Distinct functions of the major Fgf8 spliceform, Fgf8b, before and during mouse gastrulation

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The vertebrate Fgf8 gene produces multiple protein isoforms by alternative splicing. Two evolutionarily conserved spliceforms, Fgf8a and Fgf8b, exhibit distinct bioactivities, with Fgf8b having a more potent inductive activity due to higher affinity for Fgf receptors. To investigate the in vivo requirement for Fgf8b, we created a splice-site mutation abolishing Fgf8b expression in mice. Analysis of this mutant has uncovered a novel function of Fgf8 signaling before the onset of gastrulation. We show that the loss of Fgf8b disrupts the induction of the brachyury gene in the pregastrular embryo and, in addition, disrupts the proper alignment of the anteroposterior axis with the shape of the embryo and the uterine axes at embryonic day (E) 6.5. Importantly, Fgf8b-null embryos display the same phenotype as Fgf8b-deficient embryos at E6.5, demonstrating that signaling by Fgf8b is specifically required for development of the pregastrular embryo. By contrast, during gastrulation, Fgf8a can partially compensate for the loss of Fgf8b in mesoderm specification. We show that an increased level of Fgf8a expression, which leads to Fgf4 expression in the primitive streak, can also promote mesoderm migration in the absence of Fgf8b. Therefore, different Fgf signals may have distinct requirements for the morphogenesis and gene regulation before and during gastrulation. Importantly, our findings implicate Fgf8 in the morphogenetic process that establishes the defined relationship between the axes of the embryo and the uterus at the beginning of gastrulation, a perplexing phenomenon discovered two decades ago.

KEY WORDS: Fgf8, Signaling, Alternative splicing, Anteroposterior axis, Embryo, Uterus, Remodeling, Mouse

INTRODUCTION

Gastrulation is a morphogenetic process that leads to production of three different germ layers and establishes the embryonic body plan (Tam and Behringer, 1997). During gastrulation, epiblast cells traverse the primitive streak at the posterior end of the embryo and undergo an epithelial-to-mesenchymal transition. The newly generated mesoderm migrates away from distinct positions of the primitive streak to form different mesodermal lineages. When gastrulation starts at embryonic day 6.5 (E6.5), the mouse embryo exhibits a morphologically explicit anteroposterior (AP) polarity. Interestingly, the AP axis tends to be perpendicular to the longitudinal axis (from the oviduct to the cervix) of the uterine horn (Smith, 1985). The mechanism and biological significance for the defined relationship between the embryonic and uterine axes is unclear. Molecules governing the orientation of the embryonic AP axis with respect to the uterus have not yet been identified.

Recent studies have identified a series of patterning events in the mouse embryo before E6.5 (Rossant and Tam, 2004). Unexpectedly, the molecular markers that are characteristic for the anterior and posterior poles of the embryo are initially expressed at the opposite ends of the short transverse axis of the embryo between E5.75 and 6.0, when the embryo exhibits an ellipsoidal shape in the transverse plane (Mesnard et al., 2004; Perea-Gomez et al., 2004). Furthermore, the emerging AP axis does not relate with the uterine axis (Mesnard et al., 2004). After E5.75, the AP axis gradually shifts and eventually becomes parallel to the long axis of the embryo (Mesnard et al., 2004; Perea-Gomez et al., 2004). The shift of the AP axis was shown to be mainly caused by tissue remodeling, converting the short axis to long (Mesnard et al., 2004; Perea-Gomez et al., 2004). Concomitant with the remodeling, both the AP axis and the long axis of the embryo become perpendicular to the longitudinal axis of the uterine horn at E6.5 (Mesnard et al., 2004). Therefore, the embryo remodeling may be crucial for the final alignment of the AP axis with the long axis of the embryo and the uterus. Currently, little is known about the molecular mechanism underlying the morphogenetic remodeling.

Studies in chick and Xenopus embryos have demonstrated that fibroblast growth factor (Fgf) signaling plays important roles before and during gastrulation, including the induction of the primitive streak and the mesoderm, and the control of mesoderm migration (Bottcher and Niehrs, 2005). In the mouse embryo, Fgf8 is the only Fgf that is known to play an essential role during gastrulation. Fgf8 is expressed in the epiblast and visceral endoderm at E5.75, and subsequently in the emerging primitive streak at E6.5 (Crossley and Martin, 1995). In the absence of Fgf8, or a component essential for Fgf8 signaling, the mesoderm is formed but fails to migrate away from the primitive streak at E7.5 (Ciruna and Rossant, 2001; Ciruna et al., 1997; Deng et al., 1997; Garcia-Garcia and Anderson, 2003; Sun et al., 1999; Yamaguchi et al., 1994). Therefore, Fgf8 signaling is essential for mesoderm migration during mouse gastrulation. However, whether Fgf8 plays any role before gastrulation remains unanswered.

The vertebrate Fgf8 gene produces multiple protein isoforms by alternative splicing (Crossley and Martin, 1995; Fletcher et al., 2006; Gemel et al., 1996; MacArthur et al., 1995; Sato et al., 2001). Two evolutionarily conserved isoforms, Fgf8a and Fgf8b, exhibit distinct bioactivities. When they are ectopically expressed in the developing brain, Fgf8a promotes cell proliferation in the midbrain, whereas Fgf8b transforms the midbrain into a cerebellum (Lee et al., 1997; Liu et al., 2003; Liu et al., 1999; Sato et al., 2001). In Xenopus, Fgf8b is the predominant Fgf8 spliceform involved in mesoderm induction, whereas Fgf8a appears to be involved in posterior neural development (Fletcher et al., 2006). The more potent inductive activity of Fgf8b is attributed to its higher affinity than Fgf8a for FGF receptors (Olsen et al., 2006). To investigate the in vivo function of
Fgf8b, we mutated the alternative splice site of the Fgf8 gene, thereby abolishing Fgf8b expression in mice. Our analysis of this mutant has uncovered a novel function of Fgf8 signaling before the initiation of gastrulation. We show that there are different requirements for Fgf8b in embryo remodeling before gastrulation, in mesoderm specification and mesoderm migration during gastrulation.

MATERIALS AND METHODS

Generation of mouse mutants deficient for Fgf8b

By gene targeting, we changed the intron-exon junction sequence of Fgf8 exon 1D from taaagGTA to caaagGTA, abolishing the 5’ alternative splice acceptor, while the downstream alternative splice acceptor in exon 1D remained intact (Fig. 1A,D). The mutation produced a new restriction endonuclease site for Ndel. A selectable marker, neo was placed 400 bp downstream of exon 1D. We also produced a mutation, designated as Fgf8Δneo that contains only the neo cassette at the same intronic region as Fgf8b-Δneo (Fig. 1D). Targeted embryonic stem (ES) cells were identified by a combination of Southern blot analysis, PCR and restriction analysis (Fig. 1B,C), and used to generate germline chimeras. The neo cassette, which is flanked with two loxP sites was subsequently removed by breeding Fgf8+vΔneo or Fgf8Δneo mice to CMV-Cre transgenic mice, which express Cre broadly (Li et al., 2002).

Mouse breeding and genotyping

Mutant mouse strains were maintained in the CD1 (Charles River Laboratory, Wilmington, MA) background. Noon of the day on which the vaginal plug was detected was designated as E0.5 in staging of embryos. Embryos at E5.5 and 5.75 were staged using morphological landmarks (Rivera-Perez et al., 2003) and EGFP fluorescence derived from a Hex-EGFP transgene, which is expressed in the visceral endoderm at the distal at E5.5 and the proximal anterior at E5.75 (Rodriguez et al., 2001). After primitive streak formation, embryos were staged according to Downs and Davies (Downs and Davies, 1993).

Genotyping was carried out by PCR analysis. Primers Fgf8-GT-f (CAGAGGTTCAGAGGAGAGG) and Fgf8-GT-r1 (CCCGGAGTCT-AACCTGGC) were used to produce 198 bp PCR products from the wild-type allele and 290 bp from the Fgf8Δb allele, respectively. Primers Fgf8-GT-r1 and Neo-pro-R (CGGTGGATGTGGAATGTGTGC) were used to produce 300 bp PCR products from the Fgf8Δb-Neo or Fgf8Δneo allele.

Histological analysis

Embryos and uteri were recovered in PBS and fixed immediately in 4% paraformaldehyde at 4°C overnight. Serial transverse sections of the uterus were made at 7 µm across the mesometrium-antimesometrium axis. Whole-mount or section RNA in situ hybridization was performed as described previously (Li and Joyner, 2001). Detection of Hex-EGFP was performed by immunohistochemistry (rabbit anti-GFP IgG fraction, 1:1000 dilution, Invitrogen, A11122). Measurements of embryonic dimensions and axis angles were performed on digital images with ImageJ software. Student’s t-test and two-way ANOVA test were carried out with Microsoft Excel.

RESULTS

Generation of mouse mutations abolishing Fgf8b

As shown previously (Crossley and Martin, 1995; MacArthur et al., 1995), alternative splicing of the first four exons of the mouse Fgf8 gene produces at least eight Fgf8 splice variants (a-h), with Fgf8b (Fig. 1F).
being the predominant one (Fig. 1D,E). To determine the in vivo function of Fgf8b, we mutated the 5’ alternative splice acceptor of Fgf8 exon 1D, designated as Fgf8b-neo. The neo selectable marker located in the Fgf8 intron was subsequently removed to produce the Fgf8b allele (Fig. 1A,D). RT-PCR analyses revealed that only Fgf8b-null (Fgf8–/–) embryos at E7.5, whereas the Fgf8b-exon 1D, designated as Fgf8b, we mutated the 5’ function of Fgf8b spliceform with more severe gastrulation defects, similar to those found in Fgf8–/– mutants (data not shown). The amniotic membrane of all Fgf8–/– embryos appears rough and fluffy, probably due to abnormal development of the mesodermal component of the membrane (Fig. 2H,J). Analysis of region-specific markers demonstrated that the AP patterning of the neural plate of Fgf8–/– embryos is largely normal (Fig. 3). At E9.5, Fgf8–/– embryos are significantly smaller in size than their wild-type littermates and manifest multiple morphological defects, including open neural tube, malformed branchial arches and the heart tubes (data not shown). Therefore, Fgf8b is essential for proper developmental

**Fgf8**{supb}-null/neo embryos exhibit severe abnormalities at E7.5. In all embryos, a mass of cells with the morphology and molecular characters of the nascent mesoderm was detected in the posterior region, bulging into the amniotic cavity (Fig. 2C,D, Fig. 4A.D, Fig. 5). Furthermore, few mesodermal cells were evident outside of the primitive streak in the embryonic region of Fgf8b-neo/neo embryos (Fig. 2D). In the majority of Fgf8b-neo/neo embryos (23/25, 92%), embryonic mesoderm-derived structures were completely absent at E8.5, whereas the allantois and the mesodermal components of the amnion and yolk sac, which are derived from the extraembryonic mesoderm, were observed (Fig. 2F). In a few Fgf8b-neo/ neo mutants (2/25, 8%), the heart mesoderm, the somites and the headfold were evident but severely malformed (data not shown). The phenotypes of Fgf8b-neo/neo embryos are remarkably similar to those described for Fgf8-null (Fgf8–/–) embryos at the morphological and histological level (Sun et al., 1999). These observations suggest that, similar to Fgf8–/– mutants, the embryonic mesoderm fails to migrate away from the primitive streak in Fgf8b-neo/neo embryos.

Significantly, removing the neo cassette led to relatively normal gastrulation in Fgf8b/b embryos. At E8.5, the somites and cardiomesoderm were discernible in most Fgf8b/b embryos (40/51, 78.4%, Fig. 2H,J), although some mutants (11/51, 21.6%) had more severe gastrulation defects, similar to those found in Fgf8b-neo/neo mutants (data not shown). The amniotic membrane of all Fgf8b/b embryos appears rough and fluffy, probably due to abnormal development of the mesodermal component of the membrane (Fig. 2H,J). Analysis of region-specific markers demonstrated that the AP patterning of the neural plate of Fgf8b/b embryos is largely normal (Fig. 3). At E9.5, Fgf8b/b embryos are significantly smaller in size than their wild-type littermates and manifest multiple morphological defects, including open neural tube, malformed branchial arches and the heart tubes (data not shown). Therefore, Fgf8b is essential for proper developmental

**Fig. 2.** Fgf8b-neo/neo embryos have more severe defects than Fgf8b/b embryos. (A-D) Morphology and histology of murine wild-type (A,B) and Fgf8b-neo/neo (C,D) embryos at E7.5. (B,D) Hematoxylin and Eosin (H&E) analysis of sagittal sections of the embryos shown in A and B, respectively. Notice the mesodermal cells (arrowheads) accumulated at the primitive streak (brackets) in the Fgf8b-neo/neo embryo. (E,F) Lateral view of a wild-type embryo (E) and dorsal view of an Fgf8b-neo/neo embryo (F) at E8.5. (G-J) Morphology and histology of wild-type and Fgf8b/b embryos at E8.5. Arrowheads indicate the rough appearance of the amniotic membrane of Fgf8b/b embryos, compared with the control. (L,J) H&E analysis of sagittal sections of the embryos in G and H, respectively. PSM, presomitic mesoderm.
progression after E8.5. Collectively, our data demonstrate that the defect of mesoderm migration in Fgf8<sup>ab-neo</sup> embryos can be largely rescued by removing the neo cassette.

**Fgf4 and Wnt3a are expressed in the primitive streak of wild-type and Fgf8<sup>ab/ab</sup> embryos, but not in Fgf8<sup>ab-neo/ab-neo</sup> embryos**

The more severe phenotype of Fgf8<sup>ab-neo/ab-neo</sup> than Fgf8<sup>ab/ab</sup> embryos prompted us to compare and contrast the underlying molecular defects that might reveal unique developmental contributions of Fgf8a and Fgf8b. We speculated that the presence of the neo cassette might interfere with expression of Fgf8 (Fgf8a, -c, -e and -g only) from the Fgf8<sup>ab-neo</sup> locus. Indeed, a homozygous mutation for Fgf8<sup>neo</sup> that contains an identical neo insertion to Fgf8<sup>ab-neo</sup> (see Materials and methods) results in defects similar to a homozygous mutation for an Fgf8 hypomorphic allele (Meyers et al., 1998), and the defects of Fgf8<sup>neo/neo</sup> mutants are completely rescued by removing the neo (data not shown). These data demonstrate that the neo insertion impairs Fgf8 expression, while the loxP sequence that is remained after neo removal has no effect on Fgf8 function (Fig. 1A,D). A similar neo insertion has been shown to cause aberrant splicing due to cryptic splice sites in the neo cassette (Meyers et al., 1998). Indeed, our RT-PCR and sequencing analyses demonstrated that both Fgf8b and Fgf8-neo hybrid transcripts are produced from Fgf8<sup>ab-neo</sup> and Fgf8<sup>neonga</sup> alleles, whereas only Fgf8 transcripts are generated from Fgf8<sup>ab</sup> and Fgf8<sup>neog</sup> alleles (Fig. 1F and data not shown). The Fgf8-neo hybrid transcript contains stop codons in all three potential reading frames upstream of the coding sequences for the conserved FGF domain, and thus cannot produce functional Fgf8 proteins. Collectively, our genetic and molecular analyses strongly suggest that the presence of neo reduces functional Fgf8a expression from the Fgf8<sup>ab-neo</sup> allele, and thus Fgf8<sup>ab-neo</sup> embryos produce a higher level of functional Fgf8a than that in Fgf8<sup>ab-neo/ab-neo</sup> embryos. Furthermore, our data indicate that an increase of Fgf8a expression can partially compensate for the loss of Fgf8b in promoting mesoderm migration.

We next sought to investigate the molecular basis for the rescued mesoderm migration in Fgf8<sup>ab-neo</sup> embryos. Fgf4 is co-expressed with Fgf8 in the primitive streak (Niswander and Martin, 1992), and Fgf4 expression is lost in Fgf8<sup>–/–</sup> mutants at E7.5 (Sun et al., 1999). Fgf4 and Fgf8 are known to play redundant roles in limb development (Boulet et al., 2004; Sun et al., 2002). In wild-type embryos at E7.5, Fgf4 is uniformly expressed in the primitive streak from the base of allantois to cells immediately posterior to the node (Fig. 4A). In Fgf4<sup>ab/ab</sup> embryos at E7.5, Fgf4 expression was detected in an increasing gradient from the anterior to the posterior of the primitive streak (4/4, Fig. 4A). By contrast, Fgf4 expression was absent (4/6), or greatly reduced in Fgf8<sup>ab-neo/ab-neo</sup> mutants (2/6, Fig. 4A).

The interplay between Wnt and Fgf signaling has been implicated in controlling mesoderm migration (Ciruna and Rossant, 2001). To investigate whether Wnt signaling is affected in Fgf8<sup>ab-neo</sup> mutants, we analyzed expression of Wnt3a and Wnt3b, which are expressed in the primitive streak at E7.5 (Takada et al., 1994). Wnt3 expression was indistinguishable between Fgf8<sup>ab/ab</sup> and Fgf8<sup>ab-neo/ab-neo</sup> mutants (data not shown). By contrast, Wnt3a transcripts were detected in the primitive streak of wild-type and Fgf8<sup>ab/ab</sup> (5/5), but not in Fgf8<sup>ab-neo/ab-neo</sup> mutants (3/3, Fig. 4B-D).

In summary, Fgf4 and Wnt3a are expressed in the primitive streak of Fgf8<sup>ab-neo</sup>, but not in Fgf8<sup>ab-neo/ab-neo</sup> embryos. The restoration of Fgf4 and Wnt3a, resulting from an elevated Fgf8a expression, may contribute to the rescue of mesoderm migration in Fgf8<sup>ab-neo</sup> embryos.

**The remaining Fgf8a can promote mesoderm specification and regionalization of the primitive streak in Fgf8<sup>ab-neo/ab-neo</sup> embryos**

Our RT-PCR analyses showed that the transcription of Fgf8 is independent of Fgf8b proteins (Fig. 1E). In situ hybridization analysis further demonstrated that there was robust Fgf8 expression in the mesodermal cells in the posterior of Fgf8<sup>ab-neo/ab-neo</sup> embryos (Fig. 5A). To determine whether the remaining Fgf8a isoform proteins elicit Fgf8 signaling in Fgf8<sup>ab-neo/ab-neo</sup> embryos, we analyzed expression of Spry2, which is a feedback inhibitor of Fgf signaling. Spry2 expression occurs in the mesoderm at E7.5 and is lost in the absence of Fgf8 signaling (Garcia-Garcia and Anderson, 2003; Minowada et al., 1999). Significantly, Spry2 expression was detected in the primitive streak of Fgf8<sup>ab-neo/ab-neo</sup> embryos.
embryos at E7.5 (3/3, Fig. 5D). Therefore, the remaining Fgf8a isoforms activate Fgf8 signaling to a limited degree in the primitive streak of Fgf8αb-neo/αb-neo embryos.

We next sought to examine whether the residual Fgf8 signaling promotes mesoderm specification in Fgf8αb-neo/αb-neo embryos by analyzing expression of mesodermal markers at E7.5. Evx1 is expressed in the proximal part of the primitive streak (Fig. 5E), whereas Foxa2 (also known as HNF3β) is expressed in the distal end of the streak and the emerging axial mesoderm (Fig. 5G) (Ang et al., 1993; Dush and Martin, 1992; Sasaki and Hogan, 1993). Similar to that found in wild-type embryos, transcripts of Evx1 (n=5) and Foxa2 (n=3) were detected in the proximal and distal regions, respectively, of the primitive streak in Fgf8αb-neo/αb-neo embryos (Fig. 5F,H). These data suggest that the regionalization of the primitive streak in Fgf8αb-neo/αb-neo embryos is largely maintained.

T is expressed in the nascent mesoderm and in the axial mesoderm, while expression of Tbx6 demarcates the lineage of the paraxial mesoderm (Fig. 5I,K) (Chapman et al., 1996; Wilkinson et al., 1993; Dush and Martin, 1992; Sasaki and Hogan, 1993). Similar to that found in wild-type embryos, transcripts of Evx1 (n=5) and Foxa2 (n=3) were detected in the proximal and distal regions, respectively, of the primitive streak in Fgf8αb-neo/αb-neo embryos (Fig. 5F,H). These data suggest that the regionalization of the primitive streak in Fgf8αb-neo/αb-neo embryos is largely maintained.

**Fig. 4.** Fgf4 and Wnt3a are expressed in the primitive streak of murine Fgf8αb-b/b, but not in Fgf8αb-neo/αb-neo, embryos at E7.5. (A) Expression of Fgf4 in embryos of indicated genotypes. (B-D) Expression of Wnt3a in wild-type (B), Fgf8αb-b/b (C) and Fgf8αb-neo/αb-neo (D) embryos. Notice that the morphologically distinct node (arrowhead) is absent in Fgf8αb-b/b and Fgf8αb-neo/αb-neo embryos. Arrows mark the mass of mesodermal cells, which is variable and tends to be smaller than that found in Fgf8αb-neo/αb-neo embryos, at the primitive streak of Fgf8αb-b/b embryos at E7.5.

**Fig. 5.** The regionalization of the primitive streak and the specification of the mesoderm are partially maintained in murine Fgf8αb-neo/αb-neo embryos. (A-L) In situ hybridization analysis of markers (indicated to the left) characteristic for Fgf8 signaling and mesoderm in wild-type and Fgf8αb-neo/αb-neo embryos at E7.5. (A-D,I,J) Adjacent sagittal sections of wild-type (A,C,I) and Fgf8αb-neo/αb-neo (B,D,J) embryos. Inset in B shows whole-mount analysis of Fgf8 expression. Notice that axial mesoderm marked by T and Foxa2 expression is absent anterior to the streak of Fgf8αb-neo/αb-neo embryos, indicating a lack of mesoderm migration. (E,F) Asterisk indicates Evx1 expression at the proximal end of the primitive streak.
al., 1990). Prior studies have shown a great reduction of T and loss of expression of Tbx6 in embryos lacking Fgf8 signaling at E7.5 (Ciruna and Rossant, 2001; Garcia-Garcia and Anderson, 2003; Sun et al., 1999). By contrast, robust expression of T (n=9) and Tbx6 (n=3) was detected in the posterior region of Fgf8−/−neo/−neo embryos at E7.5 (Fig. 5J,L). These observations demonstrate that Fgf8 is expressed in the anterior visceral endoderm (A VE) (Fig. S1A,C,E in the supplementary material). We also analyzed expression of Cer1 where Fgf8 is expressed in the anterior visceral endoderm, while expression was absent in the distal extraembryonic ectoderm, and throughout the epiblast (see Fig. S2A in the supplementary material). The abnormalities of Fgf8 at E5.75 were found in the A VE and throughout the epiblast (measured along the proximodistal axis of the egg cylinder) among Fgf8−/− (270.42±82.91 μm, n=20), Fgf8+/− (284.35±80.20 μm, n=39) and Fgf8−/− (283.19±78.22 μm, n=26) (Fig. 6I). Therefore, loss of Fgf8 does not cause a gross delay in development.

To ascertain whether the mutant phenotype of Fgf8+/− embryos at E6.5 is specific to the loss of Fgf8, we re-examined the phenotype of Fgf8+−/− mutant (Meyers et al., 1998). Fgf8+/− embryos display identical defects in the loss of Lefty2 (3/3) and T (5/5), and abnormal orientation of the AP axis, to those observed in Fgf8+/− embryos at E6.5 (Fig. 6C,F). These results demonstrate that the Fgf8 proteins are essential for Fgf8 activity before E6.5.

**Loss of Fgf8b leads to abnormal orientation of the AP axis relative to the embryo shape at E6.5**

Given the remarkably normal development of many Fgf8+/− embryos at E8.5, it was somewhat surprising that all the mutant embryos displayed significant abnormalities at E7.5, including a lack of morphologically distinct node structure and an accumulation of mesodermal cells at the primitive streak (Fig. 6A). We examined expression of the molecular markers that are characteristic for the anterior and posterior poles of the embryos at E6.5. At the pre-streak and early streak stages, Fgf8 and Nodal are normally expressed in the posterior region of the mouse embryo, whereas Cer1 is expressed in the anterior visceral endoderm (AVE) (see Fig. S1A,C,E in the supplementary material). Transcripts of Fgf8 and Nodal were detected in the posterior side, whereas Cer1 expression was found in the AVE of Fgf8+/− embryos at E6.5. As described previously (Mesnard et al., 2004; Perea-Gomez et al., 2004), Fgf8 and Nodal are expressed at one end of the long transverse axis of wild-type embryos, whereas Cer1 is expressed in the opposite end. By contrast, Fgf8/Nodal and Cer1 expressing cells were found at the opposing ends of the short transverse axis of Fgf8+/− embryos (Fig. S1 in the supplementary material). We next performed in situ hybridization with an RNA probe that recognizes both Lefty1 and Lefty2, which are expressed in the AVE and the emerging primitive streak, respectively (Fig. 6A). We detected signals in the AVE, presumably Lefty1 expression, at one end of the short transverse axis in Fgf8+/− embryos at E6.5, whereas Lefty2 transcripts were largely absent (n=8, Fig. 6B). We also analyzed expression of T, which is a posterior marker of the pregastrular embryo. As described previously (Perea-Gomez et al., 2004; Rivera-Perez and Magnuson, 2005), T is expressed in the proximoposterior epiblast and a radial ring of cells in the distalmost extraembryonic ectoderm between E6.25 and 6.5 (Fig. 6D). Interestingly, we found that T expression was absent in the distal extraembryonic ectoderm, while T transcripts were barely detected in the posterior epiblast of Fgf8+/− (3/3) embryos at E6.5 (Fig. 6E). Taken together, our data demonstrate that in the absence of Fgf8b, the AP axis aligns with the short, rather than the long, transverse axis of the embryo at E6.5. Furthermore, Fgf8b is required for the normal induction of T and Lefty2.

To determine whether the mutant phenotypes of Fgf8+/− embryos at E6.5 result from a developmental retardation, we measured the dimensions of Fgf8+/− and control embryos after in situ hybridization analysis with the above markers. The ratio of AP versus left-right (LR) dimension is significantly greater than 1 in control (Fgf8+/+ and Fgf8+/−) embryos (1.52±0.38, n=59), but smaller than 1 in Fgf8+/− embryos (0.73±0.14, n=26), demonstrating that the loss of Fgf8b leads to abnormal alignment of the AP axis with the shape of the embryo (Fig. 6J). Importantly, no significant difference (P=0.208, two-way ANOVA test) was found in the height of epiblast (measured along the proximodistal axis of the egg cylinder) among Fgf8+/+ (270.42±82.91 μm, n=20), Fgf8+/− (284.35±80.20 μm, n=39) and Fgf8−/− (283.19±78.22 μm, n=26) (Fig. 6I). Therefore, loss of Fgf8b does not cause a gross delay in development.

**Fgf8b is not required for the establishment of the AP polarity at E5.75**

The abnormalities of Fgf8+/− embryos at E6.5 prompted us to analyze expression patterns of Fgf8 in pregastrular embryos in greater detail. At E5.5, diffuse signal of Fgf8 was detected in the epiblast (data not shown). By E5.75, Fgf8 transcripts were found in the AVE and throughout the epiblast (see Fig. S2A in the supplementary material). Between E5.75 and 6.0, Fgf8 expression was progressively confined to the proximal, and subsequently to the proximoposterior, epiblast, while the expression in the AVE was downregulated (see Fig. S2B in the supplementary material).

We next examined whether the specific expression of Fgf8 in the AVE might play a role in positioning the emerging AVE with reference to the shape of the embryo. As shown previously (Mesnard et al., 2004; Perea-Gomez et al., 2004), Lefty1 and Cer1 are expressed at one end of the short axis of wild-type embryos at E5.75 (Fig. 6G and data not shown). In Fgf8+/− embryos, the expression of Lefty1 and Cer1 was indistinguishable from that in wild type (Fig. 6H and data not shown). Therefore, proper positioning of the AVE precursors along the short axis of the embryo at E5.75 does not depend on Fgf8b.

**Loss of Fgf8b affects the orientation of the AP axis, but not the long axis, of the embryo relative to the longitudinal axis of the uterine horn**

The AP axis and the long axis of the embryo are normally parallel to each other, and both axes tend to be perpendicular to the longitudinal axis of the uterine horn at E6.5 (Mesnard et al., 2004). As the AP axis becomes proximally perpendicular to the long axis, both with respect to the uterine horn. To do so, we examined the relative position of the anterior pole of the embryo and the AVE cells with respect to the long axes of the embryo and the uterus on cross sections of E6.5 embryos within the uterus. The Fgf8+/− males used for heterozygous intercrosses were homozygous for the Hex-EGFP transgene (Rodriguez et al., 2001), so that the AVE was marked by EGFP. In roughly three-quarters (73.0%) of the embryos (group I, 27/37), the center of EGFP expression domain was near one end of the long axis of the embryo (Fig. 7B,C). In the rest of the embryos (group II, 10/37 (27.0%), the center of EGFP expression domain was close to one end of the short axis of the embryo (Fig. 7B,D). Remarkably, 27/37 (76.4±5.9, Fig. 7E). As a result, no significant difference (P=0.608, Student’s t-test) between group I (72.6±17.7, n=27) and group II (76.4±5.9, n=10) (Fig. 7E). As a result, no significant difference (P=0.608, Student’s t-test) between group I (72.6±17.7, n=27) and group II (76.4±5.9, n=10) (Fig. 7E). As a result, no significant difference (P=0.608, Student’s t-test) between group I (72.6±17.7, n=27) and group II (76.4±5.9, n=10) (Fig. 7E). As a result, no significant difference (P=0.608, Student’s t-test) between group I (72.6±17.7, n=27) and group II (76.4±5.9, n=10) (Fig. 7E).
result, the AP axis of group I embryos tends to be perpendicular to the longitudinal axis of the uterus, whereas the AP axis tends to be parallel to the longitudinal uterine axis of group II embryos (Fig. 7B-D). Based on the abnormal position of the AVE cells relative to the shape of the embryo, we suggest that the embryos of group II represent \textit{Fgf8^{\text{ab/ab}}}, whereas the embryos of group I represent wild type (\textit{Fgf8^{+/+}} and \textit{Fgf8^{+/ab}}). Indeed, the percentage of embryos of group II (27%) is close to the expected Mendelian ratio (25%) for \textit{Fgf8^{ab/ab}} embryos.

We next examined cross sections of E7.5 embryos within the uterus from the intercross of \textit{Fgf8^{+/+}} parents. At E7.5, \textit{Fgf8^{ab/ab}} embryos could be readily identified by the abnormal histology, while the orientation of the AP axis could be identified by the position of the primitive streak at the posterior end of the embryo based on histology and expression of \textit{T}. In agreement with our analysis of \textit{Fgf8^{ab/ab}} embryos at E6.5, the AP axis of \textit{Fgf8^{ab/ab}} embryos tended to be parallel with the long uterine axis, whereas the AP axis of wild-type embryos was largely perpendicular to the long uterine axis (Fig.

**Fig. 6.** \textit{Fgf8}b is required for the normal induction of \textit{Lefty2} and \textit{T}, and for proper alignment between the AP axis and the shape of the embryo. (A-C) In situ hybridization with an RNA probe detecting both \textit{Lefty1} and \textit{Lefty2} in E6.5 murine embryos of the indicated genotypes. Expression of \textit{Lefty1} in the anterior visceral endoderm (AVE; marked by arrowhead) is unaffected in \textit{Fgf8^{ab/ab}} (B) or \textit{Fgf8^{+/+}} (C) embryos, whereas \textit{Lefty2} expression in the emerging primitive streak (arrow) is missing in the mutants. Insets show distal views of the embryos in A and C. Double-headed arrow in A indicates the height of the epiblast (see J). (D-F) Analysis of \textit{T} expression in E6.5 embryos of the indicated genotypes. Arrowhead marks the \textit{T} expression domain in the distal extraembryonic ectoderm, while the arrow marks \textit{T} expression in the posterior epiblast. (G,H) Expression of \textit{Cer1} in the AVE of wild-type (G) and \textit{Fgf8^{ab/ab}} (H) embryos at E5.75. Insets show distal views of the embryo. Broken double-headed arrow marks the long axis of the embryo. (I) Schematic summary of the AP polarity with respect to the shape of wild-type and \textit{Fgf8^{ab/ab}} embryos at E5.75 and E6.5. Notice that the shift of AP axis fails to occur in the \textit{Fgf8^{ab/ab}} embryo. (J) Distribution of the ratio of AP and LR dimensions relating to the height of the epiblast (indicated by double-headed arrow in A) between E6.0 and E6.75 from intercrosses of \textit{Fgf8^{+/+}} mutants. Each dot represents one embryo. AP, anteroposterior; LR, left-right. Scale bars: 50 \textmu m in F for A-H, including insets in G and H; 50 \textmu m in inset in C for insets in A-C.
The average angle between the AP axis and the long uterine axis of control embryos (70.88±19.16, n=53) was significantly different from that of Fgf8b+/H9004b/embryos (25.40±24.24, n=21, P=1.2×10^-8). Taken together, these observations demonstrate that the loss of Fgf8b results in an almost 90 degree rotation of the AP axis relative to the longitudinal axis of the uterus. Nevertheless, the long axis of Fgf8b-deficient embryos was correctly in register with the uterine axis, suggesting that positioning of the embryo within the uterine lumen is dependent on the shape, rather than the AP axis, of the embryo.

**DISCUSSION**

Fgf8b is the major splice variant of the Fgf8 gene, and Fgf8b proteins have more potent activity than Fgf8a. In this study, by knocking out Fgf8b in the mouse embryo, we have uncovered distinct requirements for Fgf8b before and during gastrulation. We show that before the onset of gastrulation, Fgf8b is essential for Fgf8 activities in the induction of T and positioning the AP axis relative to the shape of the embryo and the uterine axis. Moreover, Fgf8b is required for the normal induction of Lefty2 in the emerging streak. During gastrulation, Fgf8a can partially compensate for the loss of
Fgf8b. Our molecular and genetic studies suggest that a higher level of Fgf8a is required for promoting mesoderm migration than that for mesoderm specification.

**Fgf8 is essential for embryo remodeling before gastrulation**

Recent studies have demonstrated that the orientation of the AP axis shifts relative to the shape of the embryo between E5.75 and 6.5 due to morphogenetic remodeling of the epiblast (Mesnard et al., 2004; Perea-Gomez et al., 2004). Our studies have revealed a key function of Fgf8 in this morphogenetic process. In Fgf8b/Δb embryos at E6.5, the AP markers are expressed at the opposite ends of the short axis, rather than the long axis, of the embryo. We have ruled out that loss of Fgf8b leads to a developmental arrest at the earlier stages, when the AP axis is parallel with the short axis. We further showed that the AP axis is correctly aligned with the short axis of Fgf8b/Δb and Fgf8a/Δa embryos have the same defect in the orientation of the AP axis at E6.5 (Fig. 6A-F). Taken together, our results demonstrate that Fgf8b is responsible for Fgf8a signaling in mediating the morphogenetic remodeling of the embryo between E5.75 and 6.5 (see Fig. 6I).

**The uterus influences positioning of pregastrular embryos according to the shape but not the AP axis of the embryo**

It was discovered almost two decades ago that the AP axis of the mouse embryo tends to be perpendicular to the longitudinal uterine axis at E6.5 (Smith, 1985). This has led to speculations that the uterus might influence formation of the AP axis, or that the positional cues for the prospective AP axis with reference to the uterus might be secured at the time of implantation (Rossant and Tam, 2004). Here, we showed that in the absence of Fgf8b the AP axis of the embryo becomes parallel, rather than perpendicular, to the longitudinal uterine axis at E6.5 and 7.5. This demonstrates that the uterus does not have an overriding role in orienting the AP axis, and suggests that the prospective AP positioning cues are not fixed at the time of implantation.

What could be the mechanism for the defined relationship between the AP axis and the long uterine axis at the onset of gastrulation? Interestingly, the orientation of the long axis of the embryo with respect to the uterine axis is unaffected in the absence of Fgf8b (Fig. 7B). These findings have two important implications. One is that the uterus imposes positioning of the embryo according to its shape but not its AP axis. The second is that embryo remodeling is not necessary for the biased positioning of the embryo within the uterine lumen. We therefore propose that the predictable relationship between the AP axis and the uterine axes at E6.5 is a coincidental outcome of two independent and concurrent events. Between E5.75 and 6.5, the embryo undergoes remodeling, leading to alignment of the AP axis with the long axis. We suggest that this morphogenetic process is driven by cues intrinsic to the embryo and is dependent on Fgf8a signaling. The fact that embryos cultured in vitro undergo similar shape changes is entirely consistent with our deduction (Mesnard et al., 2004; Perea-Gomez et al., 2004). Externally, interactions between the embryo and uterus lead to biased positioning of the embryo based on the shape of the embryo within the uterine lumen, probably due to physical constraints imposed on the developing embryo.

Does the abnormal alignment of the AP axis with the longitudinal uterine axis contribute to the abnormal development observed in Fgf8bΔ/Δb embryos? We found that about half of the Fgf8bΔ/Δb embryos (11/21, 52.4%) significantly extended along the AP axis at E7.5, with their AP:LR ratio being greater than 1 (Fig. 7H). However, the angle between the AP axis and the uterine axis did not correlate with the AP:LR ratio, or the severity of the phenotype at the histological level (Fig. 7H and data not shown). Furthermore, a significant number of Fgf8bΔ/Δb embryos were markedly normal in morphology at E8.5, although most mutants displayed an abnormal orientation with respect to the long uterine axis at E6.5. Our data, therefore, suggest that the alignment of the AP axis with the longitudinal uterine axis at the onset of mouse gastrulation is unlikely to play a crucial role in subsequent development.

**Fgf8b is involved in the initiation of T expression**

Experiments in different model organisms have demonstrated that T and other T-box genes are the downstream target as well as the immediate mediators of Fgf signaling in mesoderm induction and patterning (Bottcher and Niehrs, 2005). Analysis of Fgf8 spliceforms in Xenopus demonstrates that Fgf8b, but not Fgf8a, has robust activity in inducing Xbra, the homolog of T (Fletcher et al., 2006). We show that in the absence of Fgf8b or Fgf8, T expression is lost in the extraembryonic ectoderm and greatly reduced in the proximoposterior epiblast at E6.5. The lack of T expression in Fgf8bΔ/Δb embryos is unlikely to be caused by a gross delay in development. The evidence for this assertion is that Fgf8aΔ/Δ and wild-type embryos are comparable in size, and Nodal, Wnt3 and Fgf8a are normally induced in the posterior Fgf8bΔ/Δb embryos at E6.5 (see Fig. S1 in the supplementary material; and data not shown). Our results thus demonstrate that there is an evolutionarily conserved requirement for Fgf8 signaling in the normal induction of T in the epiblast. Interestingly, T is eventually expressed in the primitive streak of Fgf8b-deficient embryos (Fig. S1), and in a reduced level in Fgf8-null embryos between E6.5 and 7.5 (Sun et al., 1999). These findings indicate that Fgf8 is not essential for T expression in the primitive streak of gastrulas.

**Different levels of Fgf8a expression govern mesoderm specification and migration**

Mesoderm specification and morphogenetic movement of mesodermal cells are two highly coordinated processes during vertebrate gastrulation. Distinct molecular pathways have been implicated in mesoderm specification and migration (Carver et al., 2001; Nutt et al., 2001; Sivak et al., 2005; Zohn et al., 2006). It has been suggested that two Fgf inhibitors, Sprouty (Xsprouty1, 2001; Nutt et al., 2001; Sivak et al., 2005; Zohn et al., 2006) implicated in mesoderm specification and migration (Carver et al., 2001; Nutt et al., 2001; Sivak et al., 2005). Are different Fgf signals involved in coordinate mesoderm specification and migration in Xenopus embryos (Sivak et al., 2005). Are different Fgf signals involved in the differential control of specification and migration of the mesoderm? Our results show that in the absence of the Fgf8b, different levels of Fgf8a are required for promoting mesoderm specification and mesoderm migration. In Fgf8bΔ/Δb, the residual Fgf8a isoforms can compensate for the loss of Fgf8b in mesoderm specification, but not mesoderm migration (Figs 2, 4 and 5). Remarkably, the mesoderm migration defects of Fgf8aΔ/ΔbΔb-neo embryos are largely rescued by removing the neo cassette. We did not attempt to directly compare Fgf8a expression between Fgf8bΔ/Δb-neo and Fgf8bΔ/ΔbΔb embryos owing to complications arising from the variability of the mutant phenotype. However, we provided genetic and molecular evidence that the presence of the neo cassette impairs expression of Fgf8 (Fig. 1). The simplest interpretation of our results is that the Fgf8bΔ/ΔbΔb-neo embryo produces higher levels of Fgf8a than those in the Fgf8bΔ/ΔbΔb-neo embryo,
thereby leading to normal gastrulation in the absence of Fgf8b. A crucial yet unsolved question is how a higher level of Fgf8a can promote mesodermal migration. We showed that Fgf9 is expressed in the primitive streak of Fgf8b−/−/− embryos, but not in Fgf8b+/−/−/− mutants at E7.5 (Fig. 4A). Clearly, the increased Fgf9a expression is sufficient for the induction of Fgf4, which may in turn activate a different signaling pathway controlling the mesoderm migration.

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