate that FGF signaling negatively regulates recruitment of prospective PXM cells but is required for their patterning. Interestingly, we found that BMPs do not regulate the recruitment of prospective LPM cells, but they are essential for LPM patterning (this study). These observations suggest that a mechanism(s) might be present that are responsible for controlled allocation of mesoderm and thus for successful establishment of mouse body plan.

Control embryos treated with 20 μM SU5402 developed apparently normally up to the allantoic bud stage. However, Fgf8-deficient mice exhibit a marked decrease in migration of mesoderm cells out of the primitive streak during gastrulation (Sun et al., 1999). Probably, the concentration of SU5402 used here does not sufficiently inhibit FGF signaling to elicit this migratory defect.

It is unlikely that BMP signaling directly inhibits FGF signaling. In Bmpr-MORE embryos, the phosphorylation of ERK1 and ERK2, major signal transducers of FGF signaling appears at the normal level. In addition, Snail, one of target genes of FGF signaling, is expressed normally in mutant embryos (data not shown). Rather, downstream targets of BMP and FGF signaling are probably involved in antagonistic effects.
Through the analyses of Bmpr-MORE embryos, we identified novel functions of BMP signaling in gastrulation. However, many issues remain to be addressed. The mechanism that explains delayed recruitment of epiblast cells (prospective PXM) is unknown, although we showed that BMP and FGF signaling have antagonistic effects on this process. The identification of such mechanisms and the assessment of their functions are crucial for a more thorough understanding of mouse gastrulation.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/113/19/3767/DC1

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