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FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in *Xenopus*

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The relative contributions of different FGF ligands and spliceforms to mesodermal and neural patterning in *Xenopus* have not been determined, and alternative splicing, though common, is a relatively unexplored area in development. We present evidence that *FGF8* performs a dual role in *X. laevis* and *X. tropicalis* early development. There are two *FGF8* spliceforms, *FGF8a* and *FGF8b*, which have very different activities. *FGF8b* is a potent mesoderm inducer, while *FGF8a* has little effect on the development of mesoderm. When mammalian *FGF8* spliceforms are analyzed in *X. laevis*, the contrast in activity is conserved. Using a loss-of-function approach, we demonstrate that *FGF8* is necessary for proper gastrulation and formation of mesoderm and that *FGF8b* is the predominant *FGF8* spliceform involved in early mesoderm development in *Xenopus*. Furthermore, *FGF8* signaling is necessary for proper posterior neural formation; loss of either *FGF8a* or a reduction in both *FGF8a* and *FGF8b* causes a reduction in the hindbrain and spinal cord domains.

KEY WORDS: FGF, FGF8, FGF8a, FGF8b, FGF8f, Mesoderm, Neural, Patterning, Spliceforms, FGF8 isoforms, Alternative spliceforms, *Xenopus*, Hindbrain, Spinal cord

INTRODUCTION

FGF8 is alternatively spliced and has been implicated in many developmental processes; the alternative splicing of *FGF8a* and *FGF8b* is highly conserved in *Xenopus*, chick, mouse and human (Crossley and Martin, 1995; Ghosh et al., 1996; Haworth et al., 2005). As many as 74% of multi-exon human genes are predicted to be alternatively spliced (Johnson et al., 2003), and alternative splicing is found across the eukaryotic phyla (Brett et al., 2002) where it has been argued to be a source of functional complexity of the genome (Stamm et al., 2005). In this study, we have investigated the roles of *FGF8a* and *FGF8b* spliceforms in mesodermal and neural development in *X. laevis* and *X. tropicalis*.

In *Xenopus laevis*, FGF was the first identified mesoderm inducer (Kimelman and Kirschner, 1987; Slack et al., 1987; Slack et al., 1990), and disruption of FGF signaling results in the loss of most trunk and tail mesoderm (Amaya et al., 1991; Amaya et al., 1993). Although the initial view held that FGF was a mesoderm inducer, subsequent experiments have suggested it was more important in the maintenance of mesoderm through a feedback loop involving *brachyury* (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Kroll and Amaya, 1996).

FGF8 is expressed in the presumptive mesoderm by gastrulation, but only had minimal effects on mesoderm formation (Christen and Slack, 1997; Hardcastle et al., 2000). However, only the *FGF8a* spliceform was tested. In addition to its role in mesoderm formation, FGF signaling has an established role in neural patterning. Several FGFs, including *FGF8*, are expressed in the early posterior dorsal mesoderm, where they are in proximity to the presumptive neuroectoderm (Christen and Slack, 1997).

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Several studies have disrupted FGF signaling in the whole embryo to investigate its normal role. Embryos injected with the dominant-negative FGFR1 (XFD) have perturbed posterior mesoderm and neural development (Amaya et al., 1991; Amaya et al., 1993; Pownall et al., 1996; Pownall et al., 1998). In embryos transgenic for XFD and expressing it before gastrulation, early expression of posterior patterning Hox genes are inhibited, and embryos develop with posterior truncations (Pownall et al., 1998). Several experiments in explants and tissue recombinants have reported that induction of anterior neural tissue is not perturbed by inhibiting FGF signaling but that posterior neural tissue is dependent upon FGF signaling (McGrew et al., 1997; Xu et al., 1997; Barnett et al., 1998; Holowacz and Sokol, 1999; Ribisi et al., 2000). When embryos receive a transplant of presumptive neural ectoderm expressing either XFD or a dominant-negative form of the Ras GTPase, posterior neural tissue did not form, but anterior neural tissue did form, confirming that FGF signaling, specifically FGF signaling through Ras, is necessary for posterior neural tissue formation (Xu et al., 1997; Ribisi et al., 2000).

Although many FGF ligands have similar effects in *Xenopus* explants, it is not clear why they have quantitatively or qualitatively different effects in normal development. Cell culture experiments suggest that different FGF ligands activate specific FGF receptors and promote proliferation (Ornitz et al., 1996), and in oligodendrocyte cultures, different FGF-FGFR combinations have specific effects on cell proliferation and differentiation (Fortin et al., 2005). In vivo experiments have resolved differences in ligand activity as well; for example, large differences are apparent in the developing limb bud where *FGF8* secreted from the apical ectodermal ridge and *FGF10* secreted from the mesenchyme of the limb bud have distinct activities (Martin, 1998).

In addition to differences in activity between FGF ligands, there is evidence for differences between individual spliceforms of the *FGF8* gene. The mammalian *FGF8* is alternatively spliced to yield as many as seven different protein forms in the mouse and four in human (Crossley and Martin, 1995; MacArthur et al., 1995b; Gemel et al., 1996). These variants have shown different activities in cell culture experiments: for example, human and mouse *FGF8B*/*Fgf8b*, but not *FGF8A*/*Fgf8a*, robustly transform NIH3T3 cells (MacArthur

et al., 1995a; Ghosh et al., 1996), and *FGF8B*/*Fgf8b* binds multiple FGFR 'c' spliceforms expressed in BaF3 cells and induces mitosis. However, *FGF8A*/*Fgf8a* has no detectable activity (MacArthur et al., 1995b; Blunt et al., 1997). At the midbrain-hindbrain boundary, *FGF8A*/*Fgf8a* promotes midbrain fates, but *FGF8B*/*Fgf8b* transforms midbrain to cerebellar fate (Liu et al., 1999; Sato et al., 2001; Liu et al., 2003).

In this study, we characterize the activity of *FGF8a* and *FGF8b* in *X. laevis* and *X. tropicalis* early development. *FGF8b*, unlike *FGF8a*, is a robust inducer of mesodermal cell fate in both explants and whole embryos. Recently, Myers et al. (Myers et al., 2004) used a mouse *Fgf8* to induce mesoderm in *X. laevis* explants, and we show here that this mesoderm induction is due to its splicing; thus, *X. laevis FGF8b*, human *FGF8B* and mouse *Fgf8f* all have very similar activities when analyzed in *X. laevis*. *FGF8* has at a minimum a dual role in early *Xenopus* development. Strong knockdown of *FGF8* results in reduction of *xbra* and *myoD* expression, disruption of gastrulation, and a subsequent reduction in the paraxial mesoderm. The *FGF8b* spliceform is specifically needed for mesoderm development. *FGF8* is also involved in early neural development, and we expand the previous findings that *FGF8a* can posteriorize the neural plate, demonstrating that it functions to restrict the caudal boundary of anterior neural gene expression, and to expand hindbrain and spinal cord gene expression domains. Using a loss-of-function approach, we demonstrate that *FGF8* is essential for proper establishment of posterior neural fate: reduction of both *FGF8a* and *FGF8b*, as well as loss of *FGF8a* alone, causes a reduction in MHB, hindbrain and spinal cord domains in *Xenopus*.

MATERIALS AND METHODS

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 (Nieuwkoop and Faber, 1994). *X. tropicalis* embryos were collected and cultured by standard procedures (Khokha et al., 2002).

Ectodermal explants (animal caps)

Ectodermal explants (animal caps) (300-400 μ^2) were excised from stage 9 embryos with either an eye-brow knife or with the Gastromaster (XENOTEK Engineering). Animal caps were then cultured in 75% NAM until the indicated stage and either collected for RT-PCR analysis or fixed.

Whole-mount RNA in situ hybridization

RNA in situ hybridization used multibasket containers (Sive et al., 2000). Nuclear localized β-galactosidase (nβgal-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 1 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 they were rinsed in PBS + 0.1% Tween 20, 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.

Antisense RNA probes were made for the following transcripts: *xbra* (Smith et al., 1991); *myoD* (Hopwood et al., 1989); *ntub* (Good et al., 1989); *sox2* (Grammer et al., 2000); *nrp1* (Knecht et al., 1995); *otx2* (Lamb et al., 1993); *en2* (Hemmati-Brivanlou et al., 1991); *krox20* (Bolce et al., 1992); *hoxB9* (Sharpe et al., 1987); *dbx* (Gershon et al., 2000); *ntubulin* (Good et al., 1989); *collagen type II* (Amaya et al., 1993); *rx1* (Casarosa et al., 1997); *sox2* (GenBank AL680500); and *ephA4* (Smith et al., 1997). *X tropicalis* probes *en2*, *myoD*, *krox20*, *hoxB9*, *otx2*, *n-tubulin* have been described previously (Khokha et al., 2002).

DNA constructs and cloning

X. laevis FGF8a (Christen and Slack, 1997) that had been subcloned into pCS107 (Monsoro-Burq et al., 2003) was used in this study. *X. laevis FGF8b* was found in GenBank (Accession Number BG892841; IMAGE: 4084172). The coding sequence was amplified using Pfu polymerase and PCR with the following primers: U, 5′-GGATCCATGAACTACATCACCTCCATCC-3′; D, 5′-GAATTCTTACCGAGAACTTGAATATCGAGT-3′. The version with 5′ and 3′ UTRs was not as potent as the coding sequence alone. The coding sequence was cloned into the pCS108 expression vector. Mouse *Fgf8a* (Crossley and Martin, 1995) and human *FGF8B* (Ghosh et al., 1996) were subcloned into pCS108. *X. tropicalis FGF8a* and *FGF8b* spliceforms were found by BLASTing EST databases, GenBank CX742774 (*Xt FGF8a*), GenBank BC082344 (*Xt FGF8b*).

mRNA synthesis and injection

Synthetic capped messenger RNA was made using the SP6 mMessage mMachine kit (Ambion). Quantified mRNA was resuspended in RNAse-free H_2O and stored at –80° C. The following constructs were linearized with *Asc*I and used as templates for SP6 mediated in vitro mRNA synthesis: *X. laevis FGF8a* (XLFGF8a-CS7) (Monsoro-Burq et al., 2003); *X. laevis FGF8b* with 5′ and 3′ UTR (XLFGF8bfl-CS8); *X. laevis FGF8b* with only the coding sequence (XLFGF8b-CS8); mouse *Fgf8a* (Crossley and Martin, 1995) subcloned into pCS108 (mF8a-CS8); mouse *Fgf8f* (mF8f-CS7) (Myers et al., 2004); human *FGF8B* (Ghosh et al., 1996) subcloned into CS108 (HsFGF8b-CS8); *X. laevis FGF4* (Isaacs et al., 1992) subcloned into pCS107 (FGF4-CS107); *X. laevis noggin* (CS2+xnoggin) (Mariani and Harland, 1998); and *nuclear beta-galactosidase* (nβgal-CS2+) (Turner and Weintraub, 1994). Embryos were injected into one cell at the two-, four- or eight-cell stage, as indicated, in 5 or 10 nl volumes.

RT-PCR

Trizol reagent was used to isolate RNA from embryos and explants for reverse-transcriptase polymerase chain reaction (RT-PCR) (Wilson and Melton, 1994). One embryo equivalent or 15 ectodermal explants were used for each RT-PCR experiment. 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. *EF1α* or *ornithine decarboxylase* (*ODC*) were used as loading controls. RT-PCR primers for the following have been described: *EF1α* (Krieg et al., 1989); *xbra* (Isaacs et al., 1994); *muscle actin* (*MA*) (Wilson and Melton, 1994); *sox2* (Liu and Harland, 2003); *NCAM*, *en2*, *krox20* and *hoxB9* (Hemmati-Brivanlou and Melton, 1994); *otx2* (Lamb and Harland, 1995); *hoxD1* and x*cad3* (Kolm et al., 1997); *slug* (Mizuseki et al., 1998); *ODC* (Hudson et al., 1997). The primers used for detection of *Xenopus FGF8a* and *FGF8b* are: U, 5′-ATCACCTCCATCCTGGGCTATC-3′; D, 5′-TGCGAACTCT-GCTTCCAAACG-3′; *FGF8a*, 253 bp; *FGF8b*, 286 bp.

Morpholino oligonucleotide (MO) design and injection

A morpholino oligonucleotide (MO) (Gene Tools) was designed to bind the translation initiation region of the *FGF8* mRNA; the sequence of the *X. laevis FGF8* translation blocking morpholino oligonucleotide (XlMOF8) is 5′-GGAGGTGATGTAGTTCATGTTGCTC-3′. A four-mismatch oligonucleotide 5′ GGAGGTGATGTAGCTCATCCTGCCC 3′ had a fivefold lower specific activity in *X. laevis*. The splice-blocking MOs are as follows: MOSAF8a, 5′-CTCTGCTCCCTCACATGCTGTGTAA-3′; MOSDF8, 5′-AGACGGATGTTCGGGTCCATTTAAC-3′. The Gene Tools standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) conjugated to fluorescein was used as a lineage tracer. The morpholino oligonucleotides were resuspended in RNAse-free 1/20×MR. The injection volume was either 5 nl or 10 nl.

RESULTS

FGF8 expression in *Xenopus*

Surprised by the difference in a mouse *FGF*8 (Myers et al., 2004) and the reported *Xenopus FGF8*a activity, we sought to determine whether different spliceforms might have different activity. An *FGF8b* spliceform (Accession Number BG892841; IMAGE: 4084172) differs from *FGF8a* by 11 amino acids due to the use of

an alternative 3′ splice site (Fig. 1A,B). These two spliceforms are conserved in mice and humans (Crossley and Martin, 1995). These eleven amino acids reside at the N terminus of the protein after cleavage of the signal peptide and contain a potential N-linked glycosylation site (Fig. 1A); this region is highly conserved in all vertebrate *FGF8b* proteins (Olsen et al., 2006).

X. laevis and *X. tropicalis FGF8* mRNAs are not found maternally, but are detectable by RT-PCR at stage 9.5 just before gastrulation (Fig. 1C,D). *FGF8b* is expressed at higher levels than *FGF8a*, and expression of both is maintained throughout early development. In situ hybridization to *FGF8* in *X. tropicalis* confirms that expression begins circumferentially around the blastopore and becomes restricted dorsally as gastrulation proceeds (Fig. 1E,F). By late gastrula, it is expressed in the posterior dorsal mesoderm, and as neurulation proceeds it is expressed in the future midbrain-hindbrain boundary, and then in the anterior neural ridge and future pharyngeal arches and placode regions (Fig. 1G-J). This pattern is consistent with the *X. laevis* expression patterns (Christen and Slack, 1997). Because *FGF8* is expressed in the presumptive mesoderm by gastrulation and in the posterior dorsal mesoderm during early neural patterning, it is a good candidate for affecting mesoderm and neural development.

FGF8b is a robust mesoderm inducer in explants

To analyze the inductive capability of *FGF8a* and *FGF8b*, we injected embryos with *Xenopus*, mouse or human *FGF8a*, *FGF8b* or *FGF8f* mRNAs, and analyzed ectodermal explants (Fig. 2A). In confirmation of Christen and Slack (Christen and Slack, 1997), *Xenopus FGF8a* did not induce mesodermal tissue to any appreciable level, but *FGF8b* induced mesoderm as assayed by *xbra* expression (Fig. 2B, lanes 6,7). The difference in activity between

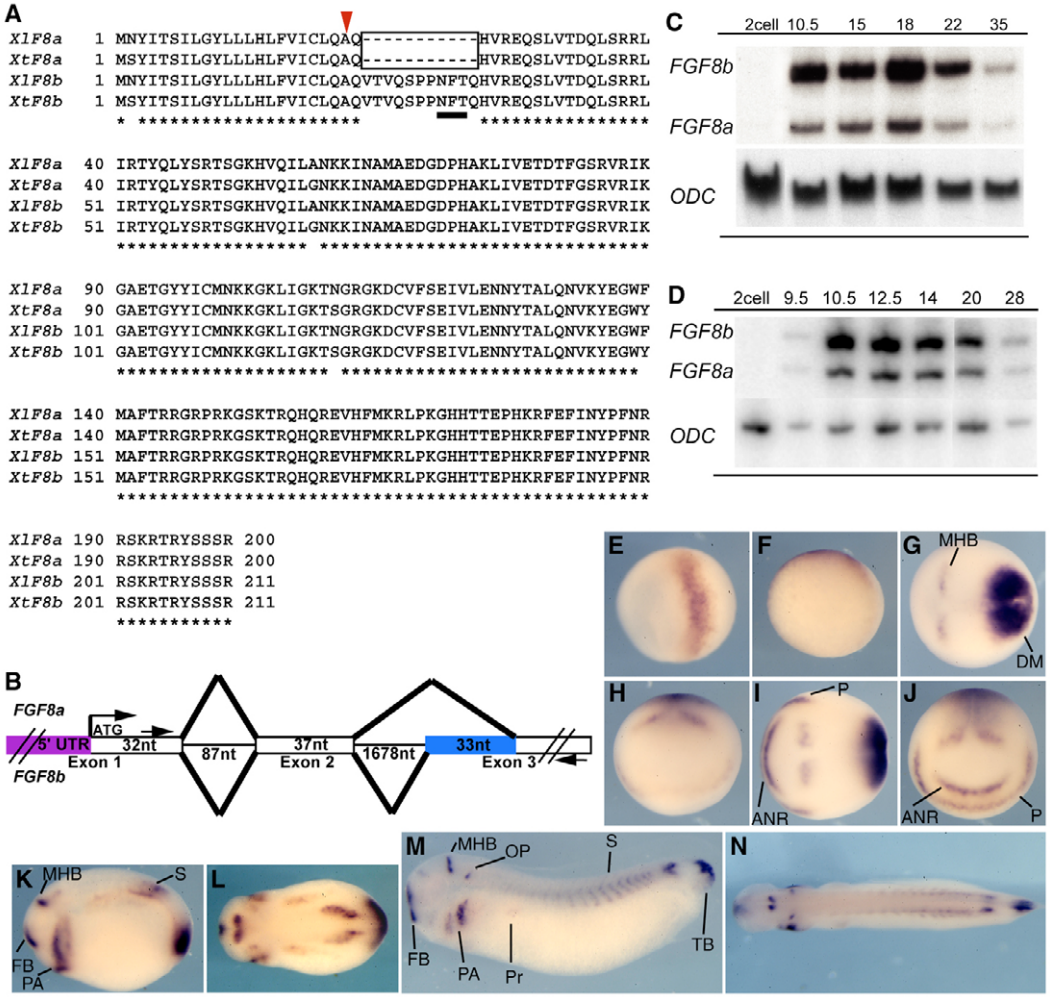

Fig. 1. *FGF8* expression. (**A**) Alignment of *Xenopus* FGF8a and FGF8b amino acid sequences (ClustalW). The signal sequence cleavage position is predicted to be after the 22nd amino acid residue (alanine) (arrowhead), determined by using the SignalP 3.0 program (Bendtsen et al., 2004). Underlining indicates the N-linked glycosylation site (NFT) (reviewed by Dempski and Imperiali, 2002). (**B**) *Xenopus FGF8a* and *FGF8b* result from alternative splicing of the third exon; *FGF8a* uses an alternative splice acceptor (3′) site. The ATG indicates the translational start; black arrows indicate primers used for PCR amplification in the RTPCR panel in C,D. (**C**) *X. laevis* and (**D**) *X. tropicalis* RTPCR analysis of whole embryos using primers to amplify both FGF8a and FGF8b simultaneously. The *FGF8a* product is 253 bp; *FGF8b* is 286 bp. (**E**-**N**) In situ hybridization profile for *FGF8* in *X. tropicalis* at the indicated stages. (E,G,I,L,N) Dorsal views with anterior towards the left; (F) a blastorporal view; (H,J) frontal views, dorsal upwards; (K,M) lateral views, anterior towards the left. FGF8 is expressed circumferentially around the blastopore (E,F) but is restricted to the dorsal posterior mesoderm as gastrulation proceeds; expression in the posterior mesoderm strengthens during neurulation and MHB expression begins (G,H). DM, dorsal mesoderm; MHB, midbrain-hindbrain boundary; ANR, anterior neural ridge; P, placode region; FB, forebrain; PA, pharyngeal arches; S, somite; OV, otic vesicle; Pr, pronephric anlage; TB, tail bud.

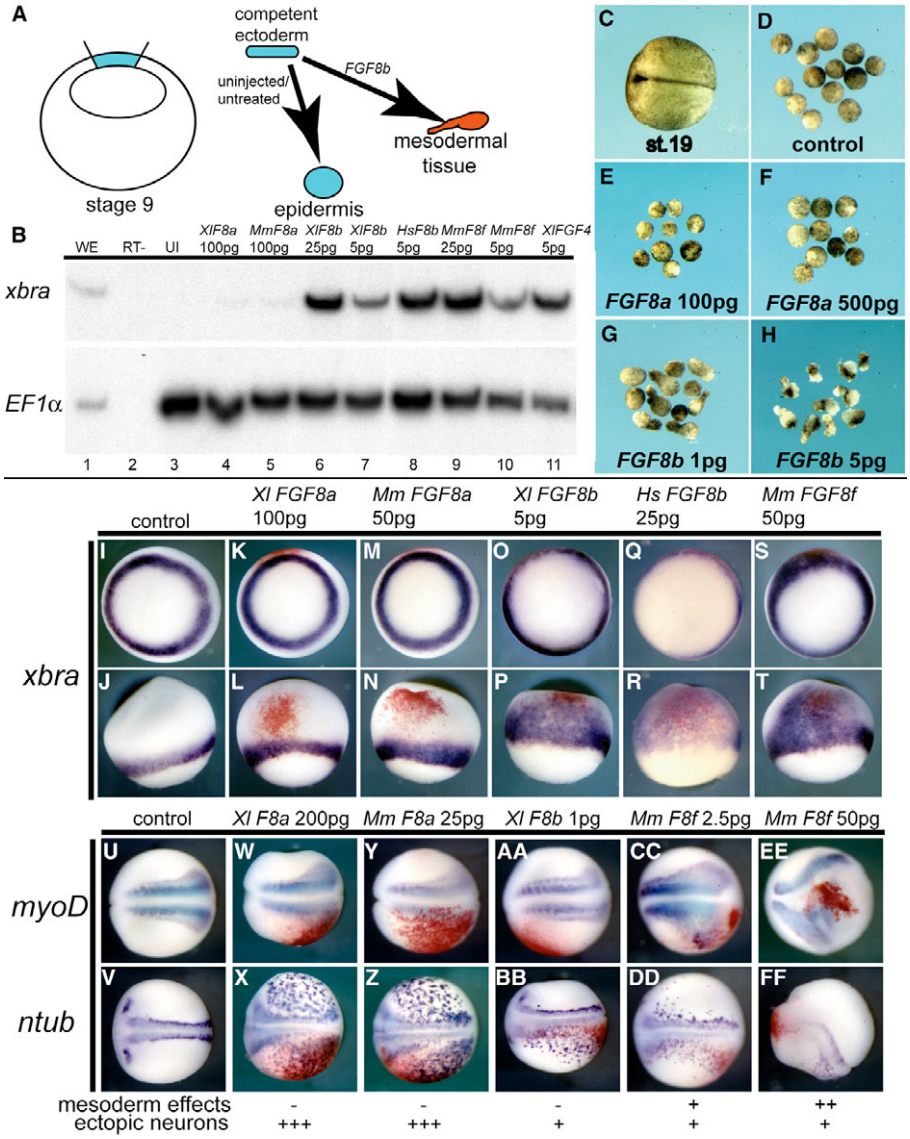

Fig. 2. *FGF8b* is a robust mesoderm inducer. (**A**) Diagram of explant assay. (**B**) RT-PCR analysis of explants injected as indicated at the one-cell stage, excised at stage 9 and cultured to stage 11; *EF1*α was used as a loading control. Lane 1, whole embryo (WE) positive control; lane 2 negative control minus reverse transcriptase (RT–); lane 3, uninjected explant lane. *FGF8b* and *FGF8f* robustly induce *xbra* expression (lanes 6-10). (**C-H**) Animal caps injected as indicated and cultured to stage 19. (I-T) Overexpression of *Xenopus FGF8b* expands *xbra* in whole embryos. Embryos were injected with mRNA, as indicated, into the marginal zone of one cell at the two-cell stage and cultured until stage 10.5. Embryos in the top row are shown from blastoporal views; the bottom row of images show the same embryos as the respective one above but from a lateral view with the blastopore down. β-*galactosidase* mRNA was injected as a lineage tracer and detected using Red-Gal substrate. (**I**,**J**) Control uninjected embryos. Neither (**K**,**L**) *XlFGF8a* (15/15 embryos) or (**M**,**N**) *MmFGF8a* (14/15 embryos) affects *xbra* expression; (**O**,**P**) *XlFGF8b* (15/15 embryos), (**Q**,**R**) *HsFGF8b* (20/20) and (**S**,**T**) *MmFGF8f* (18/18 embryos) robustly expand the *xbra* expression domain in a non-cell-autonomous manner. (**U-FF**) *FGF8a* and *FGF8b* have separable activities. Embryos were injected as indicated into one cell at the two-cell stage and processed by in situ hybridization for expression of *myoD* (top row) or *neuronal β-tubulin* (*ntub*) (bottom row) at stage 20. All are dorsal views with anterior towards the left. Effects on mesoderm and production of ectopic neurons is scored below the images; – indicates no effect. Overexpression of *XlFGF8a* and *MmFGF8a* results in massive ectopic *ntub* expression without affecting mesodermal development (W-Z). A minimum of eight embryos were examined and they showed consistent phenotypes for each injection.

FGF8a and *FGF8b* in explants is conserved. Xenopus and mouse *FGF8a* have minimal mesoderm inducing activity, whereas the longer 'b' and 'f' forms of *Xenopus,* human and mouse induce mesoderm robustly (Fig. 2B, lanes 4-10).

Ectodermal explants from uninjected embryos differentiate into epidermal derivatives and take on a spherical form (Fig. 2A,D). Explants that were injected with as little as 1pg of *X. laevis FGF8b* mRNA and cultured until the late neurula stage elongated, consistent with mesoderm formation (Fig. 2G,H). By contrast, *FGF8a* injected explants did not elongate; at the 100 pg dose, they formed only slightly oval-shaped explants, and at the higher dose of 500 pg, the explants were even more round (Fig. 2E,F).

FGF8a and FGF8b have separable activities: FGF8b expands mesoderm in the embryo

To explore the different activities of *FGF8a* and *FGF8b* in the embryo, we tested whether they would expand mesodermal tissue at the gastrula stage. Control embryos express *xbra* in a ring around the blastopore at stage 11 (Fig. 2I,J). *Xenopus FGF8a* overexpression did not increase the *xbra* expression domain (Fig. 2K,L), in agreement with Hardcastle et al. (Hardcastle et al., 2000). Injection of *Xenopus FGF8b* mRNA expanded the mesodermal territory in a non-cell-autonomous manner as *xbra* expression is expanded beyond the lineage-traced tissue (Fig. 2O,P).

This difference in activity between the two forms of *FGF8* on the whole embryo is conserved. Overexpression of mouse *FGF8a* had the same phenotype as *Xenopus FGF8a* and did not expand *xbra* expression (Fig. 2M,N). Both human *FGF8B* and mouse *Fgf8f* phenocopied the *Xenopus FGF8b* expansion of *xbra* (Fig. 2Q-T).

Hardcastle et al. (Hardcastle et al., 2000) found that overexpression of *Xenopus FGF8a* can induce ectopic neurons detectable by in situ hybridization to *neuronal β-tubulin* (*ntub*). To address further whether *FGF8a* and *FGF8b* have separable activities, embryos were injected with a range of doses and analyzed for production of ectopic neurons and effects on mesoderm. *Xenopus FGF8a* overexpression does not expand mesoderm (Fig. 2W), but it does cause massive ectopic *ntub* expression in a punctate, non-cell-autonomous manner over the entire epidermis (Fig. 2X). *FGF8b* (Fig. 2O,P) induces mesoderm strongly and disrupts gastrulation at 5 pg doses and higher. At a very low dose, *FGF8b* does not appear to affect mesoderm, but it does have a very weak ability to increase *ntub* expression (Fig. 2AA,BB), suggesting that it also possesses at

least a low level of the *FGF8a* activity. This contrasts with a recent report that *FGF8b* robustly induces ectopic *ntub* (Shim et al., 2005). At an even lower dose of *FGF8b* (0.1pg), no phenotype is observed (data not shown) and no dose mimicked *FGF8a*. Importantly, even at high doses, *FGF8a* does not expand mesoderm (Fig. 2K,L,W).

Mouse *Fgf8a* and *Fgf8f* parallel the activities of *Xenopus FGF8a* and *FGF8b*. Mouse *FGF8a* causes ectopic *ntub* expression in a non-cell-autonomous manner (Fig. 2Y,Z). Mouse *FGF8f* induces mesoderm (Fig. 2S,T) and perturbs gastrulation and *myoD* expression (Fig. 2EE), while reducing *ntub* expression (Fig. 2FF). At a low dose, mouse *FGF8f* mispatterns *ntub* with a few ectopic neurons present, and it slightly expands *myoD* expression (Fig. 2CC,DD). *Xenopus FGF4* has the same effect as both *Xenopus FGF8b* and mouse *Fgf8f* in that it affects mesoderm and does not result in the massive ectopic *ntub* expansion (Hardcastle et al., 2000).

Morpholino oligonucleotides targeted to *Xenopus* FGF8

Several morpholino oligonucleotides (MOs) (Gene Tools) were designed to prevent either translation or proper splicing of *FGF8* transcripts in *X. laevis* and *X. tropicalis* (Fig. 3A). XlMOF8 was targeted to the translational start site of *X. laevis*. The XlMOF8 inhibited *FGF8* protein synthesis in in vitro transcription and translation assays when the template had the XlMOF8 target sequence but not when the target was absent (data not shown). In animal cap assays, explants injected with *FGF8b* mRNA expressed *xbra* (Fig. 3B, lane 4), whereas uninjected animal caps did not (lane 3). When 40 ng of XlMOF8 was injected with *FGF8b* containing the target sequence, no *xbra* was induced (Fig. 3B, lane 6), and this effect was eliminated when the XlMOF8 target sequence was mutated (lane 7). Similarly, *FGF4* induced *xbra* in the presence of the XlMOF8 (lane 8).

MOs were designed to block splicing of either one or both spliceforms (Fig. 3A). Ultimately, MOSAF8a, which targeted the splice-acceptor site that yields *FGF8a* (Fig. 3E), was the only spliceform-specific MO. It prevented splicing of the FGF8a spliceform with little effect on splicing of *FGF8b* (Fig. 3F,G). MOSDF8, which targeted the exon two splice-donor, prevented splicing of both *FGF8a* and *FGF8b*, and resulted in aberrant splicing. Sequencing revealed that MOSDF8 caused skipping of exon two (Fig. 3E). This results in a frameshift and premature termination and thus eliminates both *FGF8a* and *FGF8b* (Fig. 3F,G). MOSAF8a and MOSDF8 had the same respective effect in

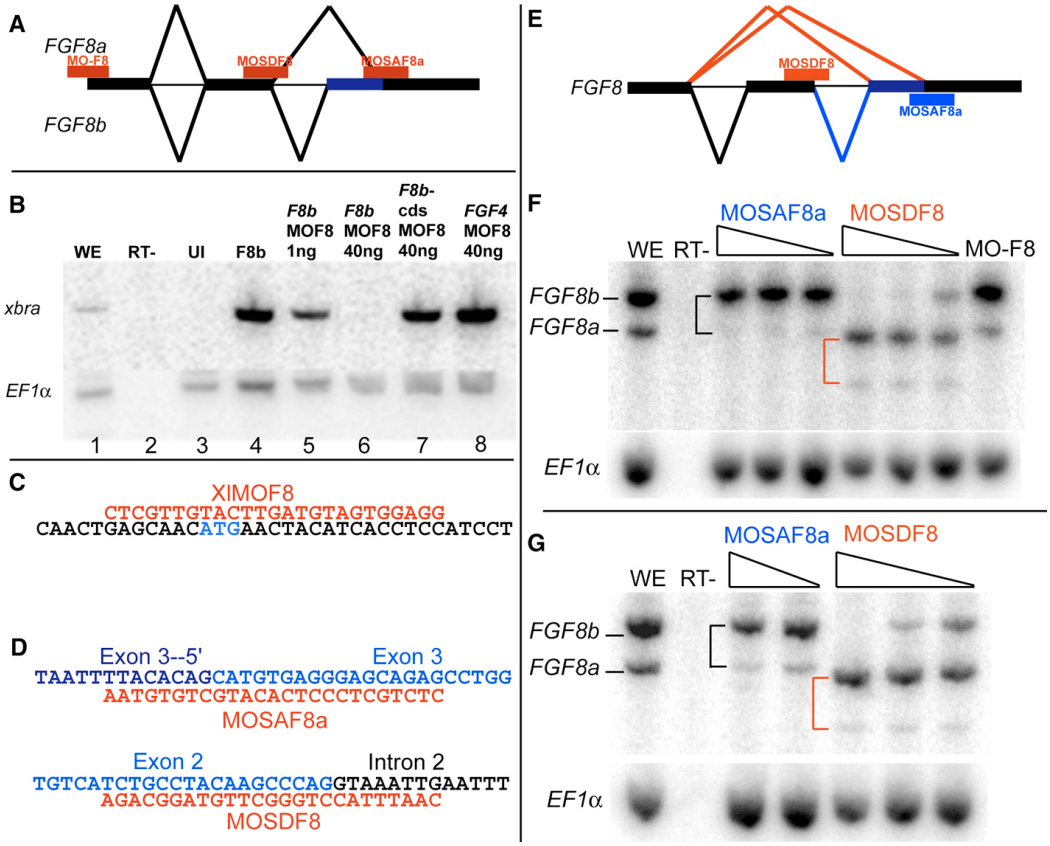

Fig. 3. Morpholino oligonucleotides (MOs) targeted to the *FGF8* mRNA. (**A**) Schematic of *FGF8* gene diagramming the position of the MOs (red); *FGF8b*-specific alternatively spliced region (dark blue). (**B**) RT-PCR analysis of explants from embryos injected as indicated above the lanes. *EF1*α, loading control; explants were examined for *xbra* expression. *F8b* (*FGF8b* with the UTRs) (200 pg) induces *xbra* in explants (lane 4); co-injection of 40 ng of XlMOF8 effectively inhibits this effect (lane 6); *xbra* expression is rescued with injection of *FGF8b*-cds (does not have the MOF8 target sequence) (lane 7); *FGF4* induction of *xbra* expression is unaffected by the MOF8 (lane 8). (**C**) XlMOF8 was designed to bind the translational start region of *X. laevis FGF8*. (**D**) Nucleotide sequence that MOSAF8a and MOSDF8 bind, respectively; MO sequence is in red. (**E**) Schematic of MOSDF8 and MOSAF8a effects on splicing. (**F**) RTPCR of *X. laevis* whole embryos injected as indicated; MOSAF8a (160, 120, 80 ng); MOSDF8 (170, 85, 43 ng); red brackets indicate MOSDF8 induced alternative splicing products that lead to premature termination; MOSAF8a results in a loss of the *FGF8a* but not *FGF8b* spliceform. (**G**) RTPCR of *X. tropicalis* embryos demonstrating the efficacy of MOSAF8a (32, 16 ng) and MOSDF8 (68, 34, 17 ng).

both *X. laevis* and *X. tropicalis* (Fig. 3F,G), and both were used to investigate the role of *FGF8* in mesoderm and neural development. Other MOs designed to block *FGF8b* selectively, by binding to the FGF8b splice acceptor, were not specific; instead, both spliceforms were reduced.

FGF8 is necessary for proper mesoderm formation: FGF8b is the FGF8 spliceform affecting mesodermal development

FGF signaling is necessary for trunk and tail mesoderm formation (Amaya et al., 1991; Amaya et al., 1993), and FGF signaling functions by maintaining *xbra* expression in the presumptive mesoderm (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Kroll and Amaya, 1996). To determine whether *FGF8* is an important ligand in mediating this process, we analyzed the effect of knocking down *FGF8*. Injection of the translation blocking MO XlMOF8 causes a severe reduction in *xbra* expression by the gastrula stage, and *xbra* expression can be restored by human *FGF8B*, mouse *Fgf8f* and *Xenopus FGF4* (Fig. 4B-E). Strongly

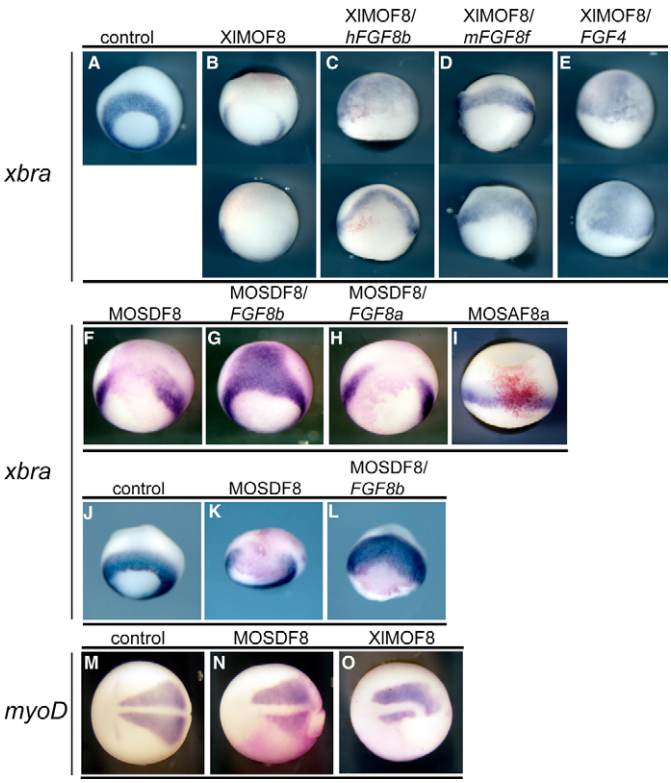

Fig. 4. Effects of FGF8 reduction on mesoderm formation. (A-I) *X. laevis* embryos; all embryos injected into the marginal zone of one cell at the two-cell stage; (F-H,K,L,N,O) pink staining for the fluorescein-conjugated control MO indicates injected side; (B-E,I) red-gal indicates injected side. (**A**) Control *xbra* expression; (**B**) XlMOF8 (40 ng) strongly reduces *xbra* expression (19/20 embryos), and this loss can be rescued by human *FGF8b* (13/17), mouse *FGF8f* (14/20) and *Xenopus FGF4* (17/23) (**C-E**). (**F**) MOSDF8 (85 ng) reduces *xbra* expression (27/31 embryos); (**G**) *FGF8b* rescues MOSDF8 phenotype (33/40); but *FGF8a* does not rescue (32/42) (**H**). (**I**) MOSAF8a (*FGF8a*-specific) (60 ng) does not affect *xbra* expression (15/16). (**J-L**) *X. tropicalis* embryos. MOSDF8 (42 ng) reduces *xbra* expression (22/28) (K). *FGF8b* rescues MOSDF8 (30/37) (L). (**M-O**) *X. laevis* embryos, dorsal views with anterior to the left. (M) Control stage 13 *myoD* expression. (N) MOSDF8 85 ng (39/39 reduced); (O) XlMOF8 (30 ng) (22/22 reduced).

reducing the *FGF8* spliceforms with the *FGF8* splice-blocking MO, MOSDF8, reduces *xbra* (Fig. 4F; blue hybridization signal, pink/red lineage tracer). This reduction can be rescued with *FGF8b* but not with *FGF8a* (Fig. 4G,H). The *FGF8a*-specific MO MOSAF8a does not diminish *xbra* expression (Fig. 4I), demonstrating that *FGF8a* is not necessary for proper mesoderm formation. Similarly, reduction of the *FGF8* spliceforms results in a reduction in *xbra* expression in *X. tropicalis* (Fig. 4K), and this can be rescued with *FGF8b* (Fig. 4L). These results demonstrate that *FGF8* is necessary for mesoderm formation in the embryo and suggests that *FGF8b* is the predominant *FGF8* spliceform involved in early mesoderm formation.

myoD is expressed from gastrulation through somite formation (Fig. 4Q), and both *bFGF* and *FGF4* induce *myoD* in explants (Harvey, 1991). FGF signaling is necessary for proper muscle formation (Amaya et al., 1991; Amaya et al., 1993) and for full expression of *myoD* (Isaacs et al., 1994). *Xenopus FGF4* is necessary for proper *myoD* expression in the embryo (Fisher et al., 2002). Because *FGF8b* induces mesoderm in explants and expands mesoderm so robustly in whole embryos, we examined the effect of knocking down *FGF8* on *myoD* expression. A strong knockdown of *FGF8* with either MOSDF8 or XlMOF8 reduces *myoD* expression, and the embryos fail to gastrulate properly (Fig. 4N,O). This demonstrates that *FGF8* plays at least a supporting role in *myoD* expression and suggests that at least partially redundant signaling by multiple FGF ligands is responsible for proper *myoD* expression in the embryo. The phenotype caused by high level knockdown of *FGF8* appears to be the same as that caused by overexpression of a dominant-negative FGFR (XFD) in the embryo (Amaya et al., 1991). At lower doses, most embryos gastrulate normally, though there are neural patterning defects. Stronger knockdowns of *FGF8* also result in apoptosis during neurula stages (not shown), so, in addition to its role in patterning, *FGF8* is important for cell survival.

Because several FGF ligands (*FGF3*, *FGF4*, *FGF8b*) are expressed around the blastoporal region in *Xenopus laevis* and have been shown to affect mesoderm formation in whole embryos (Isaacs et al., 1992; Isaacs et al., 1994; Lombardo et al., 1998; Fisher et al., 2002) (Figs 1-4), multiple FGF ligands contribute to early mesoderm formation and patterning in *Xenopus*, but *FGF8b* appears to be particularly necessary for early mesoderm formation in the *Xenopus* embryo.

FGF8a promotes posterior neural fate in explants and in the whole embryo

Whereas a strong knockdown of *FGF8* results in a reduction in mesoderm formation, a lower level knockdown of both spliceforms or loss of *FGF8a* alone causes a reduction in posterior neural tissue development with little effect on mesoderm formation. To follow up on the initial observations of Christen and Slack (Christen and Slack, 1997) that *FGF8a* caused headless tadpoles (Fig. 5C), we first analyzed how *FGF8a* mRNA overexpression affects explants and anteroposterior neural patterning in the whole embryo. Because it does not affect mesoderm formation (Figs 2, 4), we focused on the activity of *FGF8a*.

FGF signaling posteriorizes explants (Cox and Hemmati-Brivanlou, 1995), is necessary for posterior neural tissue to develop in explants (Holowacz and Sokol, 1999) and can induce posterior neural tissue directly in explants (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). In Fig. 5, we show that *FGF8a* mRNA can induce posterior neural transcripts directly in explants. *FGF8a* induces expression of hindbrain transcripts (*krox20*, *hoxD1*), spinal cord transcripts (*hoxB9* and *cad3*), and at a very high dose the

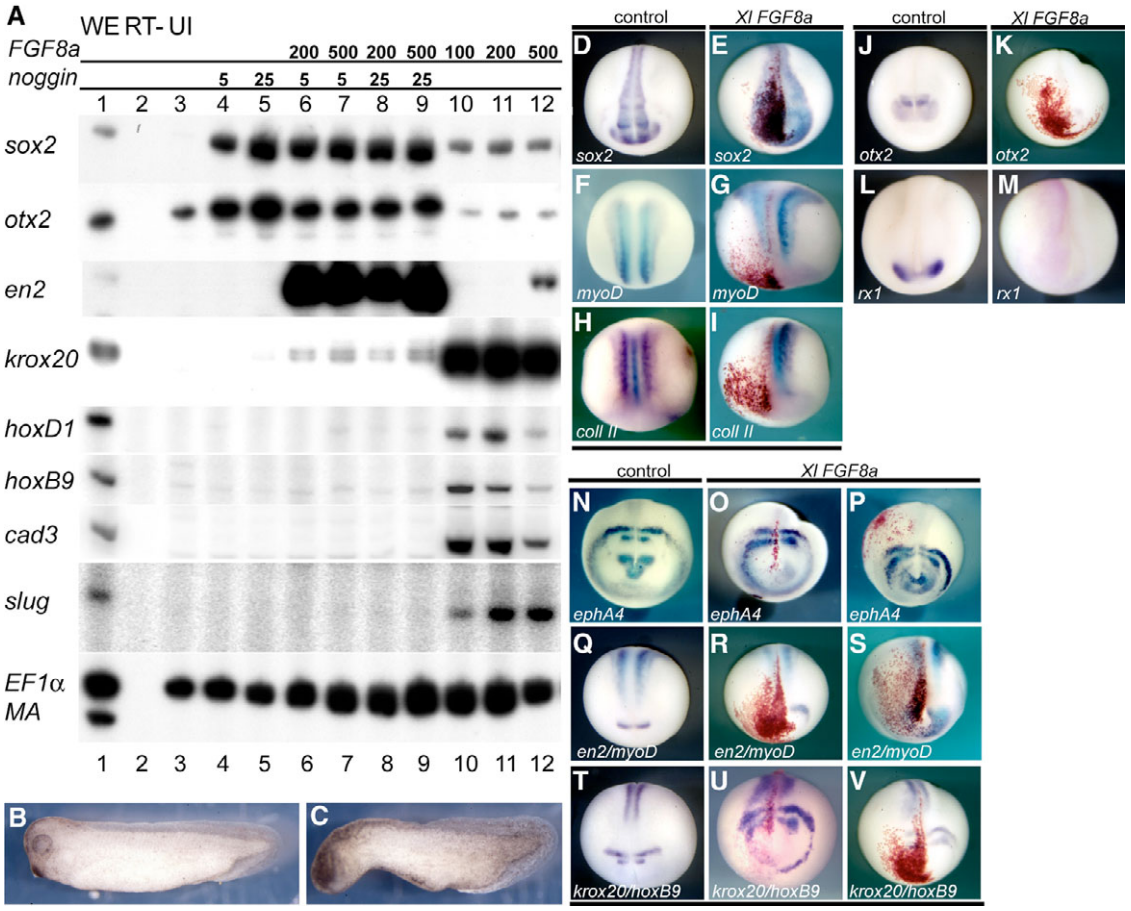

Fig. 5. Effect of *FGF8a* overexpression on neural patterning. (**A**) *FGF8a* induces posterior neural genes in ectodermal explants. Embryos were injected into the animal hemisphere at the one-cell stage. Explants were excised at stage 9 and cultured until stage 20. Whole embryo (WE) stage-control embryo sample; whole embryo lysate processed without reverse transcriptase (RT–); explants from uninjected embryo (UI). Embryos were injected as indicated along the top. *EF1α*, loading control; *muscle actin* (*MA*) is an indicator of dorsal mesoderm. Endogenous neural gene expression domains are as follows: *sox2*, general neural tissue; *otx2*, forebrain and midbrain; *en2*, MHB; *krox20*, hindbrain r3 and r5; *hoxD1*, posterior hindbrain and spinal cord; *hoxB9* and *cad3*, spinal cord. (**B**) Uninjected *X. laevis* tadpole; (**C**) *FGF8a*-injected tadpole. (D-V) Embryos displayed dorsoanteriorly; red staining indicates the lineage tracer. Embryos injected into a dorsal blastomere at the four-cell stage with 50 pg of *FGF8a* mRNA and cultured until neural tube stage 19/20. (**D-I**) *FGF8a* does not expand expression of the mesodermal genes *myoD* (32/32 embryos) or *coll II* (6/6), but *sox2* expression domains are mispatterned and expanded (25/25). (M) Fluorescein-conjugated control MO was injected with the *FGF8a* mRNA as a lineage tracer (pink). (**J-V**) *FGF8a* expands posterior neural domains and reduces anterior neural domains. The displayed phenotypes are representative of the effects of *FGF8a* mRNA quantitated as follows: (K) *otx2*, 13/14; (M) *rx1*, 17/17; (O,P) *ephA4*, 33/35; (R,S) *en2*, 25/32; (U,V) *krox20*, 45/52; *hoxB9*, 43/44.

midbrain-hindbrain boundary marker, *en2* (Fig. 5A, lanes 10-12). In combination with *noggin*, which induces expression of the anterior transcript, *otx2*, *FGF8a* causes strong expression of the MHB gene, *en2* and a slight reduction in the level of *otx2* expression (lanes 6-9), confirming the ability of *FGF8a* to act as a posteriorizing agent.

Dorsal injection of *FGF8a* mRNA causes, in a non-cell-autonomous manner, a modest expansion of the neural plate as shown by *sox2* expression in the spinal cord domains and a more pronounced anterior expansion in the normal brain regions (Fig. 5E). Expression of mesodermal *myoD* and *collagen II* is not expanded (Fig. 5G,I), consistent with other reports (Hardcastle et al., 2000).

To understand how *FGF8a* can affect neural pattern, embryos were examined for expression of a range of anteroposterior transcripts by in situ hybridization. Injection of *FGF8a* mRNA reduces anterior neural gene expression domains. The forebrain and midbrain mRNA, *otx2* (Lamb et al., 1993) and the eye-specific *rx1* (Casarosa et al., 1997) are strongly reduced in *FGF8a*-injected embryos (Fig. 5J-M). Similarly, the forebrain domain of *ephA4* expression (Smith et al., 1997) is reduced (Fig. 5O,P).

Overexpression of *FGF8a* causes posterior neural tissue domains to expand both laterally and anteriorly. *FGF8a* expands the r3 and r5 domains of *ephA4* and *krox20* (Bradley et al., 1993), resulting in an extension of hindbrain domains laterally and forward (Fig. 5O,P,U,V). The exposure to *FGF8a* signaling appears to transform the behavior of rhombomere 3 into that of rhombomere 5, with a trail of expressing cells extending towards ventral regions of the embryo. The effects are dose dependent and non-cell autonomous, as effects are seen on the injected side but also on the uninjected side. *FGF8a* expands expression of the spinal cord transcript, *hoxB9* (Sharpe et al., 1987) anteriorly (Fig. 5U,V). The midbrain-hindbrain boundary (MHB) expression of *en2* is expanded forward, sometimes to the most anterior regions of the neural plate (Fig. 5R,S), consistent with the results of Christen and Slack (Christen and Slack, 1997).

The posterior expansion explains the peculiar morphology observed when *sox2* expression is examined (Fig. 5B,D); the bulbous expansion in the anterior of the embryos reflects the expansion of the posterior neural tissue domains both laterally and towards the anterior, and this repatterning fits well with previous work showing that *FGF8a* can shift neural crest domains laterally and expand neural crest tissue around the anterior of the embryo (Monsoro-Burq et al., 2003).

FGF8 is essential for proper posterior neural specification in *X. laevis* and *X. tropicalis*

Because *FGF8a* can posteriorize the neural plate (Fig. 5) and because FGF signaling is necessary for proper posterior neural tissue formation (Amaya et al., 1991; Amaya et al., 1993; Pownall et al., 1996; Pownall et al., 1998; Ribisi et al., 2000), we were interested in determining whether *FGF8* was crucial for posterior neural fate specification.

By using low doses of the MOs that knockdown both *FGF8* spliceforms and by using the *FGF8a*-specific MOSAF8a, we have addressed how *FGF8* is involved in neural fate specification and differentiation. First, *X. laevis* embryos were injected with the indicated MOs and analyzed early in neural development to ascertain which neural fates depended upon *FGF8* signaling. XlMOF8, MOSDF8 and MOSAF8a all affect expression of *sox2* – it is still present, but it is mispatterned (Fig. 6B-D). This demonstrates that loss of the *FGF8a* spliceform or a lowering of the levels of both the *FGF8a* and *FGF8b* spliceforms prevents proper patterning of the neural plate.

FGF8 signaling is necessary to properly establish the caudal boundary of the anterior neural domain. Loss of *FGF8a* or a reduction in both *FGF8a* and *FGF8b* results in slight posterior expansion of expression of the forebrain and midbrain mRNA *otx2* (Fig. 6F-H). Establishment of the midbrain-hindbrain boundary, an important signaling center as neural development proceeds, also depends on *FGF8* signals. XlMOF8, MOSDF8 and MOSAF8a result in a severe reduction in *en2* expression, and any faint remaining *en2* expression is shifted towards the posterior of the embryo (Fig. 6J-L). XlMOF8- and MOSDF8-injected embryos also show reduced *myoD* staining at these low doses, whereas *myoD* appears only slightly affected in MOSAF8a-injected embryos. The slight mispatterning and reduction in *myoD* expression may also be in part due to the effect of *FGF8* signaling on the neural plate as the neural plate is important for somite formation (Mariani et al., 2001).

FGF8a and *FGF8b* signaling is necessary for the specification of the hindbrain and spinal cord neural plate domains. XLMOF8, MOSDF8 and MOSAF8a all result in a very strong reduction to loss of expression of the hindbrain transcript *krox20* and of the spinal cord transcript *hoxB9* (Fig. 6N-P). Additionally, the spinal cord transcript *dbx* (Gershon et al., 2000) is absent on the injected side (Fig. 6F-H). *FGF8* is also necessary for posterior neural tissue formation in *X. tropicalis* (data not shown). Because the effect on posterior neural development occurs early, it strongly suggests that *FGF8* is necessary for the initial specification of posterior neural fate – not simply to maintain posterior neural tissue.

Congruent with the analysis in early neurula stage embryos, *en2* (data not shown), *krox20* and *hoxB9* are all reduced and, if present, shifted towards the posterior of the embryo at the neural tube stage (Fig. 6Q-S). The effect of XlMOF8, MOSDF8 and MOSAF8a on posterior neural tissue can be rescued by *FGF8a* mRNA (Fig. 6T-V) where the anterior truncation of the *hoxB9* expression domain is reversed, and where *krox20* is more strongly expressed and not shifted to the posterior.

The analysis of neural tube stage embryos confirms the importance of *FGF8* to posterior neural development, but it also reveals that *FGF8* is important for placode formation (Fig. 7B,C). The placode domains (Schlosser and Ahrens, 2004) are reduced and the regionalized staining in the brain regions is perturbed bilaterally but more so on the injected side of the embryo which agrees with a recent report (Ahrens and Schlosser, 2005). The eye-field, marked by *rx1*, is expanded towards the posterior in MOSAF8a-injected

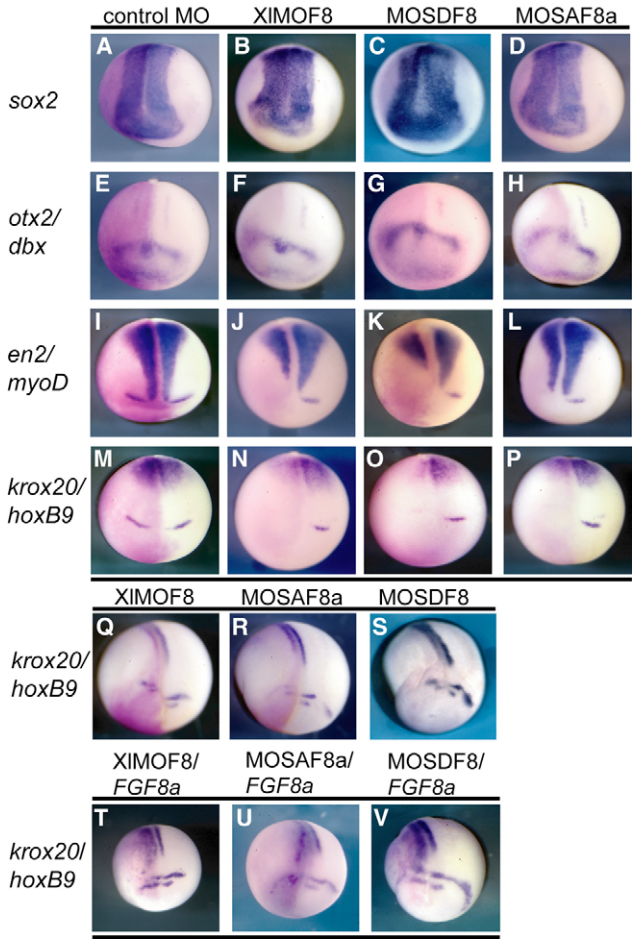

Fig. 6. Lower level reduction of *FGF8a* and *FGF8b* or strong reduction of *FGF8a* alone with XlMOF8, MOSDF8 and MOSAF8a prevents proper formation of posterior neural tissue at the early neurula stage. *X. laevis* embryos displayed dorsoanteriorly. Pink staining indicates the lineage tracer; embryos injected into one cell at the two- or four-cell stage. (A,E,I,M) Control MO (40 ng); (B,F,J,N) MOF8 20 ng; (C,G,K,O) MOSDF8 43 ng; (D,H,L,P) MOSAF8a 60 ng. (**A-D**) XlMOF8 (44/45), MOSDF8 (18/19) and MOSAF8a (38/39) cause a mispatterning of *sox2* expression. (**E-H**) *otx2* expression is expanded toward the posterior after XlMOF8 (23/25), MOSDF8 (20/20) and MOSAF8a (26/29) injection, while the posterior neural gene *dbx* is absent (MOF8, 25/25; MOSDF8, 20/20; MOSAF8a, 35/38). (**I-L**) *en2* expression is diminished and sometimes completely absent on the injected side: XlMOF8 (13/13), MOSDF8 (17/17) and MOSAF8a (32/33). (**M-P**) both the spinal cord domain (*hoxB9*) and the hindbrain domain (*krox20*) is strongly reduced and shifted toward the posterior of the embryo on the XlMOF8 (42/42), MOSDF8 (20/20) and MOSAF8a (40/40) injected side of the embryo. Effects on the uninjected side are present but much weaker. (**Q-S**) Neural tube stage 20 embryos treated as indicated; (**T-V**) FGF8a mRNA (50 pg) rescued the reduction of hoxB9 caused by XlMOF8 (32/44), MOSAF8a (19/35) and MOSDF8 (19/20).

embryos (Fig. 7E), again supporting the role of *FGF8a* in helping to limit the anterior neural domain boundary. Neuronal differentiation, marked by *ntub* (Fig. 7F-M) is reduced when *FGF8a* and *FGF8b* levels are lowered; this is complementary to the massive expansion of *ntub* expression when *FGF8a* is overexpressed (Hardcastle et al., 2000). In addition to posterior neural reductions, knockdown of *FGF8a* and *FGF8b* causes posterior truncations by the tadpole stage (Fig. 7I,K,M).

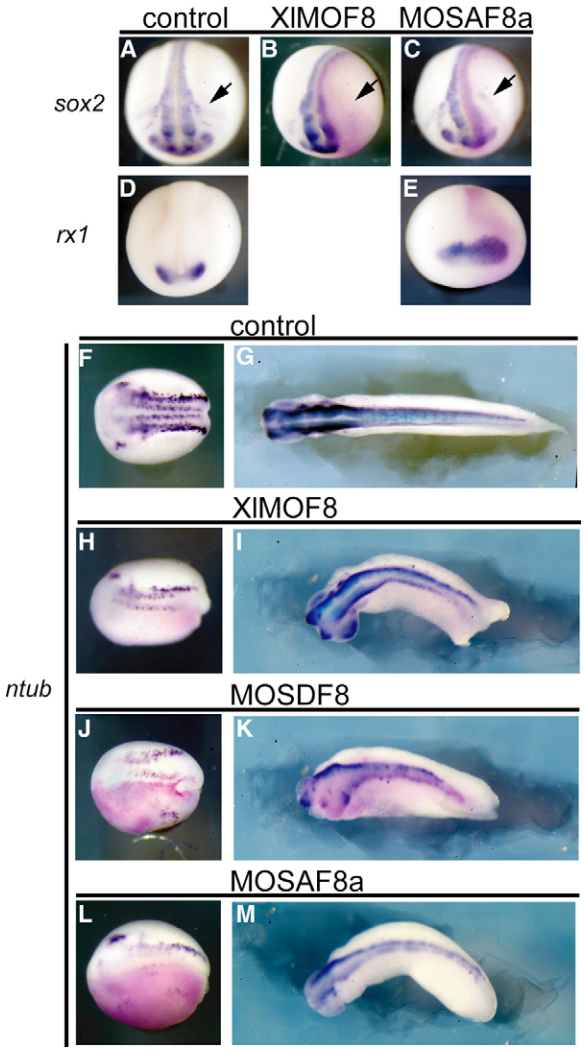

Fig. 7. Lowering *FGF8* levels causes a reduction in placode formation and neural differentiation. (**A-E**) *X. laevis* embryos displayed dorsoanteriorly. Embryos were cultured with XlMOF8 (10 ng) or MOSAF8a (60 ng) until the neural tube stage 20; lineage tracer (pink). (B,C) XlMOF8 caused *sox2* mispatterning (40/42), so did MOSAF8a but to a lesser degree (20/22). (E) MOSAF8a resulted in a slight expansion of the *rx1* domain toward the posterior (16/20). (**F-M**) The effect of *FGF8* reduction on early neuronal differentiation in *X. tropicalis*. XtMOF8 (8 ng), MOSDF8 (17 ng) and MOSAF8a (16 ng) were injected into one cell at the two-cell stage; injected side is oriented downwards. (F,H,J,L) Embryos were cultured until neurula stages; injected embryos demonstrate an early strong reduction in neuronal differentiation [XtMOF8 (20/20), MOSDF8 (9/9), MOSAF8a (16/16)]. (G,I,K,M) Embryos were cultured until the early tadpole stage; injected embryos demonstrate posterior truncations and continued reduction in differentiated neurons

DISCUSSION

Alternative splicing can add diversity to functions of the proteome, and more than 50% of human genes are alternatively spliced (Kan et al., 2001; Lander et al., 2001; Modrek et al., 2001; Johnson et al., 2003), suggesting that this is an important process for regulating morphological complexity. *FGF8* is alternatively spliced and is important in many developmental contexts. In this study, we have explored the functions of the *FGF8a* and *FGF8b* spliceforms in the early formation of mesoderm and neural tissue in *X. laevis* and *X. tropicalis*.

Our analysis confirms that *Xenopus FGF8a* is not a strong mesoderm inducer because it has almost no activity in mesoderm induction assays in explants, and it does not expand *xbra* expression in whole embryos when overexpressed (Christen and Slack, 1997). Consistent with this, an *FGF8a*-specific MO (MOSAF8a) that blocks the splice acceptor does not affect *xbra*, yet knocking down only the *FGF8a* spliceform does affect neural patterning. This contrasts remarkably with the activity of *Xenopus FGF8b*. *X. laevis FGF8b* is a robust inducer of *xbra* in explants, and in the whole embryo it expands *xbra* in a non-cell-autonomous manner (Fig. 2). A strong knockdown *FGF8a* and *FGF8b* with either a translation-blocking MO (XlMOF8) or a splice-donor blocking MO reduces *xbra* expression and, additionally, results in a reduction of *myoD* expression (Fig. 4). A low level knockdown of *FGF8a* and *FGF8b* or a strong knockdown of *FGF8a* alone causes a reduction in the specification of posterior neural tissue. Therefore, *FGF8* plays at least two separable roles in early *Xenopus* development: *FGF8* signaling is specifically required for formation of mesoderm, and this work demonstrates the important role of *FGF8b* as the primary *FGF8* spliceform involved in this process. Second, *FGF8* signaling is necessary for proper establishment of posterior neural identity. The *FGF8a* spliceform is necessary for this process, and because we see an enhanced posterior neural reduction when both spliceforms are reduced, it argues that *FGF8b* may also be contributing to posterior neural development.

An earlier analysis of *Xenopus FGF4* has shown that in addition to mesoderm inducing activity, it is necessary for full *myoD* expression in the embryo (Fisher et al., 2002). Taken with our work on FGF8, this demonstrates that both *FGF4* and *FGF8b* are necessary for proper mesoderm formation in *Xenopus*. It is interesting that a strong knockdown of *FGF8* is sufficient to perturb proper mesoderm formation; this suggests that one need only remove part of the FGF signaling to prevent the proper *xbra* feedback loop, and it suggests that these FGFs are working together to some degree.

Interestingly, FGF8 is involved in proper mesoderm formation in the mouse but in a different manner. In the mouse, homozygous *FGF8* loss-of-function mutants form mesoderm early, but cells do not migrate away from the streak and later differentiation of mesodermal derivatives does not occur (Sun et al., 1999). In zebrafish, the combination of *FGF8* and *FGF24* is needed for establishment of posterior mesoderm (Draper et al., 2003). In contrast to zebrafish, where *FGF8* appears involved in establishing dorsal identity (Furthauer et al., 1997; Furthauer et al., 2004), in *Xenopus*, *FGF8* does not induce secondary axes as it can in zebrafish.

Wnt, FGF and RA signaling have all been shown to be involved in posterior neural development (Lamb and Harland, 1995; Blumberg et al., 1997; Christen and Slack, 1997; Kolm et al., 1997; McGrew et al., 1997; Hollemann et al., 1998; Domingos et al., 2001; Kiecker and Niehrs, 2001). The *FGF8* spliceforms, even the individual *FGF8a*, are necessary for establishment of posterior

neural identity and for restriction of the anterior neural domain. Because the effect of reduction in *FGF8* spliceforms is observed early in development, we argue that *FGF8* signaling is necessary for the establishment of posterior neural fate, not simply for its maintenance. This *FGF8* signal would cooperate with other FGFs, Wnts and RA in the formation of posterior identities (Isaacs et al., 1995; Pownall et al., 1996; McGrew et al., 1997; Lombardo et al., 1998; Domingos et al., 2001; Kiecker and Niehrs, 2001). Interestingly, reduction in *FGF8* levels does not cause an expansion of anterior neural gene expression into the normal spinal cord domains; rather, there is only a limited movement of the caudal anterior neural gene expression boundary towards the posterior; this would support the idea that multiple signals are involved in limiting anterior neural gene expression.

Recent work suggests that FGF signaling is involved in the specification of all neural tissue, not just for formation of posterior neural tissue (Pera et al., 2003; Delaune et al., 2005). This may be why *sox2* expression is weakly reduced in XlMOF8- and MOSDF8-injected embryos, whereas knockdown of specifically *FGF8a* has a weaker effect on *sox2* expression levels while still strongly affecting posterior neural gene expression. Perhaps a stronger loss of more FGF signaling ligands is necessary to preclude neural tissue formation, but a more temporally precise loss of individual ligands will be necessary to discern any direct effects on neural development from early mesoderm formation.

In addition to the differences in activity between FGF spliceforms that have been observed in several cell culture assays (MacArthur et al., 1995a; MacArthur et al., 1995b; Ghosh et al., 1996; Blunt et al., 1997) and in mesodermal development in *X. laevis* (Figs 2, 4), differences in activity between *FGF8a* and *FGF8b* in neural development have also been reported in the mouse and chick. In the mouse and chick, *FGF8b* overexpression at the MHB results in expansion of the hindbrain, while overexpression of *FGF8a* at the MHB results in some expansion of the midbrain and ectopic *en2* expression (Liu et al., 1999; Sato et al., 2001; Sato et al., 2004). In addition, in chick extra-embryonic epiblast cells, *FGF8b* could induce expression of *brachyury* and the neuronal precursor gene, *cash4*, while *FGF8a* had no activity (Storey et al., 1998).

Although *FGF8a* and *FGF8b* have very different activities, they differ by only 11 amino acids in the N-terminal region of the protein (Fig. 1A). One possible explanation is that the difference in activity between *FGF8a* and *FGF8b* – specifically, that *FGF8b* can robustly induce mesoderm and expand it in the whole embryo whereas *FGF8a* cannot and that *FGF8a* can posteriorize the neural plate without affecting mesoderm – could be due to differences in the affinity of the two spliceform products for different receptors or spectrum of receptors. Recent biochemical and structural work supports the idea that a large part of the difference in activity between the two ligands at the MHB in the chick and mouse is due to differences in affinity between the isoforms for the different FGFRs, with *FGF8b* having a higher affinity than *FGF8a* (Olsen et al., 2006). This must certainly be a contributing mechanism to the differences in their activities in *Xenopus*, regardless of whether they bind a different set or combination of receptors in vivo. It is remarkable that the embryo can respond in a drastically different way to the two versions of the *FGF8* ligand. As there is evidence that in some cellular contexts, heparin sulfate can mediate FGF8b interaction with different FGFRs (Allen and Rapraeger, 2003), it would be interesting to know whether molecules such as heparin sulfate function in eliciting such biologically significant differences in activity. Furthermore, spliceform specific knockouts in the mouse, which has seven different splice variants, would be informative in understanding how the *FGF8* gene functions. Alternative splicing of *FGF8* confers specific activity to the spliceforms and is integral to the role of the gene in early mesodermal and neural development in *Xenopus*.

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