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). As many as seven different protein forms in the mouse and four in human (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, FGF8 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 FGFRs, including FGFR8, are expressed in the early posterior dorsal mesoderm, where they are in proximity to the presumptive neuroectoderm (Christen and Slack, 1997).

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., 1995b).
et al., 1995a; Ghosh et al., 1996), and FGF8B/Fgf8b binds multiple FGFR ‘c’ spliceforms expressed in BaF3 cells and induces mitosis. However, FGF8A/Fgfa has no detectable activity (MacArthur et al., 1995b; Blunt et al., 1997). At the midbrain-hindbrain boundary, FGF8A/Fgfa 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. FGF8a, 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 Fg8 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 Fg8/f 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) were excised from stage 9 embryos with either an eye-brow knife or with the Gasteromast (XENOTEK Engineering). Animal caps were then cultured in 75% NAM (XENOTEK Engineering). Animal caps were then cultured 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); ntit (Good et al., 1989); sox2 (Grammer et al., 2000); nrlf (Knecht et al., 1995); otz2 (Lamb et al., 1993); em2 (Hemmati-Brivanlou et al., 1991); krox20 (Bolce et al., 1992); hoxB9 (Sharpe et al., 1987); dbx (Gershon et al., 2000); ntitulin (Good et al., 1989); collagen type II (Amaya et al., 1993); rcl (Casarosa et al., 1997); sox2 (GenBank AL580590); and epha4 (Smith et al., 1997). X. tropicalis probes en2, myoD, krox20, hoxB9, otz2, 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 B589284; IMAGE: 4084172). The coding sequence was amplified using Phusion polymerase and PCR with the following primers: U, 5’-GATTCATGACTAATCCTAAGCTT-3’; D, 5’-GAATTCCTAGAGAATGATCTAGCT-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 Fg8a (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 (X. FGF8a), GenBank BC082344 (X. 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 H2O and stored at –80°C. The following constructs were linearized with AscI 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 (XLFGF8b-CS8); X. laevis FGF8b with only the coding sequence (XLFGF8b-CS8); mouse Fg8a (Crossley and Martin, 1995) subcloned into pCS108 (mF8a-CS8); mouse Fg8b (mF8b-CS7) (Myers et al., 2004); human FGF8b (Ghosh et al., 1996) subcloned into pCS108 (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 Weinstein, 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 un.injected 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, em2, krox20 and hoxB9 (Hemmati-Brivanlou and Melton, 1994); otz2 (Lamb and Harland, 1995); hoxD1 and 3x1d (Kolm et al., 1997); slug (Mizusaki et al., 1998); ODC (Hudson et al., 1997). The primers used for detection of Xenopus FGF8a and FGF8b are: U, 5’-ATCACCCTCATTCTGGGCTAC-3’; D, 5’-TGCGAACCCTCTGCTACCAAGC-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 (XIMO8F) is 5’-GAAGGTGTAGTATGTCGTCTGCTCTGC-3’ had a fivefold lower specific activity in X. laevis. The splice-blocking MOs are as follows: MOSAFA68a, 5’-CTCTGTCCTCCCTCAGTCTGCTTGA-3’; MOSDF8, 5’-AGACGGGTGTTCCGGCCATTAAAC-3’. The Gene Tools standard control MO (5’-CCCTTTACTCCATGATGTTTTA-3’) conjugated to fluorescein was used as a lineage tracer. The morpholino oligonucleotides were resuspended in RNase-free 1/20MR. The injection volume was either 5 nl or 10 nl.

RESULTS

FGF8 expression in Xenopus

Surprised by the difference in a mouse FGF8 (Myers et al., 2004) and the reported Xenopus FGF8a activity, we sought to determine whether different spliceforms might have different activity. An FGF8b spliceform (Accession Number B5892841; 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

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**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' H11032) 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,N) Dorsal views with anterior towards the left; (F,H,J,L) 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.
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 1 pg 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).

**FGF8b has 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 as xbra expression is expanded beyond the lineage-traced tissue (Fig. 2O,P), indicating that it also possesses at
least a low level of the FGF8a activity. This contrasts with a recent report that FGF8b robustly induces ectopic ntu (Shim et al., 2005). At an even lower dose of FGF8b (0.1 pg), 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 ntu 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 ntu expression (Fig. 2FF). At a low dose, mouse FGF8f mispatterns ntu with a few ectopic neurons present, and it slightly expands myoD expression (Fig. 2CC,DD).

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). XIMOF8 was targeted to the translational start site of X. laevis. The XIMOF8 inhibited FGF8 protein synthesis in vitro and translation assays when the template had the XIMOF8 target sequence but not when the target was absent (data not shown). In animal cap assays, explants injected with FGF8b mRNA expressed xbr (Fig. 3B, lane 4), whereas un.injected animal caps did not (lane 3). When 40 ng of XIMOF8 was injected with FGF8b containing the target sequence, no xbr was induced (Fig. 3B, lane 6), and this effect was eliminated when the XIMOF8 target sequence was mutated (lane 7). Similarly, FGF4 induced xbr in the presence of the XIMOF8 (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

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**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. EF1α, loading control; explants were examined for xbra expression. F8b (FGF8b with the UTRs) (200 pg) induces xbra in explants (lane 4); coinjection of 40 ng of XIMOF8 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) XIMOF8 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 XIMO8 causes a severe reduction in *xbra* expression by the gastrula stage, and *xbra* expression can be restored by human FGF8b, mouse Fgf8 and Xenopus FGF4 (Fig. 4B-E). Strongly 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. 4J), 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 XIMO8 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., 1996; 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
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).

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 un.injected 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.
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. XIMOF8, MOSDF8 and MOSAF8a all result in a very strong reduction to loss of the hindbrain and spinal cord neural plate domains. 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 en2x (Fig. 6F-H). Establishment of the midbrain-hindbrain boundary, an important signaling center as neural development proceeds, also depends on FGF8 signals. XIMOF8, 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). XIMOF8- 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. XIMOF8, 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 XIMOF8, 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 rxl, is expanded towards the posterior in MOSAF8a-injected...
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).

**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 (XIMOF8) 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

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**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 XIMOF8 (10 ng) or MOSAF8a (60 ng) until the neural tube stage 20; lineage tracer (pink). (B,C) XIMOF8 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. XIMOF8 (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,L) Embryos were cultured until neurula stages; injected embryos demonstrate an early strong reduction in neuronal differentiation [XIMOF8 (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.
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 because why neural tissue (Pera et al., 2003; Delaune et al., 2005). This may be because of anterior neural gene expression boundary towards the posterior; this would support the idea that multiple signals are involved in limiting anterior neural gene expression.

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 FGRs, 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 FGRs (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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