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

Qiuxia Guo and James Y. H. Li*

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, Fgf8-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 (Crosby 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., 1995; 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 taagGTA to caagGTA, 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 Fgf8neo that contains only the neo cassette at the same intronic region as Fgf8Δb-neo (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 by two loxP sites was subsequently removed by breeding Fgf8+/Δb-neo or Fgf8Δb-neo 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 (CAGAGGGTCCAGGGAGAGG) and Fgf8-GT-r1 (CCCGGAGTCT- AACTGGCAGG) 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 Fgf8Δb 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
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 allelic cassette led to relatively normal gastrulation in Fgf8^{Δb/Δb} embryos. At E8.5, the somites and cardinal and mesoderm were clearly discernible in most Fgf8^{Δb/Δb} 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 Fgf8^{Δb-neo/Δb-neo} mutants (data not shown). The amniotic membrane of all Fgf8^{Δb/Δb} 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^{Δb/Δb} embryos is largely normal (Fig. 3). At E9.5, Fgf8^{Δb/Δb} 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^{Δb-neo/Δb-neo} embryos display more severe defects than Fgf8^{Δb/Δb} embryos**

Fgf8^{Δb-neo/Δb-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 Fgf8^{Δb-neo/Δb-neo} embryos (Fig. 2D). In the majority of Fgf8^{Δb-neo/Δb-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 Fgf8^{Δb-neo/Δb-neo} mutants (2/25, 8%), the heart mesoderm, the somites and the headfold were evident but severely malformed (data not shown). The phenotypes of Fgf8^{Δb-neo/Δb-neo} embryos are remarkably similar to those described for Fgf8-null (Fgf8^{ΔΔ}) embryos at the morphological and histological level (Sun et al., 1999). These observations suggest that, similar to Fgf8^{ΔΔ} mutants, the embryonic mesoderm fails to migrate away from the primitive streak in Fgf8^{Δb-neo/Δb-neo} embryos.

Significantly, removing the neo cassette led to relatively normal gastrulation in Fgf8^{Δb/Δb} embryos. At E8.5, the somites and cardiomesoderm were clearly discernible in most Fgf8^{Δb/Δb} 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 Fgf8^{Δb-neo/Δb-neo} mutants (data not shown). The amniotic membrane of all Fgf8^{Δb/Δb} 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^{Δb/Δb} embryos is largely normal (Fig. 3). At E9.5, Fgf8^{Δb/Δb} 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. Fgf8^{Δb-neo/Δb-neo} embryos have more severe defects than Fgf8^{Δb/Δb} embryos. (A-D) Morphology and histology of murine wild-type (A,B) and Fgf8^{Δb-neo/Δb-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 Fgf8^{Δb-neo/Δb-neo} embryo. (E,F) Lateral view of a wild-type embryo (E) and dorsal view of an Fgf8^{Δb-neo/Δb-neo} embryo (F) at E8.5. (G-J) Morphology and histology of wild-type and Fgf8^{Δb/Δb} embryos at E8.5. Arrowheads indicate the rough appearance of the amniotic membrane of Fgf8^{Δb/Δb} embryos, compared with the control. (I,J) H&E analysis of sagittal sections of the embryos in G and H, respectively. PSM, presomitic mesoderm.](image-url)
progression after E8.5. Collectively, our data demonstrate that the defect of mesoderm migration in Fgf8\(^{\Delta b-neo}\) embryos can be largely rescued by removing the neo cassette.

**Fig. 3.** Pattern formation of the neural plate of murine Fgf8\(^{\Delta b}\) embryos is largely normal. (A-J) Expression of region-specific markers, as indicated to the left, in the anterior neural plate of wild-type and Fgf8\(^{\Delta b}\) embryos: Otx2, telencephalon and mesencephalon; Gbx2, thomborome 1 (r1); Fgf8, r1, En1, mesencephalon and r1; Shh, head process and axial mesoderm. Black arrows mark the midhindbrain junction. Arrowheads in A,B,I and J demarcate head process and axial mesoderm. Black arrows mark the mid-type and molecular defects that might reveal unique developmental roles, as indicated to the left, in the anterior neural plate of wild-type and Fgf8\(^{\Delta b}\) embryos. The remaining Fgf8a can promote mesoderm specification and regionalization of the primitive streak in Fgf8\(^{\Delta b-neo}\) embryos

The more severe phenotype of Fgf8\(^{\Delta b-neo}\) embryos is largely rescued by removing the neo cassette. This demonstrates that the neo insertion impairs Fgf8 expression, while the loxP sequence that is remained after neo removal has no effect on Fgf8 expression (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 Fgf8a and Fgf8-neo hybrid transcripts are produced from Fgf8\(^{\Delta b}\) and Fgf8-neo alleles, whereas only Fgf8 transcripts are generated from Fgf8\(^{\Delta b}\) and Fgf8-neo alleles (Fig. 1F and data not shown). The Fgf8-neo hybrid transcript contains stop codons in 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\(^{\Delta b-neo}\) allele, and thus Fgf8\(^{\Delta b}\) embryos produce a higher level of functional Fgf8a than that in Fgf8\(^{\Delta b-neo}\) embryos. Furthermore, our data indicate that an increase in 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\(^{\Delta b}\) embryos. Fgf4 is co-expressed with Fgf8 in the primitive streak (Niswander and Martin, 1992), and Fgf4 expression is lost in Fgf8\(^{-}\) 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 Fgf8\(^{\Delta b}\) 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\(^{\Delta b-neo}\) embryos (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\(^{\Delta b-neo}\) embryos, we analyzed expression of Wnt3a and Wnt3a, which are expressed in the primitive streak at E7.5 (Takada et al., 1994). Wnt3 expression was indistinguishable between Fgf8\(^{\Delta b}\) and Fgf8\(^{\Delta b-neo}\) mutants (data not shown). Instead, Wnt3a mutants were detected in the primitive streak and mesoderm (Fig. 5A). In Fgf8\(^{\Delta b}\) embryos at E7.5, Fgf4 expression was lost 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\(^{\Delta b-neo}\) embryos (2/6, Fig. 4A).

The remaining Fgf8a can promote mesoderm specification and regionalization of the primitive streak in Fgf8\(^{\Delta b-neo}\) 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\(^{\Delta b-neo}\) embryos. To determine whether the remaining Fgf8a isoform proteins elicit Fgf8 signaling in Fgf8\(^{\Delta b-neo}\) embryos, we analyzed expression of Spry2, which is a feedback inhibitor of Fgf8 signaling. Spry2 expression occurs in the primitive streak 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\(^{\Delta b-neo}\) 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 Fgf8/H9004b-neo/H9004b-neo embryos.

We next sought to examine whether the residual Fgf8 signaling promotes mesoderm specification in Fgf8/H9004b-neo/H9004b-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 HNF3ß) 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 Fgf8/H9004b-neo/H9004b-neo embryos (Fig. 5F,H). These data suggest that the regionalization of the primitive streak in Fgf8/H9004b-neo/H9004b-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., 1990). Prior studies have shown a great reduction of T and loss of expression of Tbx56 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 Tbx56 (n=3) was detected in the posterior region of Fgf8b-neo/db-neo embryos at E7.5 (Fig. 5J,L). These observations demonstrate that Fgf8a can partially compensate for the loss of Fgf8b in promoting the developmental program for mesoderm specification in Fgf8b-neo/db-neo embryos.

**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 Fgf8b/db/db 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. 4A,C, n=41). These observations suggest that Fgf8b may play an essential role before E7.5. To test this hypothesis, 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 Fgf8b/db/db embryos at E6.5 (see Fig. S1B,D,F in the supplementary material). However, the expression domains of Fgf8, Nodal and Cer1 with respect to the shape of the embryo were found to be strikingly different between wild-type and Fgf8b/db/db embryos. 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 Fgf8b/db/db embryos (see 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 Fgf8b/db/db 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 Fgf8b/db/db (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 Fgf8b/db/db embryos at E6.5 result from a developmental retardation, we measured the dimensions of Fgf8b/db/db 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+/db) embryos (1.52±0.38, n=59), but smaller than 1 in Fgf8b/db/db 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+/db (284.35±80.20 μm, n=39) and Fgf8b/db/db (283.19±78.22 μm, n=26) (Fig. 6J). Therefore, loss of Fgf8b does not cause a gross delay in development.

To ascertain whether the mutant phenotype of Fgf8b/db/db embryos at E6.5 is specific to the loss of Fgf8b, we re-examined the phenotype of Fgf8+/db mutants (Meyers et al., 1998). Fgf8+/db embryos display identical defects in the loss of Lefty2 (2/3) and T (5/5), and abnormal orientation of the AP axis, to those observed in Fgf8b/db/db embryos at E6.5 (Fig. 6C,F). These results demonstrate that the Fgf8b proteins are essential for Fgf8 activity before E6.5.

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

The abnormalities of Fgf8b/db/db and Fgf8+/db 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 Fgf8b/db/db 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, but not the long axis, of the embryo relative 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 Fgf8b/db/db 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.

The AP and long axes of the embryo in both groups tended to be perpendicular to the longitudinal axis of the uterus at E6.5. In Fgf8+/db/db embryos, the expression of Lefty1 and Cer1 was indistinguishable from that in wild type. In Fgf8b/db/db embryos, the expression of Lefty1 and Cer1 was indistinguishable from that in wild type. In Fgf8+/db/db embryos, the expression of Lefty1 and Cer1 was indistinguishable from that in wild type. In Fgf8b/db/db embryos, the expression of Lefty1 and Cer1 was indistinguishable from that in wild type.
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 Fgf8^+/+, whereas the embryos of group I represent wild type (Fgf8^+/+ and Fgf8^-/-). Indeed, the percentage of embryos of group II (27%) is close to the expected Mendelian ratio (25%) for Fgf8^+/+ embryos.

We next examined cross sections of E7.5 embryos within the uterus from the intercross of Fgf8^+/+ parents. At E7.5, Fgf8^+/+ 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 T. In agreement with our analysis of Fgf8^+/+ embryos at E6.5, the AP axis of Fgf8^+/+ 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. 6).

**Fig. 6.** Fgf8b is required for the normal induction of Lefty2 and 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 Lefty1 and Lefty2 in E6.5 murine embryos of the indicated genotypes. Expression of Lefty1 in the anterior visceral endoderm (AVE; marked by arrowhead) is unaffected in Fgf8^+/+ (B) or Fgf8^+/+ (C) embryos, whereas 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 T expression in E6.5 embryos of the indicated genotypes. Arrowhead marks the T expression domain in the distal extraembryonic ectoderm, while the arrow marks T expression in the posterior epiblast. (G,H) Expression of Cer1 in the AVE of wild-type (G) and Fgf8^+/+ (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 Fgf8^+/+ embryos at E5.75 and E6.5. Notice that the shift of AP axis fails to occur in the Fgf8^+/+ 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 Fgf8^+/+ mutants. Each dot represents one embryo. AP, anteroposterior; LR, left-right. Scale bars: 50 μm in F for A–H, including insets in G and H; 50 μm in inset in C for insets in A–C.
7E,F,H). 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 Fgf8bΔ/Δb embryos (25.40±24.24, n=21, P=1.2x10⁻⁸). 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 lumen, 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 Fgf8\(\Delta b/\Delta b\) 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 Fgf8\(\Delta b/\Delta b\) and Fgf8\(-/-\) 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 Fgf8 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 axes is unaffected in the absence of Fgf8b (Fig. 7B). These findings have two important implications. One is that the zona pellucida 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 Fgf8 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 Fgf8\(\Delta b/\Delta b\) embryos? We found that about half of the Fgf8\(\Delta b/\Delta b\) 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 Fgf8\(\Delta b/\Delta b\) embryos were remarkably 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 (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 Fgf8\(\Delta b/\Delta b\) embryos is unlikely to be caused by a gross delay in development. The evidence for this assertion is that Fgf8\(\Delta b/\Delta b\) and wild-type embryos are comparable in size, and Nodal, Wnt3 and Fgf8 are normally induced in the posterior Fgf8\(\Delta b/\Delta b\) 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, Xsprouty2) and Spred, which inhibit MAPK and Ca\(^{2+}/\)PLC\(^{g}\) signaling pathways, respectively, switch Fgf signal interpretation to 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 Fgf8b, different levels of Fgf8a are required for promoting mesoderm specification and mesoderm migration. In Fgf8\(\Delta b-neo/\Delta b-neo\) embryos, 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 Fgf8\(\Delta b-neo/\Delta b-neo\) embryos are largely rescued by removing the neo cassette. We did not attempt to directly compare Fgf8a expression between Fgf8\(\Delta b-neo/\Delta b-neo\) and Fgf8\(\Delta b/\Delta b\) 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 Fgf8\(\Delta b/\Delta b\) embryo produces higher levels of Fgf8a than those in the Fgf8\(\Delta b-neo/\Delta b-neo\) 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 mesoderm migration. We showed that Fgf8a is expressed in the primitive streak of Fgf8b-M1/4 embryos, but not in Fgf8b-M9/14 embryos at E7.5 (Fig. 4A). Clearly, the increased Fgf8a 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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References

Supplementary material

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