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The role of FGF signaling in establishment and maintenance of mesodermal gene expression in *Xenopus*

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Abstract

FGF signaling is important for the formation of mesoderm in vertebrates, and when it is perturbed in *Xenopus*, most trunk and tail mesoderm fails to form. Here we have further dissected the activities of FGF in patterning the embryo by addressing its inductive and maintenance roles. We show that FGF signaling is necessary for the establishment of *xbra* expression in addition to its well-characterized role in maintaining *xbra* expression. The role of FGF signaling in organizer formation is not clear in *Xenopus*. We find that FGF signaling is essential for the initial specification of paraxial mesoderm but not for activation of several pan-mesodermal and most organizer genes; however, early FGF signaling is necessary for the maintenance of organizer gene expression into the neurula stage. Inhibition of FGF signaling prevents *VegT* activation of specific mesodermal transcripts. These findings illuminate how FGF signaling contributes to the establishment of distinct types of mesoderm.

Keywords

FGF; FGF8; FGF4; mesoderm; *Xenopus*; organizer; paraxial mesoderm; gastrulation

Introduction

In *Xenopus*, the three germ layers, endoderm, mesoderm, and ectoderm, are specified early in development after zygotic transcription by a complex interplay of signals and maternal determinants. The maternal determinant *VegT* is localized to the vegetal hemisphere of the embryo and can activate transcription of several TGF β family members including *derriere*, *Xnr1,2,4,5,6*, and *activin β B*. *VegT* acts in concert with these nodal-like signals to induce endoderm and mesoderm (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997; Zhang et al., 1998; Clements et al., 1999; Kofron et al., 1999; Xanthos et al., 2001). Several studies have shown that these signals are necessary for mesoderm formation in *Xenopus*. Dominant negative Activin receptors or inhibitory *smad7* mRNA inhibit mesoderm formation (Hemmati-Brivanlou and Melton, 1992; Chang et al., 1997; Bhushan et al., 1998; Casellas and Brivanlou, 1998). Similarly, inhibition of TGF β ligands with non-cleavable precursors or by expression of nodal antagonists also prevent mesoderm formation (Sun et al., 1999; Agius et al., 2000; Cheng et al., 2000; Tanegashima et al., 2000; Eimon and Harland, 2002; White et al., 2002).

While nodal signaling is essential, FGF signaling also plays a crucial role in mesoderm formation, and an FGF ligand (*FGF2*) was the first identified mesoderm inducer (Kimelman

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and Kirschner, 1987; Slack et al., 1987; Slack et al., 1990) though since it lacks a signal sequence it may not be active in normal development (Thompson and Slack, 1992). Disruption of FGF signaling in the embryo with a dominant negative receptor causes a loss of trunk and tail tissues (Amaya et al., 1991; Amaya et al., 1993). In *Xenopus* explants, FGF signaling through MAPK is necessary for most mesoderm induction by *activin* (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Cornell et al., 1995; LaBonne et al., 1995). In vivo, multiple FGF ligands are involved in regulating mesoderm formation, including *FGF4* and *FGF8*, which are necessary for paraxial mesoderm formation (Isaacs et al., 1992; Isaacs et al., 1994; Isaacs et al., 1995; Standley et al., 2001; Fisher et al., 2002; Fletcher et al., 2006; Isaacs et al., 2007).

FGF signaling is essential for proper expression of the early mesodermal transcript, *xbra*. Disruption of FGF signaling causes a loss of *xbra* expression in embryos (Amaya et al., 1993; Delaune et al., 2005), and *xbra* itself can induce mesoderm in explants, but this activity is prevented if FGF signaling is disrupted (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Both *xbra* and *FGF4* can induce expression of the other in explants (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Thus, *xbra* and *FGF4* function in a positive feedback loop in explants, and a dominant-negative FGFR expressed after zygotic transcription reduces *xbra* expression more definitively as gastrulation proceeds (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Kroll and Amaya, 1996). For these reasons, FGF signaling has been argued to function in a maintenance role in mesoderm formation. However, it has not been determined if active FGF signaling is necessary for the initial establishment of *xbra* expression. One line of evidence supporting an early role of FGF signaling in mesoderm formation, in addition to a role in maintenance, is that at the blastula stage, MAPK is activated in the dorsal marginal zone in the same early time frame as SMAD2 (Schohl and Fagotto, 2002).

Though FGF signaling has clearly been shown to be necessary for proper *xbra* expression and formation of the paraxial mesoderm, its precise role in organizer formation is less clear. Not all types of mesoderm are sensitive to disruption of FGF signaling, as the organizer transcript, *gooseoid* (*gsc*), and the early mesodermal transcript, *eomesodermin* (*eomes*), are expressed when FGF signaling is disrupted (Amaya et al., 1993; Kumano and Smith, 2000; Delaune et al., 2005). A more systematic study of FGF signaling activity has shown that FGF signaling is not necessary for the initial expression of several organizer and anterior ectodermal transcripts suggesting that organizer formation is not dependent on FGF signaling (Delaune et al., 2005). However, for some organizer transcripts, different results have been reported, for example, *chordin* and *noggin* have been reported to be both sensitive and insensitive to FGF inhibition (Mitchell and Sheets, 2001; Delaune et al., 2005), and as these molecules are essential to organizer function (Khokha et al., 2005), the involvement of FGF signaling in their induction needs clarification. Further, although there is evidence that FGF signaling is not necessary for organizer formation, the involvement of FGF signaling in maintaining organizer gene transcription has not been addressed.

To address the necessity for FGF signaling to induce and maintain expression of different mesodermal transcripts, we used the FGFR inhibitor, SU5402, which can be applied at different times and washed out to allow recovery of signaling (Mohammadi et al., 1997; Delaune et al., 2005). To determine if FGF signaling is important for the initial zygotic transcription of *xbra*, we performed a time-course treatment of embryos. We analyzed a range of both organizer and non-organizer mesodermal gene expression patterns in the absence of FGF signaling to determine whether signaling inhibition affected organizer formation and maintenance and to understand how FGF signaling affected paraxial mesoderm development.

The way in which maternal determinants, and FGF and nodal pathways cooperate to induce mesoderm has been examined in several ways, including analysis on arrays (Kumano and Smith, 2000; Kumano and Smith, 2002; Chung et al., 2004). However, while a limited number of up- and down-regulated targets have been identified, we do not have a good understanding of how these signals cooperate to produce the final pattern of the embryo. We investigated interactions between *VegT* and FGF signaling by analyzing the ability of ectopic *VegT* to induce endoderm and mesoderm in the presence and absence of FGF signaling.

Experimental Procedures

Embryo culture

Xenopus laevis eggs were collected, fertilized and embryos cultured by standard procedures (Sive et al., 2000); embryos were staged according to Nieuwkoop and Faber (1994).

Whole mount RNA in situ hybridization

For RNA in situ hybridization multibasket containers were used (Sive et al. 2000). Nuclear localized β -galactosidase (n β gal-CS2+) mRNA was used to trace mRNAs. After fixation for 30 minutes in MEMFA and washing in PBS + 0.1% tween-20, tracer was visualized using Red-Gal (Research Organics) (Sive et al. 2000); after staining, embryos were refixed in MEMFA for an hour and dehydrated in methanol.

Embryos that were injected with the fluorescein-conjugated morpholino oligonucleotide as a lineage tracer were processed for in situ hybridization, then re-blocked, incubated with anti-fluorescein alkaline phosphatase conjugated secondary antibody, washed with MAB, and visualized with magenta phos and tetrazolium red histochemical substrates in a 10:1 ratio.

Anti-sense RNA probes were made for the following transcripts: *xbra*, (Smith et al., 1991); *myoD*, (Hopwood et al., 1989); *sox2*, (Grammer et al., 2000); *otx2*, (Lamb et al., 1993); *otx5* (Vignali et al., 2000); *hoxB9*, (Sharpe et al., 1987); *gsc*, (Cho et al., 1991); *myf5*, (Hopwood et al., 1991); *noggin*, (Smith and Harland, 1992); *chordin*, (Sasai et al., 1995); *xnr3*, (Smith et al., 1995); *sox17*, (Hudson et al., 1997); *eomes*, (Ryan et al., 1996); *VegT*, (Stennard et al., 1996); *edl*, (Sasai et al., 1996); *FGF4*, (Isaacs et al., 1992); *FGF8* (Fletcher et al., 2006).

mRNA synthesis and injection

Capped messenger RNA was synthesized using the SP6 mMessage mMachine kit (Ambion). Quantified mRNA was resuspended in RNase-free H₂O. The following constructs were linearized and used as templates for SP6 mediated in vitro mRNA synthesis: *VegT* (a version of *antipodean* expression cloned from a gastrula library by Wenge Zhang, unpublished); (Stennard et al., 1999); *FGF8b* (XLFGF8b-CS8) (Fletcher et al., 2006); *FGF4* (Isaacs et al., 1994); *nuclear β -galactosidase* (Turner and Weintraub, 1994). Embryos were injected into one cell at the two-cell stage in 5 or 10 nL volumes.

RT-PCR

Proteinase K/SDS lysis, DNase, and phenol/chloroform extraction were used to isolate RNA from embryos for reverse-transcriptase polymerase chain reaction (RT-PCR) (Wilson and Melton, 1994). To assay for DNA contamination in RT-PCR experiments, an uninjected control embryo was processed without reverse transcriptase and labeled as the RT minus lane in each experiment. *EF1 α* and *ornithine decarboxylase (ODC)* were used as loading controls. RT-PCR primers for the following have been described: *EF1 α* (Krieg et al., 1989); *xbra* (Isaacs et al., 1994); *ODC*, (Hudson et al., 1997); *FGF8* (Fletcher et al., 2006); *FGF4*,

(Isaacs et al., 1994); *sox17beta*, (Hudson et al., 1997); *endodermin*, (Sasai et al., 1996); *xnr1*, *xnr2* (Kofron et al., 1999); *xnr3*, (Smith et al., 1995; Eimon and Harland, 2002).

Morpholino oligonucleotide (MO) design and injection

The morpholino oligonucleotide (MO) (Gene Tools, LLC) binds the translation initiation region of the *FGF8* mRNA; the sequence of the *X. laevis FGF8* translation blocking morpholino oligonucleotide (XIMOF8) is 5'-GGAGGTGATGTAGTTCATGTTGCTC-3' (Fletcher et al., 2006). The Gene Tools, LLC standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') conjugated to fluorescein or nuclear β -galactosidase mRNA was co-injected as a lineage tracer. The morpholino oligonucleotides were resuspended in RNase-free 1/20 X MR. The injection volume was either 5 nL or 10 nL.

FGFR inhibitor SU5402 treatments

The FGFR inhibitor, SU5402 (Mohammadi et al., 1997), was resuspended in DMSO to make a master stock of 50 mM concentration and diluted in 1/3X MR in treatments to a final concentration of 90–100 micromolar. Although different batches have slightly different activities, we generally found that *xbra* expression was absent at an 80 micromolar dose; for all batches we used a higher dose than was needed to block *Xbra* expression to insure that FGF signaling was prevented.

Results

FGF signaling is necessary for initiation of *xbra* transcription

We used the FGFR inhibitor SU5402 to block FGF signaling (Mohammadi et al., 1997). Application of the inhibitor after zygotic transcription and the onset of *xbra* expression caused a reduction of *xbra* expression that was more severe the earlier the drug was administered (Figure 1B,C). This confirms the importance of FGF signaling in maintaining *xbra* expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Washing out the inhibitor before zygotic transcription begins can reverse its effects (Figure 1D). These results mirror the time-dependent effects that Delaune et al., (2005) found on subsequent expression of neural markers. Since it is well established that FGF signaling is necessary for *xbra* expression at gastrulation, we assayed a dose-response to the inhibitor SU5402 by analyzing gastrula stage embryos that had been treated since cleavage stages for *xbra* expression. *Xbra* expression was very strongly reduced but still detectable at the 60 micromolar dose (Supplemental Figure 1A). Additionally, we applied the inhibitor at a range of doses during gastrulation and observed the expected reduction in posterior neural tissue that was more severe at higher doses (Supplemental Figure 1B). Therefore, we used a dose of 90–100 micromolar for all drug-treatment experiments to ensure full inhibition of FGF signaling. This is about half the lethal dose.

To determine if FGF signaling is necessary for the initial activation of *xbra* expression or only for its maintenance, we examined closely spaced intervals of embryogenesis, after embryos were treated with SU5402 from the 8- to 16-cell stage. Embryos were collected and fixed at thirty-minute time intervals beginning at 6 hours post-fertilization during mid-blastulation. *Xbra* expression was not detected by in situ hybridization until 7.5 hours post-fertilization in a fraction of the carrier-treated embryos (Figure 1F). In the control embryos, *xbra* expression always first appeared on the dorsal side of the embryo in the marginal zone (Figure 1F,G), consistent with earlier work (Smith et al., 1991). Expression of *xbra* was not detected in the SU5402-treated embryos at any time-point (Figure 1K–P). This demonstrates that active FGF signaling is necessary for the initial establishment of *xbra* expression and not only for its maintenance through a positive feedback loop.

The effects of FGF signal inhibition on early endodermal and mesodermal gene expression

Consistent with the view that FGF signaling may be most important in patterning the animal part of the marginal zone (Kumano et al., 2001), FGF signaling does not induce endoderm (Cornell et al., 1995). However, it is necessary for proper expression of *Mix1* in both the endoderm and mesoderm (Cornell et al., 1995; LaBonne and Whitman, 1997). To insure that the inhibitor was not arbitrarily causing general transcript degradation or unspecifically preventing transcription, expression of several endodermal transcripts was examined. Unlike *xbra* expression, the endodermal gene transcripts *bix4* and *sox17* are not reduced when FGF signaling is inhibited (Figure 2D,F,T; Table 1). Similarly, embryos injected with the dominant negative version of FGFR1 (XFD) do not show a decrease in *sox17* expression on the injected side (Figure 2Q).

The endodermal transcript *endodermin* (*edd*) is still expressed in SU5402-treated embryos (Figure 2H), though its expression does not extend as far ventrally around the blastopore as in control embryos. This may be due to the reduction in proper cell movements in FGF inhibited embryos and to the apparent 30-minute delay in development that can be seen by observing the blastocoel in inhibited embryos (Figure 1H, O). There may also be a difference in regulation between endodermal transcripts which reflects the intricate network of gene regulation involved in endoderm formation reported in array experiments (Dickinson et al., 2006; Sinner et al., 2006).

When embryos are treated with the inhibitor SU5402 and analyzed at the early gastrula stage, *xbra* expression is absent (Figure 2J,T), but expression of *eomes* is not affected (Figure 2L,T). Likewise, XFD recapitulates these effects (Figure 2R,S), consistent with previous work showing that *eomes* is resistant to XFD's effects (Kumano et al., 2001).

Since both *FGF4* and *FGF8b* are important ligands in mesoderm formation in *Xenopus*, we analyzed the expression of both in embryos treated with SU5402. *FGF4* expression is severely reduced but not absent (Figure 2N), while *FGF8* expression is not reduced, indeed there is a slight increase in *FGF8* expression (Figure 2P). This result suggests that the positive feedback loop between *xbra* and *FGF4* is important in maintaining *FGF4* expression, but there is a fraction of *FGF4* expression that is not dependent upon the feedback loop and could be due to direct or indirect effects mediated by *VegT*. Conversely, *FGF8* expression does not appear to be dependent on a positive feedback loop with *xbra* over this time scale.

FGF signaling is essential for establishment of paraxial mesoderm and is necessary for maintenance of axial mesoderm

To help clarify how FGF signaling affects organizer formation, we analyzed expression of the organizer genes *goosecoid* (*gsc*), *chordin*, *xnr3*, and *noggin* (Cho et al., 1991; Smith and Harland, 1992; Sasai et al., 1995; Smith et al., 1995) as well as *otx2* and *otx5* (Lamb et al., 1993; Vignali et al., 2000) which are expressed in the dorsal mesendoderm prior to expression in the anterior neural plate. Embryos were analyzed at the early gastrula stage.

Gsc and *chordin* are expressed in SU5402-treated embryos though their expression domains are wider than in the control embryos due to the inhibition of convergence and extension movements (Figure 3B,D). Expression of *gsc* is reduced though still present in SU5402-treated embryos (Figure 3B). *Xnr3* expression appears stronger in the SU5402-treated embryos, but this may also be due to delayed development, and inhibition of involution of the mesoderm, where *Xnr3* normally turns off (Figure 3F). The expression of *noggin* is reduced in SU5402-treated embryos (Figure 3H); the effects were varied: the majority of embryos treated demonstrated the strong reduction shown while a few showed no *noggin* expression (not shown). Expression of both *otx2* and *otx5* is relatively unaffected in

SU5402-treated embryos at the gastrula stage (Figure 3J,L). A reduction in *FGF8* levels by antisense morpholino injection resulted in the same effects as inhibition with SU5402 but to a lesser degree; i.e. *xbra* and paraxial mesoderm is strongly reduced, but organizer gene transcripts are expressed (Supplemental Figure 2, Supplemental Table 1).

There has been a discrepancy in the findings on whether *noggin* and *chordin* expression require FGF signaling (Mitchell and Sheets, 2001; Delaune et al., 2005). In this study, we find that *noggin* expression is weaker or absent in a large percentage of treated embryos, while *chordin* expression remains at early gastrulation despite inhibition of FGF signaling. Overall, our results are consistent with a prior study showing that most axial, organizer genes are relatively independent of FGF signaling for initiation of transcription (Delaune et al., 2005). While the axial, organizer genes are largely unaffected by inhibition of FGF signaling, the paraxial mesodermal transcripts *myoD* and *myf5* are absent in inhibitor-treated embryos (Figure 3N,P).

To determine subsequent effects of FGF inhibition, embryos were examined at the neurula stage. A wide range of disruptions to FGF signaling result in failure to form trunk and tail mesoderm (Amaya et al., 1993; Fisher et al., 2002; Delaune et al., 2005; Fletcher et al., 2006), and tadpoles that have strongly inhibited FGF signaling demonstrate a loss of some axial tissue as well based on morphology (Amaya et al., 1993; Delaune et al., 2005). Examination of early neurula embryos allowed us to address whether FGF signaling is necessary to maintain organizer gene transcription throughout gastrulation.

Although several organizer genes are activated in the absence of FGF signaling, their expression is not maintained when FGF signaling is continually inhibited through gastrulation. Thus, *chordin* and *gsc* are almost completely absent relative to controls (Figure 4F,N). *Noggin* expression is no longer detectable by the neurula stage (Figure 4H). Expression of *otx5* and *otx2* remains in inhibitor-treated embryos (Figure 4J,L) though it is restricted to an anterior domain and is extinguished in the mesoderm. As expected, expression of *xbra* and *myoD* remained absent (Figure 4B,D). Expression of the endodermal *edd* is maintained into the neurula stage, again illustrating the specificity of SU5402 treatment, though the expressing cells are not internalized in treated embryos (Figure 4P).

The morphology of early tadpoles that had been treated with an equivalent dose of the inhibitor were examined by in situ hybridization with a cocktail of probes to *otx2*, *krox20*, and *hoxB9*, which are normally expressed in the forebrain and midbrain, hindbrain, and spinal cord, respectively. These embryos show a more severe phenotype than that caused by injection of dominant-negative FGFR1 (XFD) RNA, but the effect is similar (Figure Q–S; Supplementary Figure 3). These tadpoles have some anterior head structures as well as a cement gland, and *otx2* is expressed in the anterior region of these tadpoles (Figure 4Q–V; Supplementary Figure 3). The difference in the degree of head formation in embryos injected with the dominant negative construct RNA or treated with SU5402 could be because RNA injections are more localized and the inhibitor may provide a more thorough blockade of the entire signaling pathway over time. However, there is the possibility that the inhibitor has off-target effects, though the specificity of its effects on mesoderm and neural tissue is consistent with what is known about FGF signaling.

While early and continuous exposure to SU5402 has strong effects on organizer transcripts, inhibition of FGF signaling at stage 10.5 during gastrulation has much milder effects. Overall, organizer transcripts are weaker in expression, but *gsc*, *chordin*, and *noggin* expression persists in early neurula embryos (Figure 4Y–Z', ZC). This contrast in the time-dependent severity of SU5402 effects suggests that FGF signaling is particularly critical before and at the onset of gastrulation for the maintenance of organizer gene transcription.

VegT induction of *xbra* expression depends on FGF signaling

Several endodermal and mesodermal transcripts are direct targets of *VegT*, and many are also maintained or induced by the nodal or FGF targets of *VegT*. To examine the interactions between FGF signaling and *VegT*-mediated induction of mesoderm, we first determined the dose at which *VegT* overexpression would robustly induce *xbra*. *VegT* induction of both *sox17* and *xbra* expression was dose dependent; lower *VegT* amounts induced *xbra*, while higher doses were required to induce *sox17* (Figure 5A–L). At the 100 pg dose *VegT* robustly expanded *xbra* expression, but the most intense *xbra* expression was at the periphery of the injection site (Figure 5L), while endodermal *sox17* expression was centered at the injection site (Figure 5K). The dose-dependent effects observed are consistent with one of the first studies of *VegT*, which documented that it had dose-dependent effects on the type of mesoderm induced in animal cap explants, inducing more dorsal mesoderm at higher doses and more ventral mesoderm at lower doses (Horb and Thomsen, 1997).

To assay whether *VegT*-mediated induction of *xbra* was dependent upon FGF signaling, embryos were injected with 100 pg of *VegT* and treated with either the carrier DMSO or the FGFR inhibitor SU5402. Embryos were treated from early cleavage stages until the early gastrula stage and examined for marker gene expression by in situ hybridization or RT-PCR of whole embryos. *VegT* induction and expansion of the *xbra* expression domain is prevented by inhibition of FGF signaling (Figure 5S), while expansion of *sox17* by *VegT* overexpression is not affected (Figure 5T). The effectiveness of the SU5402 inhibition of FGF signaling was assessed by overexpression of *FGF8b*, which is a strong mesoderm inducer and expands *xbra* expression into ectodermal domains (Figure 5U); in the presence of SU5402, all *xbra* expression is absent (Figure 5W). Notably, *FGF8b* overexpression does not induce *sox17* expression nor does it appear to reduce the endogenous expression of *sox17* (Figure 5V). These results are confirmed by analyzing whole embryo lysates by RT-PCR (Figure 5Y). Not only is *xbra* absent and *sox17* unaffected, but also *FGF4* expression is reduced in embryos injected with *VegT* and treated with the inhibitor relative to control, *VegT*-injected embryos (Figure 5Y). In contrast, *FGF8* expression is much less affected by inhibition of FGF signaling (Figure 5Y), just as it is less affected in embryos treated only with SU5402 (Figure 2O,P). Replication of these experiments using an antisense morpholino targeted to *FGF8* demonstrates similar results highlighting the importance of *FGF8* in mediating some of the mesoderm inducing effects of *VegT* overexpression (Supplementary Figure 4).

Discussion

We have explored the role of FGF signaling in the induction and maintenance of mesodermal gene expression. We confirm that FGF signaling is required to maintain a positive feedback loop with *xbra* expression as previously reported (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), but we also find that it is necessary for the initiation of *xbra* expression (Figure 1). We confirm that FGF signaling is necessary for the establishment of the paraxial mesoderm and that it is not needed for establishment of the axial, organizer mesoderm (Figure 3). In addition, we find that FGF signaling is required to maintain expression of several of the organizer transcripts into neurulation (Figure 4), providing insight into why development of the chordamesoderm fails in the absence of sustained FGF signaling (Amaya et al., 1991).

In contrast to the large effects FGF signaling has on mesoderm development, we did not observe much effect on endodermal gene expression. Our observation that inhibiting FGF signaling does not increase *edd* expression differs from other results in *Xenopus*, where RT-PCR of explants and whole embryos injected with the dominant negative FGFR1 (XFD) or treated with SU5402 showed an increase in *endodermin* expression (Cha et al., 2004); it

could be that different degrees of pathway inhibition have different effects on *endodermin* expression. The relative similarity between expression of endodermal genes (*endodermin*, *sox17*, *bix4*) in control and SU5402-treated embryos in our work highlights a difference between *Xenopus* and zebrafish in the role of FGF signaling. In zebrafish, FGF signaling plays an important role in limiting endodermal development and promoting mesodermal fate (Mizoguchi et al., 2006; Poulain et al., 2006). However, in *Xenopus*, if FGF signaling limits endoderm development, it is to a much lesser degree than in zebrafish, at least with regard to the three endodermal transcripts analyzed.

The function of FGF signaling in *Xenopus* and zebrafish again diverges with regard to establishment of dorsal mesodermal fate. In zebrafish, FGF signaling functions to establish more dorsal cell fate in the embryo because a reduction in *FGF8* enhances the ventralization seen in *chordino* mutants (Furthauer et al., 2004); furthermore, FGF signaling is necessary for proper establishment of dorsal mesoderm in the fish, since β -catenin mediated induction of both *gsc* and *chordin* is absent when FGF signaling is inhibited (Maegawa et al., 2006). In *Xenopus*, however, FGF overexpression does not induce axial, organizer gene expression though it does expand early non-axial mesodermal domains and posteriorize the embryo (Isaacs et al., 1994; Pownall et al., 1996; Christen and Slack, 1997; Lombardo et al., 1998; Pownall et al., 2003; Fletcher et al., 2006). In *Xenopus*, early FGF signaling is necessary to establish the paraxial mesoderm, to limit formation of more ventral mesoderm such as the blood islands, and to posteriorize the embryo (Amaya et al., 1991; Amaya et al., 1993; Kumano and Smith, 2000; Kumano et al., 2001; Kumano and Smith, 2002; Pownall et al., 2003; Fletcher et al., 2006; Isaacs et al., 2007).

VegT is the major maternal transcription factor that activates induction of the endoderm and mesoderm. To address which *VegT* targets depend upon FGF signaling, *VegT*-overexpressing embryos were treated with the FGF inhibitor SU5402 or a translation-blocking MO to *FGF8*. While many *VegT* targets are direct, *VegT* activation of *xbra* transcription is dependent on FGF signaling, and *FGF4* expression is strongly reduced when FGF signaling is inhibited (Figure 5). Antisense morpholino knockdown of *FGF8* leads to an early reduction in *xbra* expression and a loss of paraxial mesoderm (Fletcher et al., 2006), but inhibition of FGF signal transduction does not reduce *FGF8* mRNA expression (Figure 2P). Further, *VegT*-mediated expansion of *FGF4* expression is dependent upon *FGF8* because when *FGF8* is reduced, *FGF4* expression is strongly diminished. When taken with the evidence that *VegT* is required for *FGF8* expression (Xanthos et al., 2001), these results show that *FGF8* is a more proximal target than *FGF4* and serves to reinforce mesoderm development by activating *FGF4*.

There have been differing reports on the role of FGF signaling in the expression of organizer signaling molecules. The requirement for FGF in maintaining expression of organizer transcripts may account for some of these differences. For instance, Delaune et al. (2005) reported that *noggin* expression was absent at gastrulation in SU5402 treated embryos; we find that it initiates, but is very weak and diminishes to absence as gastrulation proceeds. On the other hand, our results differ from the findings of Mitchell and Sheets (2001) that expression of *chordin* at the onset of gastrulation is dependent upon FGF signaling. Thus our results lead us to conclude that the establishment of the organizer, or at least a field containing expression of genes with known organizer activities, occurs to a major extent in the absence of any FGF signaling. However, expression of these axial mesodermal transcripts diminishes as gastrulation proceeds.

FGF signaling can induce expression of *xbra* and *myoD*, but Activin and Nodals are capable of inducing mesoderm of different dorsal-ventral character depending on the dose in *Xenopus* (Green and Smith, 1990; Agius et al., 2000; Takahashi et al., 2000). It has been

shown clearly that the ability of Activin to induce *xbra* and *myoD*, but not *gsc*, is dependent on active FGF signaling in explants (Cornell and Kimelman, 1994). This difference in the specification of axial and paraxial mesoderm is also apparent in the embryo. One early view defined FGF signaling as a competence factor for TGF β signaling mediated induction of mesoderm. As more molecular interactions have been revealed over the last decade, we think the evidence has illuminated a more complex interaction: FGF and TGF β signaling converge to activate and repress a range of genes to specify different types of mesoderm. While *VegT* activity and active TGF β signaling is necessary for formation of all mesoderm, both ventral and dorsal, FGF signaling is necessary to induce expression of *xbra* and *myoD*, and in its absence paraxial mesoderm is not specified. On the other hand, FGF signaling is not necessary for the initial induction of most axial, organizer mesodermal gene expression.

This study therefore highlights the role of FGF signaling in the maintenance of organizer gene expression through gastrulation in addition to its established functions in paraxial mesodermal development. Prevention of FGF signaling during gastrulation has immediate effects on patterning of the embryo, resulting in posterior truncations and reductions in paraxial mesoderm and the spinal cord domain, where FGF signaling is essential for the onset of *myoD*, *myf5*, and *hoxB9* expression (Pownall et al., 1996; Slack et al., 1996; Standley et al., 2001; Fisher et al., 2002; Fletcher et al., 2006; Isaacs et al., 2007).

In addition to the need for continued FGF signaling during gastrulation, there is also an early sensitive period for signaling beginning in the late blastula stage with respect to axial, organizer gene expression, since early and continuous exposure to a high dose of SU5402 leads to the loss of organizer transcripts by the end of gastrulation that is much more pronounced than the reduction of organizer transcripts that occurs if inhibition begins during gastrulation. These results parallel the findings of Delaune et al., (2005) who found that neural induction also fails after early exposure to SU5402. This finding is consistent with previous work that shows a continuing need for blockage of BMP signals during gastrulation to induce and maintain neural tissues (Hartley et al., 2001; Khokha et al., 2005). In addition to effects mediated through the organizer, the late tadpole phenotype is at least partly due to the involvement of FGF signaling in maintaining cell viability; embryos that have been treated with a sustained dose of SU5402 are smaller at the tadpole stages, and reduction of FGF signaling results in TUNEL-positive cell death during neurula and tailbud stages (not shown).

In the direct activity of FGF signaling in specification of paraxial mesoderm identity, the FGF signals must also cooperate with BMP antagonists, which are required to dorsalize mesoderm in order to specify muscle (Eimon and Harland, 1999; Khokha et al., 2005). Therefore, FGF signaling has multiple roles in gastrulation with regard to dorsal mesodermal development: FGF signals specify paraxial mesodermal identity, with a clear requirement for positive feedback, in part through *FGF4*, in maintaining FGF signaling and function; and, secondly, early FGF signaling is necessary to maintain axial, organizer gene expression, which is consistent with the lack of neural tissue in tadpoles observed after a short, early incubation with SU5402 (Delaune et al., 2005). At this level of FGF signaling inhibition the early expression of organizer transcripts is sufficient to specify anterior fates, in contrast to more severe suppression of organizer transcript function, which completely eliminates anterior development (Khokha et al., 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. FGF signaling is necessary for initiation of *xbra* expression

All embryos were processed by RNA in situ hybridization for expression of *xbra*. Embryos were treated with SU5402 (90–100 μ M) (A–D, K–P) or DMSO alone (E–J). (A–D) The bar represents time from fertilization until eleven hours post-fertilization with each hour represented by hash marks; the black region represents the time the embryos were in carrier (DMSO) only, and the blue region represents the time frame when embryos were treated with the inhibitor SU5402. (A–D) lateral views. (A) DMSO only; (B,C) initially treated with DMSO, then SU5402 was added at 10 and 9 hours post-fertilization, respectively. (D) Embryos were first treated with SU5402 then the inhibitor was washed out after 4 hours. (E–P) Embryos were treated with either DMSO alone or SU5402 from the 8–16-cell stage until the indicated time point post-fertilization and *xbra* expression was analyzed in these blastula stage embryos. Embryos were cleared in BB:BA (2:1). The two views in each panel are of the same embryo with an animal pole view above and a lateral view below.

Figure 2. FGF signaling effects on endoderm and mesoderm

These embryos have been processed by in situ hybridization for expression of the indicated transcript above each column. Embryos were treated with DMSO or SU5402 from the 8- to 16-cell stage as indicated on the side. (A,B) are lateral views; embryos were collected at the blastula stage, 7.5 hours post-fertilization; (C–S) gastrula stage; (C–N) are blastoporal views. (O,P) are lateral views with vegetal to the bottom. (Q–S) Embryos were injected into one cell at the two-cell stage with the dominant negative FGFR1 construct XFD (500 pg); the red staining is the lineage tracer $\eta\beta$ gal. (T) Whole embryos were treated with either DMSO or SU5402, collected at the time indicated above the lanes, and analyzed by RT-PCR for expression of the indicated markers along the side. *ODC* was used as a loading control.

Figure 3. The involvement of FGF signaling in organizer and paraxial mesoderm development
 Embryos were treated with either DMSO or SU5402 from the 8- to 16-cell stage and analyzed at the gastrula stage for expression of the indicated transcripts. (A,B,E–H,M–P) embryos are displayed in lateral blastoporal view; (C,D,I–L,Q,R) embryos are displayed from lateral view with blastopore toward the bottom. Axial/organizer genes are present, but expression of paraxial mesodermal transcripts is absent in inhibitor treated embryos.

Figure 4. Early FGF signaling is necessary to maintain organizer gene expression

Embryos were treated with either DMSO or SU5402 and analyzed at the early neurula stage for expression of a range of mesodermal, posterior and anterior, and endodermal genes, except for (Q–V) which were analyzed at stage 28. (A–H, W–ZD) are dorsal views with anterior to the left; (I–M) are anterior views; (O) is a posterior view; (J, L, N, P) are dorsal blastoporal views. (Q–U) are lateral views, anterior to the left; (V) is a dorsal view. (A–P) embryos were treated from the 8- to 16-cell stage until the early neurula stage; (R) was injected with XFD RNA at 250 pg into each blastomere at the 4-cell stage. (S, U, V) were treated from the 8- to 16-cell stage until stage 28; (W–ZD) embryos were treated from mid-

gastrulation until the early neurula stage. (Y',Z') embryos demonstrating the range of effects from inhibitor treatment.

Figure 5. *VegT* activity in the absence of FGF signaling

VegT has dose-specific effects in the whole embryo. (C–L) Embryos were injected with different doses of *VegT* mRNA into one cell at the two-cell stage marked by the lineage tracer *nuclear β-gal* in red and analyzed at the mid-gastrula stage. All embryos are displayed in lateral views with the blastopore toward the bottom. At a very low dose, *VegT* mRNA has no detectable effect (C,D). At the 10 pg dose, *VegT* induces ectopic *xbra* expression but not *sox17* while higher doses induce ectopic *sox17* at the focal point of injection and *xbra* at the periphery (E–L). (M–X) FGF signaling is necessary for *VegT*-mediated expansion of *xbra* but not *sox17*. Embryos were either uninjected, or injected with *VegT* mRNA (250pg) or *FGF8b* mRNA (10 pg) and treated with either DMSO or SU5402 as indicated (M–X). At the early gastrula stage embryos were analyzed for *xbra* and *sox17* expression. (Y) is an RTPCR experiment on whole embryos treated as indicated along the top and analyzed for a range of markers. *EF1α* and *ODC* were used as loading controls.

Table 1

Summary of the effects of SU5402 treatment on endodermal and mesodermal marker gene expression.

<i>Markers/Gastrula stage</i>	<i>SU5402 (100 µM)</i>	<i>VegT (100–250 pg)</i>	<i>VegT/SU5402</i>
<i>VegT</i>	8/8 (WT)		
<i>bix4</i>	28/28 (WT)		
<i>edd</i>	7/7 ^{**} (–)		
<i>eomes</i>	19/19 (WT)		
<i>sox17</i>	32/32 (WT)	38/38 (+++)	12/12 (+++)
<i>xbra</i>	107/107 (---)	35/38 (+++)	29/29 (---)
<i>FGF4</i>	23/23 (---)	16/20 (++)	
<i>FGF8</i>	8/8 (WT)	28/28 (++)	
<i>gsc</i>	26/26 ^{**} (–)		
<i>chordin</i>	24/24 (WT)		
<i>noggin</i>	44/44 ^{**} (–)		
<i>xnr3</i>	19/19 ^{**} (WT/+)		
<i>otx2</i>	19/20 (WT)		
<i>otx5</i>	6/7 (WT)		
<i>myoD</i>	11/11 (---)		
<i>myf5</i>	11/11 (---)		
<i>sox2</i>	28/28 (---)		
Markers at early neurula	SU5402 (100 µM) 16-cell to EN	SU5402 (100 µM) gastrula to EN	
<i>xbra</i>	36/36 (---)	12/12 (---)	
<i>myoD</i>	31/31 (---)	12/12 (---)	
<i>chordin</i>	21/21 (---)	7/15 (–) 8/15 (–)	
<i>noggin</i>	21/21 (---)	3/15 (---) 5/15 (–) 7/15 (–)	
<i>otx5</i>	19/19 ^{**} (–)	16/16 ^{**} (–)	
<i>otx2</i>	19/20 ^{**} (–)	14/14 ^{**} (–)	
<i>gsc</i>	22/22 (---)	6/15 (–) 9/15 (–)	
<i>edd</i>	19/19 ^{**} (–)	13/13 ^{**} (–)	

(WT), wild-type in appearance; (---), strong reduction to absence of marker expression; (–), reduction of marker expression; (–), marker is noticeably still present but slightly weaker in expression or smaller in domain than WT; (+), is slightly higher than WT in intensity and/or area; (++) moderate increase in expression; (+++), very strong increase in expression;

^{**} refer to the text for a more precise description.