FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis

Matthias B. Wahl1, Chuxia Deng2, Mark Lewandoski3 and Olivier Pourquié1,4.*

Fibroblast growth factor (FGF) signaling plays a crucial role in vertebrate segmentation. The FGF pathway establishes a posterior-to-anterior signaling gradient in the presumptive mesoderm (PSM), which controls cell maturation and is involved in the positioning of segmental boundaries. In addition, FGF signaling was shown to be rhythmically activated in the PSM in response to the segmentation clock. Here, we show that conditional deletion of the FGF receptor gene Fgfr1 abolishes FGF signaling in the mouse PSM, resulting in an arrest of the dynamic cyclic gene expression and ultimately leading to an arrest of segmentation. Pharmacological treatments disrupting FGF signaling in the PSM result in an immediate arrest of periodic WNT activation, whereas NOTCH-dependent oscillations stop only during the next oscillatory cycle. Together, these experiments provide genetic evidence for the role of FGF signaling in segmentation, and identify a signaling hierarchy controlling clock oscillations downstream of FGF signaling in the mouse.

KEY WORDS: FGF, Somite, Segmentation, Clock, Oscillation, Vertebra

INTRODUCTION

The striking segmented pattern of the human spine is established during embryogenesis when somites are rhythmically added to the forming posterior part of the embryo. Current models of somitogenesis are based on the clock and wavefront model in which a temporal periodicity generated by the clock in presomitic mesoderm (PSM) cells is translated into the periodic array of somites at the wavefront level (Cooke and Zeeman, 1976; Pourquié, 2003). In the mouse, the clock is a molecular oscillator driving periodic pulses of notch, fibroblast growth factor (FGF) and Wnt signaling in the PSM, with a periodicity matching that of somite production (Aulehla et al., 2003; Dequeant et al., 2006; Palmeirim et al., 1997). The wavefront has been shown to involve a posterior gradient of Wnt and FGF/MAPK activity opposed to a retinoic acid (RA) gradient, which regresses posteriorly in concert with the formation of posterior structures (Aulehla et al., 2003; Diez del Corral and Storey, 2004; Dubrulle et al., 2001; Dubrulle and Pourquié, 2004b; Sawada et al., 2001). This traveling gradient defines a threshold of FGF signaling (the determination front) in the PSM, below which cells become competent to respond to the clock signal (Dubrulle and Pourquié, 2004a). When cells reach the anterior PSM, the FGF-mediated repression is relieved, allowing activation of genes controlling the segmentation program, such as Mesp2, in response to the clock signal (Delfini et al., 2005). In this model, the size of a segment depends on the distance traveled by the wavefront during one oscillation cycle. Therefore, interference with the FGF gradient results in modification of somite size (Diez del Corral et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001; Vermot and Pourquié, 2005). Thus far, this model is based essentially on gain-of-function experiments or pharmacological blockade of the FGF/MAPK pathway in chick, frog and fish (Diez del Corral et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001; Vermot and Pourquié, 2005). No direct genetic evidence for the clock and wavefront model has been provided, partly due to the fact that the FGF pathway is required during gastrulation. Thus, null mutation of genes, such as Fgfl8 or Fgfr1 in the mouse, results in a severe gastrulation defect and the quasi absence of paraxial mesoderm, thus precluding studies of the segmentation process (Deng et al., 1994; Sun et al., 1999). Here, we analyze the effect of a conditional deletion in the mesoderm of Fgfr1, the only FGF receptor expressed in the mouse paraxial mesoderm. We show that this mutation disrupts normal cyclic gene expression in the PSM and results in abnormal segmentation of somites and vertebrae. Also, we observe that inhibition of the FGF/MAPK pathway in cultured mouse embryos blocks oscillations of the Wnt and Notch cyclic genes with different kinetics. These experiments provide genetic evidence for the role of FGF signaling in positioning the determination front in mouse, and suggest that FGF acts upstream of the Wnt and Notch pathways to control the segmentation clock oscillations.

MATERIALS AND METHODS

Generation of mutant embryos

Males carrying one conditional allele for Fgfr1 (Fgfr1fl/fl) (Xu et al., 2002) and positive for the T-Cre transgene (Perantoni et al., 2005) (both on a C57BL/6 genetic background) were mated to homozygous floxed Fgfr1 females (Fgfr1fl/fl) in order to generate Fgfr1fl/fl: T-Cre progeny. To analyze the status of RA signaling, mice were crossed to the RARE (also known as Rare1 – Mouse Genome Informatics)-lacZ mice (Rossant et al., 1991) and β-gal staining was performed using X-Gal as substrate. The floxed Fgfr1 allele was genotyped using primer F: CTGGTATCCT- GTGCCCTATC and primer R: CAATCTGAT CCCAAGACCAC; in response to-the-gene expression and ultimately leading to an arrest of segmentation. Pharmacological treatments disrupting FGF signaling in the PSM result in an immediate arrest of periodic WNT activation, whereas NOTCH-dependent oscillations stop only during the next oscillatory cycle. Together, these experiments provide genetic evidence for the role of FGF signaling in segmentation, and identify a signaling hierarchy controlling clock oscillations downstream of FGF signaling in the mouse.

Accepted 23 August 2007
Mouse tail culture
E9.5 embryo tails were cultured in 10% FBS in DMEM-F12 or 50% rat serum in DMEM-F12 at 37°C in 5% CO2 (Correia and Conlon, 2000) either in 0.1% DMSO or in the presence of the pharmaceutical inhibitors U0126, 100 μM (Promega) or SU5402, 100 μM (Pfizer) in 0.1% DMSO. Explants were cultured for periods ranging from 1-6 hours and were then fixed in 4% formaldehyde and processed for in situ hybridization.

Real-time PCR
RNA from the posterior region of E8.5 mouse tails at the 5- to 9-somite stage was extracted using Trizol reagent (Invitrogen). Embryos were cut in the middle of the PSM and the posterior part was used for RNA isolation and the remaining embryos was subject to genotyping. cDNA was synthesized using SuperScript II (Invitrogen) and for each gene, three independent real-time PCR reactions (each in duplicate) were performed using TaqMan (7900 Fast System, Applied Biosystems) with probes for 

**RESULTS**
Conditional deletion of Fgfr1 in the paraxial mesoderm disrupts segmentation
A difficulty in genetically studying FGF loss of function is the high redundancy of FGF pathway members. For example, the genes coding for the FGF ligands Fgf3, Fgf4, Fgf8 and Fgf17 are expressed in the mouse PSM or its precursors in the primitive streak and tail bud (Crossley and Martin, 1995; Mansour et al., 1993; Maruoka et al., 1998; Niswander and Martin, 1992). Accordingly, conditional deletion of Fgf8 in the primitive streak and/or PSM does not lead to a segmentation phenotype, suggesting that these other ligands might act redundant in this process (Peranonti et al., 2005). Here, we have carefully compared the expression of Fgf3, Fgf4 and Fgf17 to that of Fgf8 in the mouse PSM (Fig. 1A-D). We observed that only Fgf4 is not expressed in a gradient in the posterior PSM (Fig. 1B). Fgf4 expression is restricted to a small cell population in the tail bud, which also expresses the other FGF ligands. By contrast, Fgfr1 is the only known FGF receptor we could detect by in situ hybridization in the PSM (Fig. 1E). Since mice homozygous for a null Fgfr1 allele do not form PSM or somites because of a

![Fig. 1. Expression of Fgf ligands. In situ hybridization for (A) Fgf3, (B) Fgf4, (C) Fgf8, (D) Fgf17 and (E) Fgfr1, in E9.0 embryos. Fgf3, Fgf8 and Fgf17 are expressed in a gradient in the posterior PSM. Fgf4 expression is restricted to a small cell population in the tail bud, which also expresses the other FGF ligands.](image)

**Fig. 2. Progressive disruption of segmentation in the Fgfr1<sup>fl/+;T-Cre</sup> mutant embryos.** (A,B) Skeletons stained with Alizarin Red (bone) and Alcian Blue (cartilage) were performed as described previously (Kessel et al., 1996). All probes for in situ hybridization were either amplified by RT-PCR [Fgfr1 (primer F: ATGCACTCCCATCCTGGGAGAA, primer R: GGAAAGAAGGAAAAAGTTTGCATCA, primer R: TTTTTAAAGCAGCGAGGAGAA, primer R: CCTCCCCGATTTGCTCACAT); Raldh2 (primer F: ACTCAGAGAGTGGGAGAGTG, primer R: AATTGACGAGGAGAA, primer R: CCTCCCCGATTTGCTCACAT); Paraxis (primer F: TGCTGAGCGAGGAGGAGAA, primer R: CACACTCTGTGGCCTGG); Msgn1 (primer F: ATGGACAACCTGGGTGAGAC, primer R: TCAACAGACAAAACTCAGGG); Mesp2 (primer F: ATGGACAACCTGGGTGAGAC, primer R: TCAACAGACAAAACTCAGGG); Mesp1 (primer F: ATGGACAACCTGGGTGAGAC, primer R: TCAACAGACAAAACTCAGGG); Sef (primer F: CAGGAACAGCGGACTGATCTTTTGCGCATCG; primer R: GCCACAGAAATCTTGCAGGA)] or have been previously described in the literature (Axin2, Cyp26, Dkk1-intronic, Dll1, Dll3, Dusp6, Erm, Fgf3, Fgf4, Fgf8, Fgf17, Gbx2, Lfng, Lfng-intronic, Mesp2, Notch1, Pea3, Shh, Snail1, T, Uncx4.1, Wnt5a). Mouse Genome Informatics lists some of the above genes with different names and symbols; they are, Paraxis as Tcfl15, Raldh2 as Aldh1a2, Sef as Il17rd, Cyp26 as Cyp26a1, Erm as Etv5 and Pea3 as Etv3. (Fig. 2A, B) In situ hybridization in the PSM (Fig. 1E). Since mice homozygous for a null Fgfr1 allele do not form PSM or somites because of a

![Fig. 2. Progressive disruption of segmentation in the Fgfr1<sup>fl/+;T-Cre</sup> mutant embryos.](image)
gastrulation defect (Deng et al., 1994), we used a conditional \textit{Fgfr1} allele in which exons 9-13 are flanked by LoxP sites (Xu et al., 2002). This mouse line was crossed to the \textit{T-Cre} line in which \textit{Cre} is controlled by the \textit{T} primitive streak enhancer, which promotes recombination in most primitive streak descendants including somites, PSM and tail bud (data not shown) (Perantoni et al., 2005). Fetuses homozygous for the floxed allele of \textit{Fgfr1} and positive for the \textit{T-Cre} transgene (hereafter called \textit{Fgfr1}^{f/f};\textit{T-Cre}) survive up to birth, but die neonatally. Skeletal preparations from \textit{Fgfr1}^{f/f};\textit{T-Cre} fetuses and neonates clearly showed, in all specimens, axial truncations in the sacral and tail regions, whereas the anterior segments were formed (Fig. 2A,B). We also observed rudimentary hind limbs as previously reported (data not shown) (Verheyden et al., 2005). Although cervical vertebrae appeared to be normal, more posteriorly, vertebrae and ribs became progressively fused (Fig. 2B and data not shown). Irregular skeletal elements corresponding to fused lumbar and sacral vertebral elements were present, but the caudal region did not form. These observations are consistent with a progressive disruption of the segmentation process in \textit{Fgfr1}^{f/f};\textit{T-Cre} mutants.

To trace the origin of these defects, we analyzed segmentation in detail in mutant embryos between E8.5 and E10.5. Somite formation was first examined using the \textit{Uncx4.1} probe which marks the posterior compartment of formed somites (Mansouri et al., 1997). Whereas approximately the first 10-13 somites appeared relatively normal in the mutant embryos (Fig. 2C-F and data not shown), larger irregular somites were often seen in \textit{Fgfr1}^{f/f};\textit{T-Cre} embryos at the level of somites 10-15 \((n=6/6);\) Fig. 2G,H, arrowheads). Surprisingly, \textit{Uncx4.1} expression was not always found in the posterior compartment of these larger somites (Fig. 2H). Posterior to this region, no clear segmented structures were observed (Fig. 2D,F and data not shown), and the region appeared abnormal, with an enlarged neural tube expressing \textit{Sox2} and a thinner PSM (Fig. 3A,B and data not shown). \textit{Shh} expression was weaker, but nevertheless detected.

\textbf{Fig. 3. Expression of different marker genes in E9.0} \textit{Fgfr1}^{f/f};\textit{T-Cre} mutant embryos. Expression of (A,B) \textit{Sox2}, (C,D) \textit{Wnt3a}, (E,F) \textit{T}, (G,H) \textit{Uncx4.1/Msgn1}, (I,J) \textit{Fgf8}, (K,L) \textit{Dll1}, (M,N) \textit{Notch1}, (O,P) \textit{Dll3}, (Q,R) \textit{Paraxis} and (S,T) \textit{Mesp2} in \textit{Fgfr1}^{105f};\textit{T-Cre} control and \textit{Fgfr1}^{f/f};\textit{T-Cre} mutant embryos, respectively.
all along the notochord and the floor plate, suggesting a normal differentiation of axial structures (data not shown). Wnt3a expression was maintained in the tail bud (Fig. 3C,D), and its downstream targets involved in PSM patterning, including T (Fig. 3E,F), Msgn1 (Fig. 3G,H), Fgf8 (Fig. 3I,J) and Dll1 (Fig. 3K,L) were expressed in the posterior PSM. The expression domain of Msgn1 was, nevertheless, much smaller than in wild-type embryos and was confined to the posterior-most region of the PSM (Fig. 3G,H). Dll1 is normally expressed in an anterior-to-posterior gradient in the PSM (Fig. 3K) but in the mutant embryos, the expression gradient was reversed, with the strongest expression found in the tail bud (Fig. 3L), suggesting that FGF represses Dll1 transcription. Mesp2, which marks the future segment territory at the determination front level was severely downregulated in the region failing to form somites in the mutants (Fig. 3S,T). Interestingly, the somitic marker Uncx4.1 is nevertheless expressed in the posterior unsegmented region, indicating that paraxial mesoderm maturation still proceeds in the absence of somite formation (Fig. 2D,F).

Fig. 4. Progressive downregulation of FGF target genes in Fgfr1<sup>ff</sup>; T-Cre mutant embryos after the 6-somite stage. (A-D) Pea3 is normally expressed in control (A) and Fgfr1<sup>ff</sup>; T-Cre (B) embryos at the 5-somite stage, and in 7-somite control embryos (C), but it becomes progressively downregulated in the posterior PSM/tail bud of 7-somite Fgfr1<sup>ff</sup>; T-Cre mutant embryos (D). (E) Real-time PCR for FGF target genes Erm and Pea3 in the posterior tail of Fgfr1<sup>ff</sup>; T-Cre mutant embryos and Fgfr1<sup>ff</sup>; T-Cre control embryos at somite stages 5 (n=1/1), 6 (n=3/5), 7 (n=2/2) and 9 (n=1/1). Both genes become progressively downregulated from somite stages 6 onward. Levels of Erm and Pea3 were normalized to the housekeeping gene Hprt and values are given as the mean change in crossing points (CT) in Fgfr1<sup>ff</sup>; T-Cre mutant embryos. (F-O) For other target genes (F,G) Erm, (H,I) Gbx2, (J,K) Dusp6, (L,M) Spry2 and (N,O) Sef, the expression in the posterior PSM and the tail bud of E8.75 Fgfr1<sup>ff</sup>; T-Cre mutant embryos is lost, whereas the expression in the anterior PSM and the adjacent structures is normal.
To relate the somitogenesis defect to FGF activation, we first carefully mapped the timing of loss of FGF signaling in the conditional mutants by in situ hybridization for the known FGF target Pea3 (Fig. 4A-D) (Chatteau-Lelievre et al., 2001). Between the 5- to 7-somite stage, the expression level of Pea3 became strongly downregulated in the posterior PSM and tail bud, suggesting that FGF signaling in the posterior paraxial mesoderm begins to decrease around this time (Fig. 4A-D and data not shown). To confirm this, we analyzed the expression levels of the two FGF target genes Pea3 and Erm in the posterior embryo, including the posterior PSM and the tail bud, by quantitative real-time PCR. Expression of these genes decreased progressively from the 5-somite stage onward (Fig. 4E). We also analyzed the expression of other known FGF target genes including Erm, Gbx2, Dusp6, Spry2 and Sef by in situ hybridization and observed that they are also downregulated in the posterior PSM and tail bud of Fgfr1<sup>f/f</sup>;T-Cre embryos after the 5- to 8-somite stage (Fig. 4F-O). Together, this suggests that FGF signaling becomes progressively downregulated in the paraxial mesoderm posterior to somite 5, approximately. These observations indicate that the progressive failure of somite boundary formation in these mutants parallels the progressive loss of FGF signaling in the forming PSM.

**Loss of FGF signaling does not alter the positioning of the RA-responsive domain in the PSM but disrupts cyclic gene oscillations**

The posterior FGF signaling gradient has been shown to be antagonized by an opposing RA gradient resulting from the production of RA by its biosynthetic enzyme RALDH2 in the segmented region of the embryo (Diez del Corral et al., 2003). In Fgfr1<sup>f/f</sup>;T-Cre embryos, expression of the transcript for the RA-degrading enzyme Cyp26 that is normally found in the tail bud region, is strongly downregulated (Fig. 5A,B). This is expected to lead to a gain of function of RA signaling and hence, a posterior extension of the RA-responsive domain. However, no significant difference in the positioning of the RA-responsive domain was detected between the wild-type and mutant mice using the RARE-LacZ mouse reporter (Rossant et al., 1991) (Fig. 5C,D). Consistently, expression of genes normally expressed in the RA-responsive domain, such as Paraxis (Fig. 3Q,R) or Raldh2 (Fig. 5E,F) was not significantly disrupted in the Fgfr1<sup>f/f</sup>;T-Cre embryos. A progressive shrinking of the Msgn1-positive, Uncx4.1-negative domain in the posterior PSM is nevertheless observed after the E9.5-somite stage (compare the posterior Uncx4.1-negative domain in Fig. 2E,F with the size of the Msgn1 domain in Fig. 3G,H). Therefore, FGF signaling is not necessary to position the RA-responsive domain in the anterior PSM.

Oscillations of FGF signaling targets, such as Spry2 in the mouse PSM, have recently implicated this pathway in the segmentation clock mechanism, and this pulse of FGF signaling occurs in phase with Notch signaling (Dequeant et al., 2006). However, in chick embryo cultures, short-term treatments with pharmacological inhibitors of FGF signaling or the MAPK pathway do not block oscillations of the Notch cyclic genes (Delfini et al., 2005; Dubrulle et al., 2001), and Spry2 expression remains dynamic in the mouse Notch mutant for RBPjk9 (also known as Rbpj – Mouse Genome Informatics) (Dequeant et al., 2006). This suggests that whereas NOTCH and FGF oscillate synchronously, their oscillations are controlled largely independently. To evaluate this further, we examined the expression of cyclic genes in Fgfr1<sup>f/f</sup>;T-Cre mutant embryos. Prior to the 8-somite stage when FGF signaling is maintained in the posterior PSM of Fgfr1<sup>f/f</sup>;T-Cre mutant embryos, the NOTCH cyclic gene Lfng, the WNT cyclic genes Dkk1 and Axin2, as well as the FGF cyclic genes Spry2 and Snail1, show different expression patterns, suggesting that the clock function is essentially normal (data not shown). However, in embryos with more than 10 somites, all cyclic genes show abnormal expression patterns (Fig. 6A-H). Lfng (n=15, Fig. 6B), Spry2 (n=10, Fig. 6D) display an anterior-to-posterior expression gradient with no expression in the tail bud. Axin2 (n=18, Fig. 6F), Dkk1 (n=7, data not shown) and Snail1 (n=4, Fig. 6H) have an opposite expression pattern, with strong staining in the tail bud but virtually no expression in the PSM. Disruption of the NOTCH cyclic gene oscillations is accompanied by abnormal expression in the PSM of several genes of the NOTCH pathway including Dll1 (Fig. 3K,L), Dll3 (Fig. 3O,P) and Notch1 (Fig. 3M,N). Both Notch1 and Dll1 are upregulated in the tail bud and posterior PSM, while Dll3 is severely downregulated, showing a faint ‘salt-and-pepper’ expression (Fig. 3O,P). Therefore, FGF signaling is required for oscillations of cyclic genes of the WNT, NOTCH and FGF pathway in the PSM.

**Pharmacological inhibition of FGF signaling in mouse embryos disrupts Wnt and Notch oscillations with different kinetics**

To further confirm the disruption of cyclic gene oscillations in the absence of FGF signaling, we cultured mouse tails in the presence of either the FGF receptor 1 inhibitor SU5402 (Mohammadi et al.,

---

**Fig. 5. FGF signaling is not sufficient to position the RA-responsive domain in the PSM.** (A,B) Cyp26 expression in the posterior region of the Fgfr1<sup>f/f</sup>;T-Cre (B) mutant is downregulated compared with that in Fgfr1<sup>f/+;</sup>T-Cre control (A) embryos. (C,D) There is a lack of significant change in RA activity, as detected by crossing to RARE-lacZ reporter mice, in Fgfr1<sup>f/+;</sup>T-Cre mutant (D) compared with the Fgfr1<sup>f/+;</sup>T-Cre control (C) embryos. (E,F) Expression of Raldh2 is not significantly changed in the PSM of Fgfr1<sup>f/f</sup>;T-Cre mutant embryos (F) compared with control Fgfr1<sup>f/+;</sup>T-Cre (E) embryos.
or the MKK1 inhibitor U0126 (DeSilva et al., 1998) (which blocks ERK phosphorylation) and analyzed the dynamic expression of Spry2, Axin2 and Lfng (Table 1 and data not shown). We used two different culture conditions (i.e. hanging drop culture in 10% FBS in DMEM-F12 or hanging drop culture in 50% rat serum in DMEM-F12) with E9.5 mouse tails (Correia and Conlon, 2000) and examined, by in situ hybridization, the expression of FGF target genes as a control for each batch of cultured embryos. As expected, the FGF target Spry2 is rapidly downregulated in the posterior PSM after SU5402 or U0126 treatment for 2 hours, whereas control tails cultured in DMSO maintained dynamic expression patterns for Spry2 (n=18, data not shown). Distinct patterns of Axin2 were evident in DMSO-cultured control tails, but the expression of Axin2 in the PSM of the treated embryos was downregulated after 2 hours in more than 80% of the explants, whereas it was always expressed in the tail bud (Table 1 and data not shown). This rapid downregulation of Axin2 in the PSM, which occurs during the first oscillation cycle after treatment, suggests that Axin2 might be directly regulated by FGF signaling through the MAPK pathway. Strikingly, a different situation was observed for Lfng. Whereas no significant change in expression was detected after 2 hours in culture, most embryos treated with SU5402 or U0126 began to show a similar pattern after 3 hours, a time corresponding to one clock oscillation period in these culture conditions (Table 1 and data not shown). This pattern was evident as a single stripe located in the anterior PSM and resembling phase III of the normal cycle (Pourquie and Tam, 2001) (data not shown). Therefore, blocking FGF signaling in vitro using pharmacological inhibitors disrupts the first cycle of Axin2 oscillations but acts only after a one cycle delay on Lfng oscillations.

**DISCUSSION**

Here, we show that a conditional deletion of Fgfr1, the only FGF receptor expressed in the mouse PSM, blocks somite formation. Therefore, this provides a genetic demonstration for the role of FGF signaling in vertebrate segmentation. In the mutants, however, FGF signaling remains active during formation of the first somites that appear essentially normal (Fig. 2D, Fig. 7). This delay is somehow surprising because the T-Cre driver has been shown to be active from the earliest stages of gastrulation (Perantoni et al., 2005). The delayed progressive onset of the phenotypes observed could be explained by the stability of the Fgfr1 transcript and protein. Following the formation of the first five somites or so, gradual downregulation of the FGF targets is observed in the PSM, indicating a progressive downregulation of the pathway activation. In the mutants, however, the first 10-13 somites appear essentially...
normal. In the mouse, the PSM contains around six presumptive somites (Tam, 1986), meaning that the precursors of somites 10-13 were already located in the posterior PSM when the downregulation of FGF signaling began. This suggests that enough FGF signaling was still available to allow proper specification of these somites. Posterior to somites 10-13, transient formation of a few larger irregular somites was observed (Fig. 2H), a phenotype similar to that observed in fish or chick following treatments with drugs blocking FGF signaling, such as SU5402 (Dubrulle et al., 2001). Such a phenotype is predicted by the clock and wavefront model, since downregulating FGF signaling triggers a posterior shift of the wavefront, which is expected to lead to the formation of larger somites (Dubrulle and Pourquie, 2004a). Surprisingly, Unca4.1 whose expression is normally restricted to the posterior compartment of the somites, was sometimes found in the middle or in the anterior part of these larger somites, supporting the idea that posterior somite formation in Raldh2-null mutants can be rescued by early RA treatment (Sirbu and Duester, 2006). However, these observations are difficult to reconcile with the fact that expression of Raldh2 in the segmented region and of Cyp26 in the tail bud extend all along the AP axis (Fujii et al., 1997; Niederreither et al., 1997). Moreover, a Cyp26 null mutation in the mouse leads to axis truncation at the lumbar level, suggesting that RA plays a role in the formation of posterior somites as well (Sakai et al., 2001). In the chick embryo, FGF signaling has been shown to antagonize the RA gradient and to maintain the undifferentiated state of cells in the posterior part of the embryo throughout somitogenesis (Diez del Corral and Storey, 2004; Mathis et al., 2001; Vermot and Pourquie, 2005). Experiments in chick and frog have led to the proposal that in the PSM these mutually antagonistic gradients are necessary for the appropriate positioning of the determination front (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Vermot and Pourquie, 2005). This hypothesis, however, is challenged by the observation that mouse Raldh2 null mutants and vitamin A-deficient quail embryos (which cannot synthesize RA) form smaller, yet reasonably normal somites (Maden et al., 2000; Niederreither et al., 1999). Thus, in amniotes, RA

---

**Table 1. Summary of the expression of FGF target genes in tail cultures in the absence (DMSO control) and presence of either the FGFR1 inhibitor SU5402 or the MAPK inhibitor U0126**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
<th>Phase inhibitor</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lrng</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h DMSO</td>
<td>33</td>
<td>39</td>
<td>28</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>2 h SU5402</td>
<td>30</td>
<td>39</td>
<td>58</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>3 h DMSO</td>
<td>28</td>
<td>18</td>
<td>55</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3 h SU5402</td>
<td>10</td>
<td>20</td>
<td>70</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>4 h DMSO</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>4 h SU5402</td>
<td>10</td>
<td>20</td>
<td>70</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>6 h DMSO</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>6 h SU5402</td>
<td>6</td>
<td>12</td>
<td>82</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

Percentage of tails in Phases I, II and III are given for each combination at 2, 3, 4 and 6 hours. For Axin2, which shows an abnormal expression pattern after inhibitor treatment (data not shown), an additional column (Phase inhibitor) is introduced.

---

**Fig. 7. Summary of the onset of the phenotypes observed in Fgfr1<sup>fl/fl</sup>;T-Cre mutant embryos.** FGF target genes become downregulated in the posterior PSM of Fgfr1<sup>fl/fl</sup>;T-Cre mutant embryos at the 5- to 7-somite stage, followed by the arrest of cyclic gene expression between somites 8 and 10. Normal somites and corresponding vertebrae elements are observed up to somites 10 to 13; however, abnormal skeletal elements derived from paraxial mesoderm posterior to somite 13 were present.
signaling plays a role in refining the positioning of the determination front but is not critically required for boundary formation. Our results indicate that up to E9, the Raldh2- and the RARE-lacZ-positive domains in the PSM are not significantly extended posteriorly in Fgfr1 conditional mutants despite the absence of the RA-degrading enzyme CYP26 in the posterior part of the embryo. This argues that in contrast to the situation in chick and frog, in the mouse FGF signaling antagonism is insufficient to explain the anterior positioning of the RA signaling domain (Diez del Corral et al., 2003; Moreno and Kintner, 2004). WNT signaling has also been shown to establish a posterior-to-anterior gradient that plays a role in the positioning of the determination front in the mouse (Aulehla et al., 2003). In the conditional mutants, Wnt3a (Fig. 3.C,D) and its downstream targets T (Fig. 3.E,F) and Axin2 (Fig. 6.E,F) are still expressed posteriorly, suggesting that WNT signaling is still active in the PSM. Thus, WNT signaling could act redundantly with FGF signaling to antagonize RA signaling in the PSM. Alternatively, the smaller somite size observed in Raldh2 mutants has been proposed to result indirectly from an early antagonistic effect of RA on FGF signaling in the node and posterior neural plate (Sirbu and Duester, 2006). Such an effect is likely to be intact in the Fgfr1 mutants, because the T promoter fragment does not drive expression in the node at these stages, and thus could account for the lack of effect on the positioning of the later RA domain seen in Fgfr1 conditional mutants (Perantoni et al., 2005).

Oscillations of downstream targets of FGF signaling, such as Spry2 or Dusp6 (Dequante et al., 2006), combined with our observations that FGF signaling is required for oscillations of cyclical genes of the WNT, NOTCH and FGF pathway in the PSM, provide evidence for a cyclic activation of the pathway in the PSM. On the other hand, graded distribution of the ligands and of the downstream effectors such as phosphorylated ERK (Delfini et al., 2005; Sawada et al., 2001) and AKT (Dubrulle and Pourquie, 2004b) shows that FGF signaling is also activated in a graded fashion along the PSM. A similar situation is also observed for WNT signaling which was shown to be periodically activated in the PSM and forms a signaling gradient in the tissue (Aulehla et al., 2003). Although at first glance these observations seem difficult to reconcile, several possible explanations can be envisioned to account for this situation. First, it could be that the pathway shows an overall graded yet periodic activation in the posterior PSM (Aulehla et al., 2003). These fluctuations could be sufficient to elicit periodic transcriptional output, but not to be detected biochemically using tools such as anti-phosphorylated ERK antibodies. We previously showed that phosphorylated ERK is extremely unstable in the mouse embryo PSM and hence, detecting small cyclic fluctuations might be technically very challenging (Delfini et al., 2005). Alternatively, it could be that FGF signaling is distributed uniformly in a graded fashion and is essentially required permissively for cyclic gene oscillations and its periodic transcription would be controlled independently of FGF signaling.

Oscillations of Lfng, Spry2 and Axin2 are also disrupted in cultures of mouse tails in the presence of pharmacological inhibitors of FGF signaling. In these experiments, the WNT cyclic gene Axin2 and the FGF cyclic gene Spry2 are rapidly downregulated in the PSM after inhibitor treatment, whereas Lfng expression continues to oscillate for one cycle. The observation that Lfng oscillations are halted in the vestigial tail mouse mutant led to the suggestion that in the mouse, the WNT pathway acts upstream of NOTCH oscillations (Aulehla et al., 2003). These data are therefore consistent with FGF indirectly controlling NOTCH oscillations via the WNT pathway. In summary, these data provide direct genetic evidence supporting the role of FGF signaling in the wavefront, which is involved in positioning somite boundaries in the PSM and in establishing a hierarchy in the NOTCH, WNT and FGF signaling pathways involved in the control of oscillatory expression of cyclical genes in the PSM.

We thank members of the Pourquie lab for discussions and comments, Stowers Institute Core Facilities, Joanne Chatfield for help on the manuscript and Silvia Esteban for artwork. This work was supported by Stowers Institute for Medical Research. O.P. is a Howard Hughes Medical Investigator.

References
domain of fibroblast growth factor receptor in complex with inhibitors. Science 276, 955-960.


