

ORIGINAL ARTICLE

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Evolutionarily conserved and divergent expression of members of the FGF receptor family among vertebrate embryos, as revealed by FGFR expression patterns in *Xenopus*

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Abstract Fibroblast growth factors (FGFs) mediate many cell-cell signaling events during early development. While the actions of FGFs have been well-studied, the roles played by specific members of the FGF receptor (FGFR) family are poorly understood. To characterize the roles played by individual FGFRs we compared the regulation and expression of the three *Xenopus* FGFRs described to date (XFGFR-1, XFGFR-2, and XFGFR-4). First, we describe the expression of *Xenopus* FGFR-4; XFGFR-4 is present as a maternal mRNA and is found in the embryo through at least the tadpole stage. XFGFR-4 and XFGFR-1 mRNAs are present at comparable levels, arguing that both mediate FGF signaling during early development. Second, the expression of XFGFR-4 in animal caps differs from the expression of XFGFR-1 and XFGFR-2, suggesting that the FGFRs are independently regulated in ectoderm. Third, using whole-mount in situ hybridization, we show that XFGFR-1, XFGFR-2, and XFGFR-4 are expressed in dramatically different patterns, arguing that specific FGF signaling events are mediated by different members of the FGFR family. Among these, FGF signaling during the induction of neural crest cells is likely to be mediated by XFGFR-4. Comparison of our results with previously reported FGFR expression patterns reveals that FGFR-1 expression is highly conserved among vertebrate embryos, and FGFR-2 expression shows many features that are conserved and some that are divergent. In contrast, the expression pattern of FGFR-4 is highly divergent among vertebrate embryos.

Key words *Xenopus* fibroblast growth factor receptor · *Xenopus* fibroblast growth factor receptor-4 · Induction · Neural crest

Introduction

The vertebrate body plan is established largely through inductive interactions that occur early in development. Among the signaling molecules that mediate these interactions, members of the fibroblast growth factor (FGF) family play a number of roles (reviewed by Goldfarb 1996; Slack et al. 1996; Yamaguchi and Rossant 1995). FGFs have been implicated in mesoderm induction in *Xenopus*, because FGFs induce mesoderm from isolated animal cap tissue, and inhibition of FGF signaling disrupts the development of some mesodermal derivatives (reviewed by Slack 1994; Smith 1995). However, the precise role of FGFs in mesoderm induction remains unclear. FGF signaling is necessary, though not sufficient, for the expression of many mesoderm-specific genes (Cornell and Kimelman 1994; LaBonne and Whitman 1994), and FGF maintains expression of the transcription factor *Xbra* (Isaacs et al. 1994; Schulte-Merker and Smith 1995), which is necessary for the development of some mesodermal derivatives (Cunliffe and Smith 1992).

FGFs play multiple roles in later development. Disruption of FGF signaling leads to the failure of posterior cells to undergo proper gastrulation movements. This phenotype is observed in *Xenopus* embryos expressing a dominant negative FGF receptor (Amaya et al. 1991, 1993; Isaacs et al. 1994; Kroll and Amaya 1996), and in mouse cells lacking FGFR-1 (Ciruna et al. 1997). FGFs may posteriorize neural tissue (reviewed by Doniach 1995). In *Xenopus*, FGF overexpression results in disruptions of the anterior-posterior axis that are consistent with a posteriorizing role in normal development (Isaacs et al. 1994). Finally, FGFs are involved in many later cell interactions, acting at the midbrain-hindbrain boundary (Joyner 1996), in the initiation and maintenance of limb outgrowth, and in myogenesis, lung morphogene-

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sis, eye and lens development, bone growth, hair growth, and tooth formation (see Goldfarb 1996).

Vertebrates have four related FGF receptors (FGFRs). Different receptors show different affinities for different FGFs and are capable of forming heterodimers (reviewed by Johnson and Williams 1993). Thus, the outputs from different FGFR homodimers and heterodimers can, in principle, differ depending upon the relative affinity of the ligand for different receptors, and the relative amounts of the different receptor isoforms present on the surface of a target cell. While the roles of FGFs during development are well-documented, the roles played by specific FGFRs in mediating FGF signals are poorly understood. To understand FGF signaling in the embryo it is necessary to know not only where different FGFs are found, but also where different FGFRs are found, and whether the intracellular signals generated by the different FGFRs differ from one another. This point is underscored by recent observations that FGFRs may be activated by ligands other than classical FGFs (reviewed by Goldfarb 1996; Green et al. 1996).

Here we report on the characterization of *Xenopus* FGFR-4. The isolation of *Xenopus* FGFR-4 cDNAs has been reported by others (Riou et al. 1996; Shiozaki et al. 1995). We extend the findings of these groups and show that XFGFR-4 is expressed maternally and during the period of mesoderm induction. XFGFR-4 is expressed in a strikingly restricted pattern during the period of neural induction and patterning. We also compare the expression patterns of the three *Xenopus* FGFRs described to date; the XFGFRs show dramatically different expression patterns during early development. Comparison of FGFR expression in *Xenopus* embryos with previously published reports on other vertebrate embryos reveals that the expression patterns of FGFR-1 and FGFR-2 in different embryos show features that are highly conserved, while the expression patterns of FGFR-4 are highly divergent.

Materials and methods

DNA isolation and sequencing

A stage 11 cDNA library was screened at low stringency with probes encoding the tyrosine kinase domains of a *sevenless* cDNA from *Drosophila* (Bowtell et al. 1988) and a *Xenopus* FGFR-1 cDNA (Friesel and Dawid 1991). One class of clones corresponded to *Xenopus* FGFR-4. Longer clones were isolated by rescreening the cDNA library with probes from the 5'-end of the clone. DNA was sequenced using oligonucleotide primers.

Animal caps and FGF treatment

Embryos were staged according to Nieuwkoop and Faber (1967). Animal caps were removed at stage 8 and cultured in vLCMR solution (very low Ca^{2+} - Mg^{2+} Ringer's; Lamb and Harland 1995) either untreated or with 50 ng/ml recombinant *Xenopus* bFGF.

RNA isolation and blotting

RNA was isolated by the proteinase K/LiCl method (Sive et al. 1999). RNA blots were as described (Sive et al. 1999), with 2 μg

RNA loaded per lane. ^{32}P -labeled DNA probes were generated by random priming of plasmid inserts (Pharmacia, Piscataway NJ). The XFGFR-4 probe consisted of the 3'-most 2 kb of the XFGFR-4 cDNA. The rRNA probe was from pXlr101A, a *Xenopus* ribosomal RNA repeat (Trendelenburg and Gurdon 1978).

RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described by Wilson and Melton (1994), with the following modifications. For whole embryos RNA was isolated from ten embryos. One embryo equivalent of DNase-treated RNA was reverse-transcribed, one-tenth of the RT reaction was PCR-amplified, and one-half of the RT-PCR product was loaded onto the gel. For animal caps, RNA was isolated from five caps, DNase treated and reverse-transcribed. One-tenth of the RT product was PCR-amplified, and one-half of the RT-PCR product was loaded onto the gel. XFGFR-1 and XFGFR-4 were simultaneously amplified in a single reaction; EF-1 α was amplified separately. The number of amplification cycles used is noted in figure legends. Gels were stained with ethidium bromide.

Primers used were XFGFR-1 upstream: 5'-TACAACCTATG TGTGCC-3', downstream: 5'-GGAAGCTCAGAGCAAGAAT-3' (PCR product: 539 bp); XFGFR-4 upstream: 5'-GCCTTTGATAT CACCAAG-3', downstream: 5'-AGAGGATGGCACTGGATC-3' (PCR product: 656 bp). Controls verified that the primer sets are specific to each XFGFR subtype. EF-1 α primers are from Wilson and Melton (1994). Because XFGFRs are expressed at relatively constant levels, we used EF-1 α (whose expression is well-characterized) to verify that we would clearly observe changing mRNA levels during development.

Whole-mount in situ hybridization

Whole-mount in situ hybridization of albino embryos was as described by Sive et al. (1999); the substrate was BM Purple (Boehringer-Mannheim, Indianapolis, Ind., USA). Probes were XFGFR1-ec, encoding the extracellular domain of XFGFR-1; XFGFR2-ec, encoding the extracellular domain of XFGFR-2; and F37, a partial (3'-most 2 kb) XFGFR-4 cDNA clone, encoding the transmembrane and intracellular domains of XFGFR-4 and 791 bp of 3' untranslated region (UTR). Some specimens were cleared; some were embedded in paraffin and sectioned.

Results

Isolation and sequence analysis of a *Xenopus* FGF receptor-4 cDNA

Because changes in competence occur during gastrulation (Kengaku and Okamoto 1995; Servetnick and Grainger 1991), we reasoned that such changes might be mediated at least in part, by changes in cell surface receptors expressed at that time. To identify such receptors we screened a stage 11 cDNA library with probes encoding the tyrosine kinase domains of the *Drosophila sevenless* and *Xenopus* FGFR-1 cDNAs. One of the cDNA clones identified, which we describe here, is a member of the FGF receptor family.

The sequence of the FGFR cDNA, which we call XFGFR-4C, includes 350 bases of 5' UTR, a 2484-base coding region, and 791 bases of 3' UTR sequence. The XFGFR-4C sequence is very similar to the XFGFR-4B sequence recently reported by Riou et al. (1996). The

predicted protein encoded by XFGFR-4C differs from that encoded by XFGFR-4B at ten residues. (The differences are: F18L, R34A, S175H, P335T, M408V, I572V, V715G, Y718V, deletion of S207, and insertion of V after amino acid 762. Differences are denoted as changes from the XFGFR-4B sequence.) Both XFGFR-4C and XFGFR-4B are similar to, but distinct from, another *Xenopus* FGFR-4 clone (Shiozaki et al. 1995).

Both XFGFR-1 and XFGFR-4 mRNAs are present in the unfertilized egg (Friesel and Dawid 1991; Musci et al. 1990; Riou et al. 1996; Shiozaki et al. 1995; this report). However, the absence of functional FGFR protein on the oocyte surface (Musci et al. 1990) argues that both XFGFR RNAs are translationally repressed during oogenesis. XFGFR-1 mRNA contains a translational inhibitory element in its 3' UTR (Robbie et al. 1995). The XFGFR-4C 3' UTR sequence shows no obvious similarity to the XFGFR-1 translational inhibitory element. It will be interesting to determine whether the mechanism of translational control is similar for the two XFGFRs.

The intracellular portions of the three known *Xenopus* FGFRs differ, suggesting that the FGFRs generate intracellular signals that differ from one another, at least in subtle ways. For example, of 13 tyrosine residues in the XFGFR-1 intracellular domain, all are found at homologous positions in XFGFR-2, but 3 are missing from XFGFR-4.

Temporal expression of FGFR-4 RNA during development

To determine the temporal expression pattern of XFGFR-4 mRNA, RNA blots were hybridized to an XFGFR-4C probe. Because the probe used recognizes all three XFGFR-4 RNAs described to date, we refer to the expression of XFGFR-4 mRNA; the expression pattern is shown in Fig. 1a. XFGFR-4 RNA is present at stage 2, reflecting a maternal pool of XFGFR-4 mRNA. The level of XFGFR-4 mRNA remains relatively constant through at least the tadpole stage (Fig. 1a, b).

To compare the levels of XFGFR-1 and XFGFR-4 mRNAs we performed RT-PCR analysis using total embryo RNA, simultaneously amplifying XFGFR-1 and XFGFR-4 mRNAs. Figure 1b shows that the two mRNAs are present at roughly equal levels during early development, in agreement with results of RNA blots (data not shown).

Expression of XFGFR mRNAs is independently regulated in animal caps

XFGFR-1 and XFGFR-2 mRNAs are expressed in distinct temporal patterns in cultured animal caps (Friesel and Brown 1992; Friesel and Dawid 1991; Musci et al. 1990; Riou et al. 1996). To determine whether XFGFR-4 expression differs from that of XFGFR-1 and XFGFR-2, we removed animal caps from stage 8 embryos, cultured the caps in vitro either in the presence or absence of exoge-

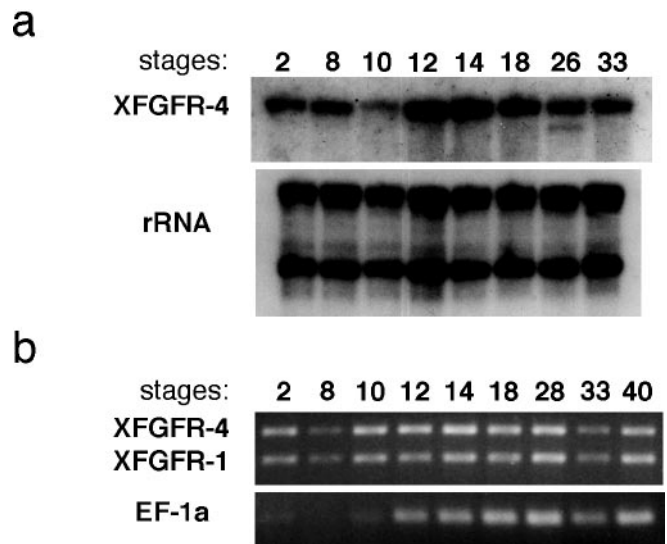


Fig. 1a, b Expression of XFGFR-4 mRNA during development. **a** RNA blot analysis. Two micrograms of whole embryo RNA was loaded per lane (stages are indicated above each lane). The blot was probed for XFGFR-4 (upper panel), then stripped and re-probed for *Xenopus* ribosomal RNA to verify that lanes contain equal amounts of RNA (lower panel). **b** RT-PCR analysis. Whole-embryo RNA was analyzed by RT-PCR (stages of development are indicated above each lane). cDNA was PCR-amplified with primers for both XFGFRs (25 amplification cycles); the same cDNA was amplified separately with EF-1 α primers (23 cycles). EF-1 α is first transcribed at stage 8; the reduced XFGFR RNA levels seen at stage 8 are not observed in all experiments

nous *Xenopus* FGF-2, isolated RNA at various times, and assayed for XFGFR-1 and XFGFR-4 mRNAs by RT-PCR.

The results (Fig. 2a) show that XFGFR-4 mRNA is initially present in stage 8 animal caps, at a level roughly equivalent to that of XFGFR-1 mRNA. In untreated caps over the next 6 h the level of XFGFR-4 mRNA increases, before undergoing a large decrease between 6 and 24 h of culture (observed in four of five experiments). This expression pattern is distinct from that of both XFGFR-1 and XFGFR-2. The expression of XFGFR-1 mRNA, which was coamplified with XFGFR-4, confirms the expression pattern previously described for this mRNA.

In cultured animal caps treated with recombinant *Xenopus* FGF-2 the expression of XFGFR-4 mRNA differs in two ways from that observed in untreated caps (Fig. 2b). First, XFGFR-4 mRNA expression is *maintained* in FGF-treated caps for at least 24 h (three experiments), as is XFGFR-1; second, the increase in XFGFR-4 mRNA observed between 2 h and 6 h does *not* occur in the presence of FGF (three of four experiments).

Spatial expression of XFGFR-4 mRNA

The pattern of XFGFR-4 mRNA expression during development was visualized by whole-mount in situ hybridization (Fig. 3). At stage 10, XFGFR-4 mRNA is widely expressed in the animal cells of the embryo. In

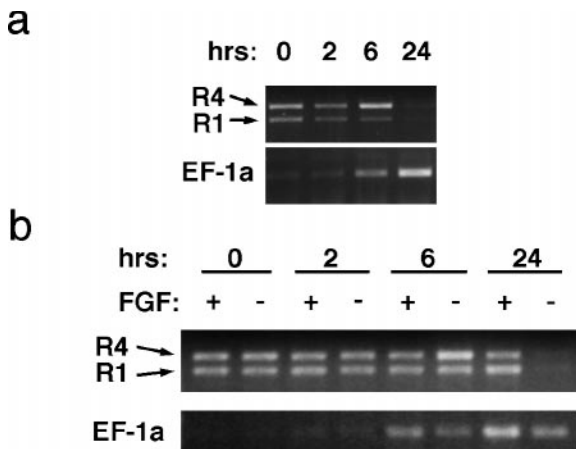


Fig. 2a, b XFGFR-1 and XFGFR-4 mRNA expression in cultured animal caps. **a** Animal caps were removed at stage 8 and cultured in vitro. At the indicated times after isolation, animal caps were frozen. RNA was isolated and analyzed by RT-PCR. cDNA was amplified with primers for both XFGFRs (25 cycles); the same cDNA was separately amplified with EF-1 α primers (28 cycles). **b** Animal caps were removed and cultured in the presence or absence of 50 ng/ml recombinant *Xenopus* FGF-2. RNA was isolated and analyzed by RT-PCR as in **a**. (XFGFRs: 25 cycles, EF-1 α : 25 cycles)

many embryos there is a markedly higher level of expression on the dorsal side of the embryo; one such embryo is shown in Fig. 3a, b. By stage 12, XFGFR-4 mRNA is expressed in an arc located roughly along the anterior border of the forming neural plate (Fig. 3c–e). XFGFR-4 mRNA is notably absent from posterior regions of the embryo at this stage (Fig. 3d). By neural plate stages the XFGFR-4 mRNA expression domain has begun to resolve into two distinct regions of expression, seen in dorsoanterior view (Fig. 3h). Observation of cleared embryos at stages 12–14 indicates that the staining is confined to the ectoderm (not shown). At stage 19 (neural tube closure), XFGFR-4 mRNA is present in several regions of the embryo. In the head, staining still forms an arc about the anterior end of the closed neural tube (Fig. 3k); XFGFR-4 is expressed in the developing eye region, and perhaps in other sense-organ primordia as well. Staining of the closed neural folds is discontinuous, forming a series of stripes of variable widths (Fig. 3k). The neural tube is stained throughout its length (Fig. 3i).

In later neurula stage (stages 22–25) whole mounts, staining is apparent in the developing eyes (Fig. 3n) and throughout the neural tube, with the strongest staining in the anterior (Fig. 3l, m). In strongly stained embryos, staining of the pronephros is visible (see Figs. 3i, l, m, 4a). Variable levels of staining can be observed along the flank of the embryo at these stages (electronic supplement, Fig. 1a).

In sections of stage 22–25 embryos (see electronic supplement, Fig. 1), anterior neural staining is concentrated in the dorsal part of the neural tube. Sectioned embryos also reveal several domains of XFGFR-4 mRNA expression that are not readily apparent from whole

mounts. Staining is observed in head mesenchyme, the forming cement gland, deep ectodermal cells in the region of the otic placode, ventral mesoderm in the region that will give rise to the heart, and lateral mesoderm in the region of the future pronephros.

In tailbud embryos XFGFR-4 mRNA is expressed in the anterior nervous system, the eye, and brain (Fig. 3o, p). Within the brain, a well-defined gap in staining along the anterior-posterior axis (Fig. 3o, p) appears to correspond to the future midbrain. Staining is also seen in both the pronephros and the heart region (also see electronic supplement, Fig. 1b). In stage 40 tadpoles, staining remains quite strong in the brain and eyes (though not in the lens; Fig. 3q, r). Within the brain the unstained region observed at stage 30 is now located just anterior to the midbrain-hindbrain boundary (Fig. 3q, r). A dorsal view of the hindbrain shows a repeating series of stripes within the developing rhombomeres (Fig. 3q). The otic vesicles also stain lightly (Fig. 3q, r).

In summary, XFGFR-4 is expressed in the developing brain and eyes, particularly at the anterior border of the neural plate during neurulation. After neurulation, staining is observed in the developing spinal cord. XFGFR-4 is expressed in the region of the developing heart, the pronephros, the developing otic placode, and later in the otic vesicles.

Expression of FGFR-1 mRNA during development

We next examined the expression pattern of *Xenopus* FGFR-1 mRNA by whole-mount in situ hybridization. At stage 10, the animal cap stains only lightly; this staining is generally even (Fig. 4a), though a slight dorsal bias in staining can be observed in some cleared embryos (not shown). By stage 12, staining is stronger on the dorsal side of the embryo (Fig. 4c). In some embryos, a region of less intense staining, corresponding roughly to the forming notochord, appears along the dorsal midline (Fig. 4b).

By stage 14, there is a distinct contrast between the lightly stained medial cells and more strongly stained lateral cells (Fig. 4d, e). Two observations argue that this pattern is due to staining of paraxial mesoderm. First, a posterior view of cleared whole embryos shows XFGFR-1 staining in paraxial mesoderm (Fig. 5a); the ectoderm and notochord show much less staining in this view. Second, in stained embryos cut to reveal interior tissues, paraxial mesoderm stains much more strongly than ectoderm or endoderm (Fig. 5b). Thus, XFGFR-1 is expressed in paraxial mesoderm, with significantly lower expression in notochord and ectoderm.

By stage 19 (neural tube closure), staining is apparent in the anterior of the embryo (Fig. 4f–h). This staining is somewhat variable among different embryos. In some embryos this expression domain is U-shaped; in others (as in Fig. 4f–h) it is broken up into two stained regions on either side of the midline, with the more anterior of these joined by a narrow strip of staining. The neural

Fig. 3a–r Spatial expression of XFGFR-4 mRNA during *Xenopus* development. **a, b** Stage 10, animal (**a**) and lateral (**b**) views. **a** Dorsal is up. **b** The animal pole is up, dorsal is to the right. This embryo shows pronounced dorsal-ventral asymmetry in XFGFR-4 expression. **c–n** Stained embryos at various stages. Each row shows a dorsal view (anterior at right); lateral view (dorsal up, anterior at right); and dorsoanterior (**e, h, k**) or anterior (**n**) view (dorsal up). **c–e** stage 12½. The yolk plug is indicated by the arrow in **c**. Note the arc of staining in the anterior of the embryo. **f–h** Stage 14. **i–k** Stage 19. Arrow in **i** shows early staining of the pronephric region. **k** A complex series of stripes is present in the anterior neural tube. **l–n** Stage 22–23. Note staining in the neural tube, pronephric region (arrows in **l, m**), developing eyes and cement gland. **o–r** Each row shows a dorsal view of the head, and a lateral view (anterior at right). **o, p** Stage 30 (different individuals). Note the distinct gap in staining along the neural tube in **o**. Arrow (**p**) shows staining in the heart region. **q, r** Stage 40. Note the series of stripes in the hind-brain (**q**). Staining is visible in the eyes (but not lenses) and otic vesicles. Arrow in **r** indicates a lighter region of neural staining dorsal to the eyes

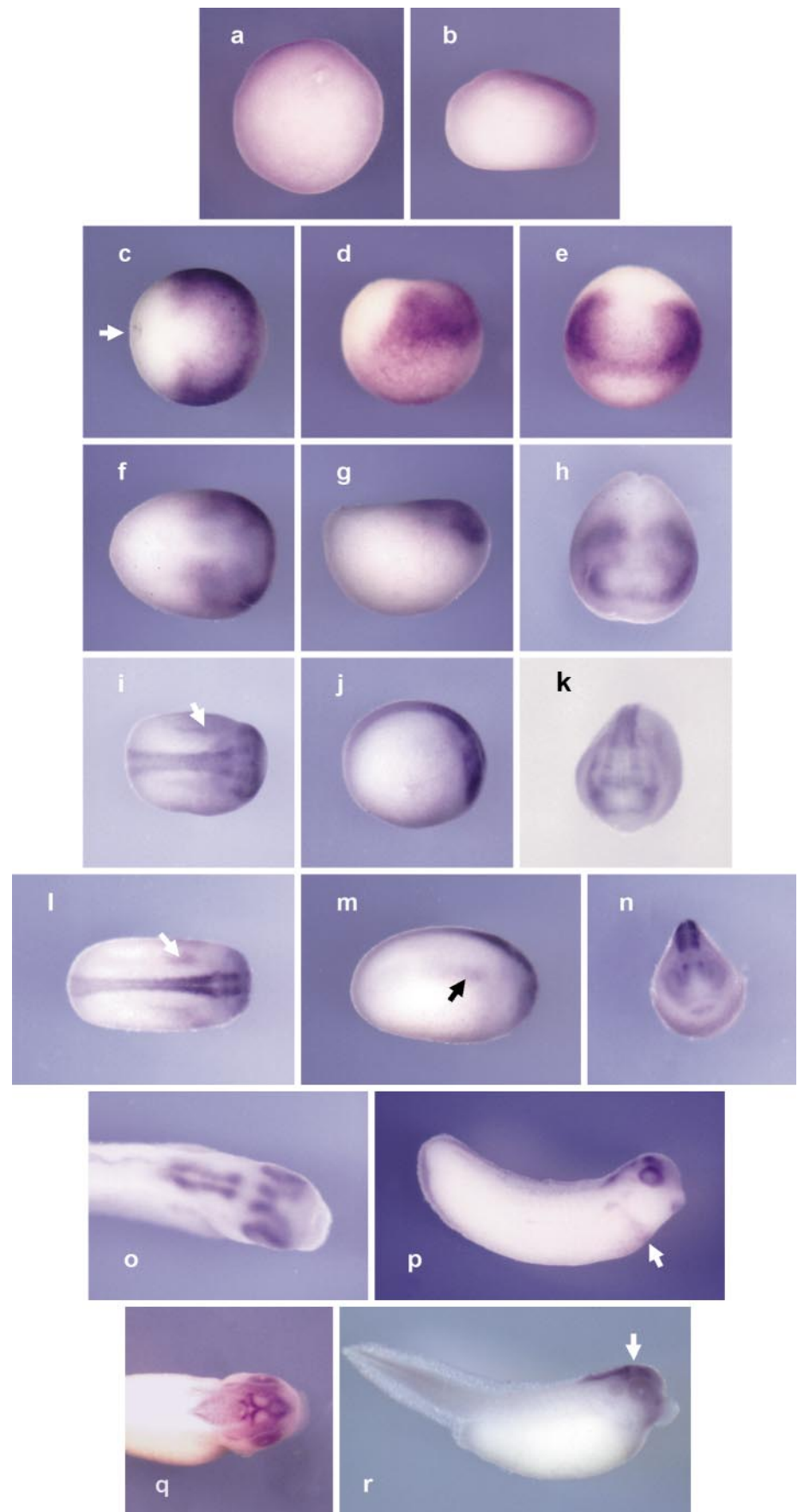
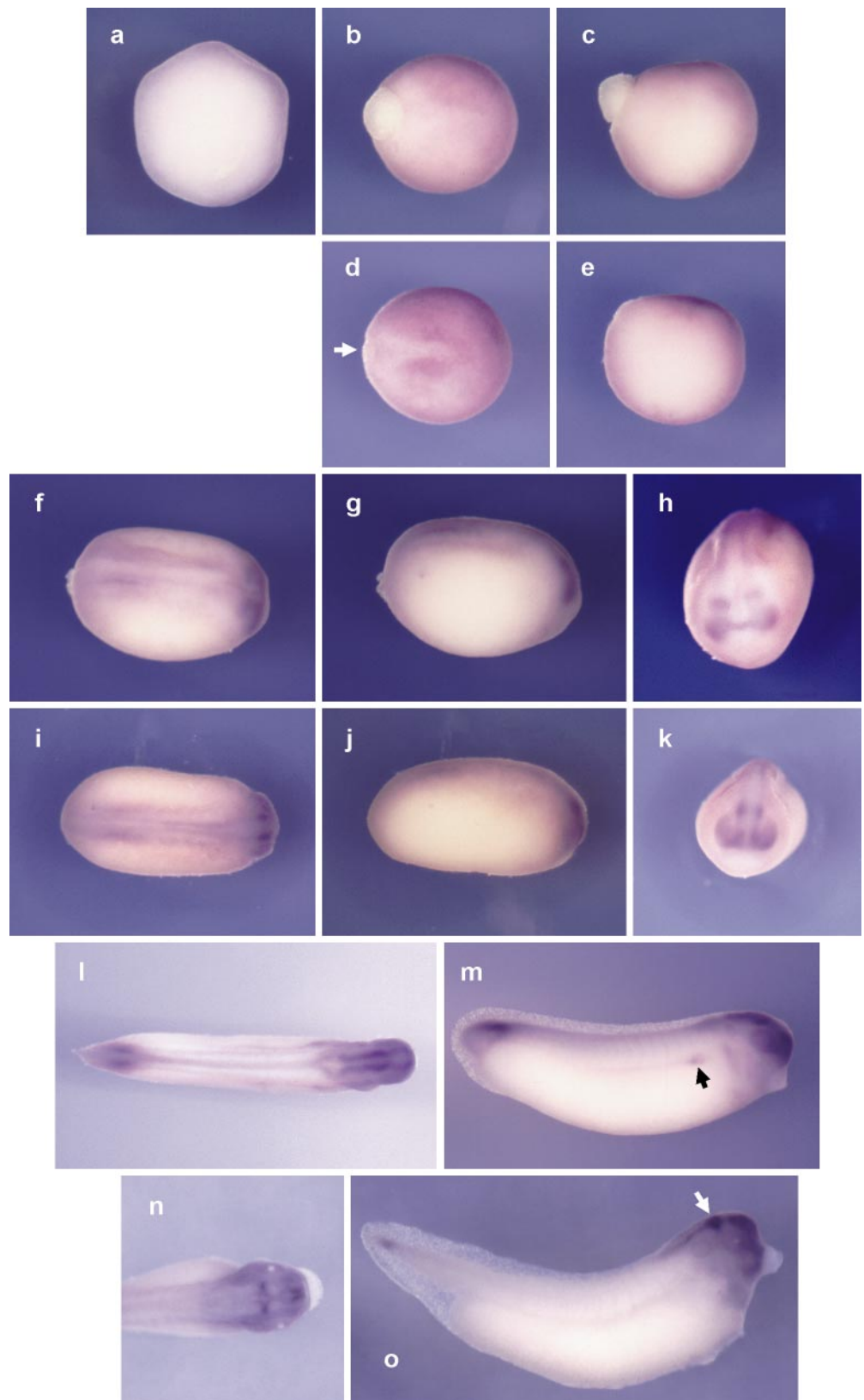


Fig. 4a–o Spatial expression of XFGFR-1 mRNA during *Xenopus* development. **a** Stage 10, animal view. Only light staining is observed. **b,c** Stage 12, dorsal (**b**) and lateral (**c**) views. The prominent yolk plug is at left. Within the dorsal region, staining is weaker along the dorsal midline. **d,e** Stage 14, in dorsal (**d**) and lateral (**e**) view. *Arrow* (**d**) indicates the yolk plug. Staining is much stronger dorsally, though staining along the dorsal midline is weak. **f–k** Stained embryos at various stages. Each row shows a dorsal view, lateral view, and dorsoanterior (**h**) or anterior (**k**) view. **f–h** Stage 19. Strong staining is apparent in anterior neural cells. **h** Staining is seen in two discrete areas (see text for details). Staining is also visible in the posterior paraxial region (**f,g**). **i–k** Stage 23. Staining is similar to stage 19. Anterior staining is in the developing eye. **l,m** Stage 30 embryos (different individuals), in dorsal (**l**) and lateral (**m**) views. Staining is in anterior neural tissue, posterior paraxial tissue, and, weakly, in the pronephric region (*black arrow*). **n,o** Stage 40 embryos (different individuals), in dorsal (**n**) and lateral (**o**) views. Strong staining is seen in a stripe at the midbrain-hindbrain boundary (*white arrow* in **o**). Posterior staining is restricted to the tip of the growing tailbud



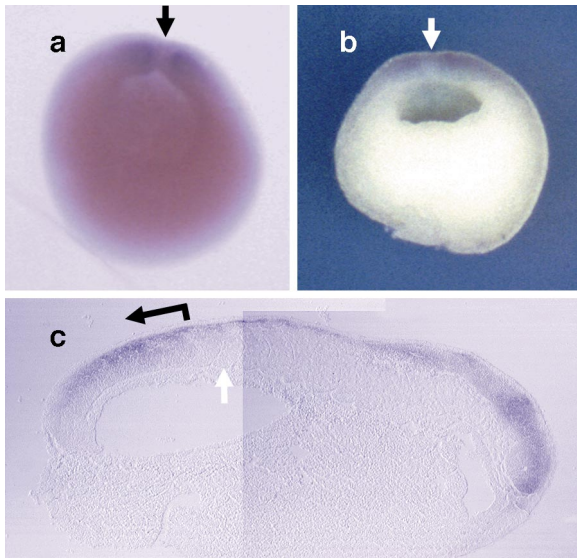


Fig. 5a–c XFGFR-1 mRNA expression in paraxial mesoderm. **a** Posterior view of a whole, cleared stage 14 embryo. Dorsal is up; the archenteron is visible. Paraxial cells stain strongly, but midline, notochord cells (arrow) stain weakly, if at all. Note the sharp border between the staining mesodermal cells and the more superficial ectoderm cells. **b** A stained (uncleared) stage 14 embryo was cut transversely. The staining pattern is similar to that observed in **a**. **c** Parasagittal section through a stage 24 embryo. Anterior is at right. Staining is apparent throughout the neural tube, including the developing eye. White arrow the last formed somite; black arrow presomitic mesoderm. Staining is absent from the formed somites but is strong in presomitic paraxial mesoderm

tube is also lightly stained. Finally, XFGFR-1 is expressed paraxially toward the posterior of the embryo (Fig. 4f, g).

The overall pattern seen at stages 18–19 persists through stages 22–24. Sections of stained embryos at these stages show that the posterior staining is in paraxial mesoderm in the region that has not yet formed definitive somites (Fig. 5c). Comparison of embryos from stages 14 to 30 suggests that XFGFR-1 mRNA is expressed in a wave along the paraxial mesoderm, in the mesoderm just posterior to the formed somites (see Fig. 4d, f, i, l); a similar wave of FGFR-1 mRNA expression has been observed in mouse embryos (Yamaguchi et al. 1992).

In tailbud embryos, staining remains strong in the eyes and brain, with lighter staining of the neural tube. The paraxial staining is now very far posterior, just anterior to the developing tailbud. Staining is also observed in the pronephros (Fig. 4m). At stage 40, there is diffuse staining throughout the head, including the eyes, though staining is absent from the lens (Fig. 4n). In the brain, there is a strong transverse stripe of staining at the midbrain-hindbrain boundary (Fig. 4n, o); some embryos show strong staining along the midline of the hindbrain (not shown). Staining in the posterior of the embryo is restricted to the tip of the developing tail. In overstained specimens pronephric staining is still observed, though it is restricted to the anterior pronephros (not shown).

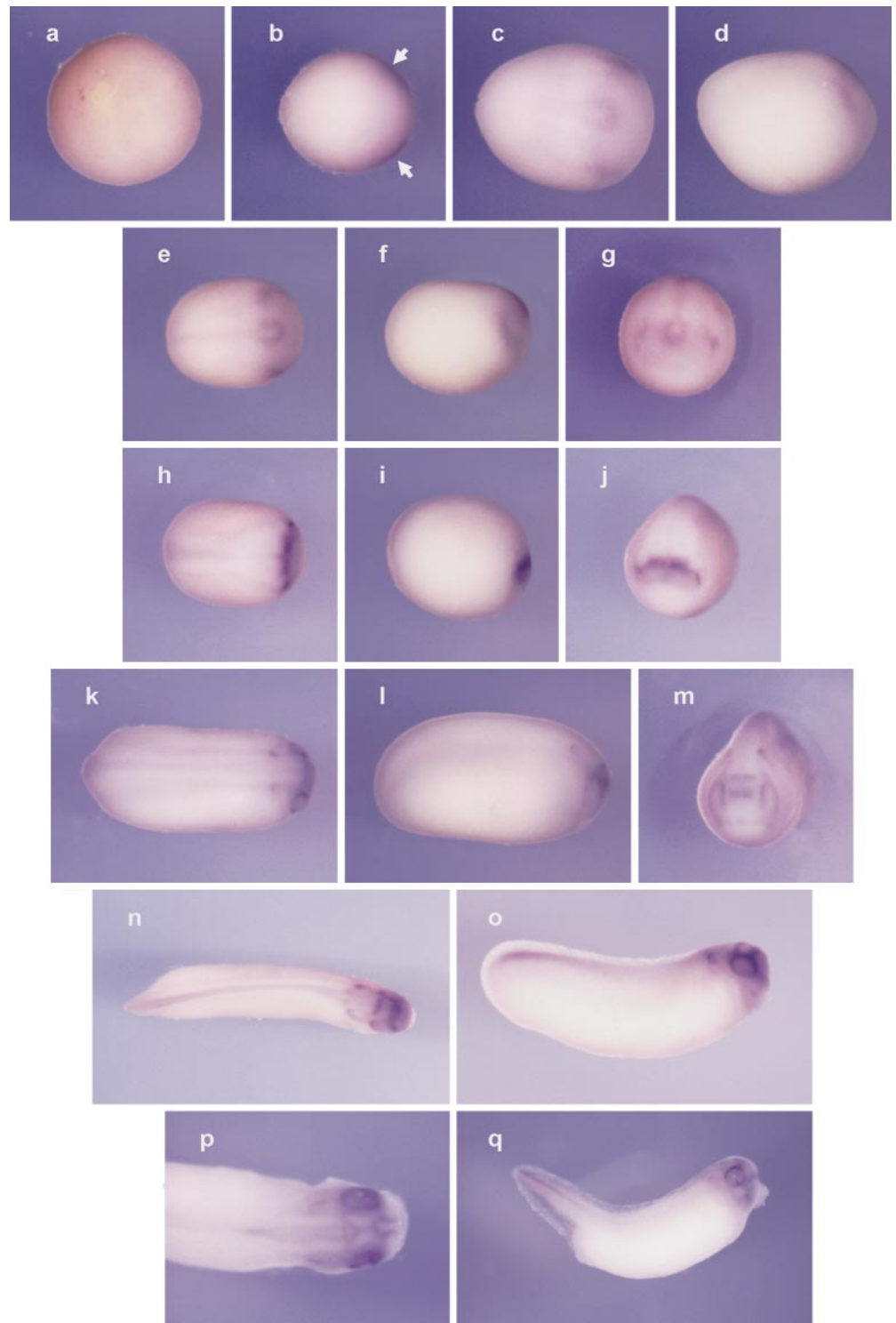
Expression of FGFR-2 mRNA during development

We analyzed FGFR-2 expression by whole-mount in situ hybridization. The probe that we used should hybridize to all XFGFR-2 RNA splicing variants; therefore, as with the other XFGFRs, we refer simply to XFGFR-2 mRNA expression.

We first detect XFGFR-2 mRNA at midgastrula stages, in two areas in the future dorsoanterior region (Fig. 6b). Based on the fate maps of Keller (1975), these regions are just lateral to the eye-forming regions of the anterior neural plate. The stain is somewhat stronger by the early neurula stage (stage 14; Fig. 6c, d), and at about this time staining also appears in the far anterior midline of the neural plate. Observation of cleared embryos indicates that this staining is ectodermal (electronic supplement, Fig. 2). By late neurula (stage 18/19), the staining areas are closely apposed (Fig. 6e–g). After the neural folds have fused, staining forms a line extending from the ectoderm adjacent to the neural plate on one side of the embryo, across the neural plate, and into the ectoderm on the other side (Fig. 6h–j). The line of staining is broader (in the anteroposterior direction) in the neural tube than in the ectoderm. By stages 22–25, this pattern has changed, forming two transverse stripes in the developing brain (Fig. 6m). The stripe observed at stage 19 (Fig. 6j) appears to form the more anterior of the two stripes observed later. The more posterior stripe initially appears, faintly, very close to the first stripe, between stages 19 and 22; the posterior stripe then becomes stronger, and the distance between the two stripes increases. At these stages, staining is also clearly visible in the developing eyes and the otic vesicles (Fig. 6k–m); otic vesicle staining is strongest in the posterior part of the vesicle (Fig. 6k, l, electronic supplement Fig. 2). Weak staining is also observed in the posterior midline.

In tailbud embryos the anterior neural stripes are still apparent (Fig. 6n, o), though the stripes appear to be more posterior than they were earlier, probably due to morphogenetic movements during development. The posterior stripe appears to be at the midbrain-hindbrain boundary, as observed by Friesel and Brown (1992). Staining persists in the eyes, otic vesicles, and in the posterior neural tube, as observed in both whole mounts (Fig. 6n, o; electronic supplement Fig. 2) and sections (not shown). At stage 40 (tadpole), XFGFR-2 staining within the brain (Fig. 6p, q) is broadly similar to the pattern observed for XFGFR-4 at this stage, although XFGFR-2 staining is weaker overall, and there is very little staining in the hindbrain (no XFGFR-2 stripes are seen in the rhombomeres). XFGFR-2 staining is also found in the eyes and, weakly, in the otic vesicles (Fig. 6p, q). Posterior staining at this stage (Fig. 6q) is primarily in the neural tube.

Fig. 6a–q Spatial expression of XFGFR-2 mRNA during *Xenopus* development. **a** Stage 10, animal view. **b** Stage 12, dorsal view. Anterior is at right; the yolk plug is at left. Staining is detected only in two regions at the anterior of the embryo (arrows). **c, d** Stage 14, in dorsoanterior (**c**) and lateral (**d**) views. **e–m** Stained embryos at various stages. Each row shows dorsal view (anterior at right), lateral view, and anterior view. **e–g** Stage 18 (**g** is a different embryo from that in **e, f**). **h–j** Stage 21. **k–m** Stage 23. **n, o** Stage 27, dorsal (**n**) and lateral (**o**) views. **p, q** Stage 40, in dorsal (**p**) and lateral (**q**) views. See text for details



Discussion

We compared the expression patterns of the three *Xenopus* FGFRs identified to date (XFGFR-1, XFGFR-2, and XFGFR-4). Our results show that the XFGFRs are expressed in very different patterns, arguing that different XFGFRs mediate distinct signaling events during *Xenopus*

development. The three XFGFRs are also expressed in different temporal patterns in isolated animal caps, further arguing that the three XFGFR genes are regulated independently of one another. Our analyses of XFGFR expression patterns extend previous studies on XFGFR-4 and XFGFR-2 (Friesel and Brown 1992; Riou et al. 1996); this is the first study that details the spatial expression of XFGFR-1.

Why are we interested in comparing the expression of different FGFRs? Based on current data, invertebrates have a single FGFR gene (in *Drosophila* this gene has recently duplicated), while vertebrates have four FGFRs (Coulier et al. 1997). The expansion of the FGFR family in the vertebrate lineage and the conservation of all four FGFR genes in the vertebrate genome suggest that the four FGFRs play distinct roles during vertebrate development. A first step in analyzing the roles of specific FGFRs is a description of the expression of the different FGFR family members. Additionally, the individual FGFRs may generate intracellular signals that differ from one another, at least in subtle ways. Thus, FGFR diversity may be essential for FGFs to generate diverse effects during development.

We first discuss what the FGFR expression patterns reveal about the potential roles of specific FGFRs during development. We then compare the expression patterns of *Xenopus* FGFRs with FGFR expression patterns in other organisms; this comparison yields insights into the evolution of FGFR expression patterns among vertebrate embryos.

Roles of different FGF receptors during early development

Ectoderm cells and mesoderm induction

The three *Xenopus* FGFRs described here are differentially regulated, as shown by different expression patterns in isolated animal caps and by their distinct spatial expression patterns during development. The animal cap experiments underscore the need for FGFR expression in early development. FGF and Xbra form an autoregulatory loop in early *Xenopus* embryos, maintaining one another's expression (reviewed by Slack et al. 1996). This loop requires that FGF *receptor* expression be maintained as well, and indeed our data show that FGF leads to continued high expression of XFGFR-1 and XFGFR-4 mRNAs (XFGFR-2 is expressed even in untreated animal caps; Friesel and Brown 1992). The temporal expression of XFGFR-1 and XFGFR-4 is consistent with these receptors transducing FGF signals during the period of mesoderm induction. In contrast, XFGFR-2 is expressed at very low levels during blastula stages (Friesel and Brown 1992; our observations).

Neural induction and patterning

Many reports argue that FGFs are involved in the formation of posterior neural tissue in *Xenopus*, either by directly inducing posterior neural tissue from uninduced ectoderm (Kengaku and Okamoto 1993, 1995; Lamb and Harland 1995) or by posteriorizing previously induced anterior neural tissue (Cox and Hemmati-Brivanlou 1995; Holowacz and Sokol 1999; Lamb and Harland 1995). Which FGFRs are involved? The expression pat-

terns suggest that XFGFR-1 and XFGFR-4, which are expressed in animal cells during gastrulation, might mediate FGF signaling during neural induction. However, XFGFR-1 mRNA is found at much lower levels in ectoderm than in mesoderm during neural plate stages (though this does not preclude the possibility that there is sufficient XFGFR-1 in ectoderm at these stages to transduce FGF signals). XFGFR-1 and XFGFR-4 are both expressed at high levels in the anterior neural plate during neurulation; these FGFRs may be involved in patterning the anterior neural plate.

Rhombomeres

XFGFR-4 is expressed in a repeating pattern within the forming rhombomeres (see Fig. 3q). While adjacent rhombomeres are known to interact (for example, Graham and Lumsden 1996), the possible role of FGF signaling between rhombomeres has not been characterized. The striking expression pattern of FGFR-4 in both frog and zebrafish embryos (see below) suggests that FGFs act in signaling between adjacent rhombomeres.

Midbrain-hindbrain boundary

The midbrain-hindbrain boundary (MHB) acts as a signaling center in specifying and patterning brain structures. FGF-8 and Wnt-1, among others, are necessary for MHB function (reviewed by Joyner 1996). FGF-8 is expressed at the MHB in many vertebrates and presumably acts through FGFRs. Our data show that all three XFGFRs are expressed in this region, though the expression patterns differ in detail. A stripe of XFGFR-2 expression can be observed in this region as early as stage 21 (Fig. 6h-j). XFGFR-1 is expressed throughout the anterior CNS by tailbud stages; XFGFR-4, while expressed in anterior neural tissue, is notably absent from the future midbrain. Whether these differences play a role in establishment of the MHB, or in neural patterning in general, remains to be clarified.

Neural crest cells

The expression pattern of XFGFR-4 in early neurulae (Fig. 3c-e) is dramatic, forming an arc at the anterior border of the forming neural plate. This pattern suggests that XFGFR-4 plays a role in developing neural crest cells, which form from this region. XFGFR-4 is also expressed later in head mesenchyme cells, which are derived from cranial neural crest, supporting the idea that XFGFR-4 plays a role in cranial neural crest cell development. Mayor et al. (1997) showed that FGF signaling is required for the expression of *Xslug*, a neural crest cell marker. *Xslug* is normally expressed only in the anterior half of the embryo, with a posterior boundary similar to the posterior boundary of FGFR-4 expression (see

Fig. 3f). *Xslug* is not expressed posteriorly because FGFRs are normally absent from that region; FGFR overexpression causes the *Xslug* expression domain to expand posteriorly (Mayor et al. 1997). The expression of XFGFR-4 in a region that corresponds closely to the future neural crest, together with a demonstrated requirement for FGF signaling in *Xslug* induction, argues strongly that the FGFR normally mediating neural crest induction is FGFR-4. Neither XFGFR-1 nor XFGFR-2 is expressed in the region of the future neural crest.

The evolution of vertebrates included the specialization of head structures; many of these specialized head structures develop from cranial neural crest cells (Gans and Northcutt 1983). We speculate that the appearance of neural crest cells during vertebrate evolution might have, at least initially, involved a specific FGFR, FGFR-4, which is not found in invertebrates. Thus, the appearance of a new cell type, the neural crest, may have occurred concomitantly with the appearance of a new gene function, FGFR-4. The phenotype of FGFR-4 knockouts in frogs or zebrafish, both of which express FGFR-4 in the neural crest region, would be useful in exploring this idea.

Eyes

The three FGFRs examined are all expressed in the developing eye; we did not examine the expression patterns in detail. Since FGF signaling has been implicated in many diverse aspects of eye development (for example, Hyer et al. 1998; Robinson et al. 1995; Schulz et al. 1993), the three FGFRs may, again, be mediating distinct signals during eye development.

Somites

We observe XFGFR-1 expression in unsegmented paraxial mesoderm, just posterior to the newly formed somites. XFGFR-1 is downregulated once somites have formed, though some XFGFR-1 expression is still observed in the somite region. (A similar FGFR-1 expression pattern is observed in mouse embryos; this is discussed in more detail below.) The presence of FGFR1 mRNA in presomitic mesoderm, and its downregulation in newly formed somites, suggests that FGFR-1 may play a role in the formation and patterning of somites in *Xenopus*; a similar role has been proposed in mouse embryos.

Other structures

In addition to the structures described above, FGFRs may be playing roles in other organ primordia. The developing pronephros expresses XFGFR-1 and XFGFR-4, but not XFGFR-2. Heart mesoderm expresses only XFGFR-4, arguing that FGFR-4 plays a role in heart de-

velopment. Finally, XFGFR-4 is expressed in the region of the otic placode and later in the otic vesicle itself. XFGFR-2 is also expressed in the otic vesicle, with stronger expression in the posterior of the vesicle. Thus, XFGFR-4 may be involved early in otic vesicle formation, while XFGFR-2 may act in later patterning of the otic vesicle.

Dominant negative FGFR analysis of *Xenopus* development

Finally, these studies raise questions concerning the use of dominant negative receptors to study FGFR function in the early *Xenopus* embryo. A dominant negative FGFR-1, called XFD, causes severe embryo malformations in lateral and posterior regions, but head development is largely unaffected (Amaya et al. 1991; Kroll and Amaya 1996). If development of XFD-expressing embryos is sufficiently normal to allow high levels of XFGFR expression in the future head, these locally high FGFR levels may render those cells refractory to the effects of the dominant negative construct. That is, in cells that express a high level of endogenous FGFR (e.g., those in the anterior neural plate), there may be a high enough level of normal FGFR that FGF signaling is no longer blocked by XFD, and dorsoanterior development can proceed normally. If so, this would require a reassessment of dominant negative FGFR studies. It will be necessary to determine whether regions of the embryo that develop normally, even after overexpression of XFD, are in fact able to transduce FGF signals.

Comparison of FGFR expression among vertebrate embryos

Here we compare the expression of the *Xenopus* FGFRs with previous reports of FGFR expression in other vertebrate embryos.

FGFR-4

The FGFR-4 subfamily differs in several ways from FGFR-1, FGFR-2, and FGFR-3. First, FGFR-4 is the only FGFR that does not undergo alternative splicing of the third Ig-like extracellular domain (reviewed by Goldfarb 1996). Second, while mutations in FGFR-1, FGFR-2, and FGFR-3 can lead to inherited human skeletal disorders, no such mutations in FGFR-4 have been described (reviewed by De Moerloozee and Dickson 1997). Finally, FGFR-4 sequences have diverged from one another to a greater extent than have members of the other FGFR subfamilies (Coulier et al. 1997). These observations suggest that FGFR-4 plays roles that are distinct from those of the other FGFRs.

FGFR-4 expression has been previously described, at various levels of detail, in mouse, chicken, newt, and ze-

brafish embryos; FGFR-4 expression is strikingly divergent in these organisms. In mice, high levels of FGFR-4 are observed in the developing gut and myotomes; transcripts are absent from the central nervous system (CNS) and heart (Stark et al. 1991). In chicken embryos, FGFR-4 (FREK) mRNA is, again, found in the myotome and skeletal muscle precursors. However, transcripts are not detected in the developing gut (as in mice), and except for early expression in the epiblast during gastrulation, broad CNS expression is not seen (Marcelle et al. 1994). Zebrafish FGFR-4 expression differs from the mouse and the chick, and most closely resembles the expression pattern observed in *Xenopus*. In the pregastrula zebrafish embryo, broad staining is seen in the animal pole; during gastrulation this resolves into two broad stripes in the epiblast (linked by an anterior loop of staining cells). The two stripes of expression are eventually found in the forebrain and hindbrain, with FGFR-4 mRNA expressed in a regular series of stripes in the rhombomeres. FGFR-4 is not expressed in the future midbrain (Thisse et al. 1995). We have observed all these features of FGFR-4 expression in *Xenopus*. There are also differences between the two species: these include FGFR-4 expression in zebrafish in prechordal mesoderm, and at the posterior of each somite. *Xenopus* shows staining in the developing pronephros; this is not observed in the zebrafish. In summary, FGFR-4 mRNAs are expressed strongly in the gut in mice (but none of the other embryos), in muscle precursors in mice and chicks (but neither frogs nor fish), and in the anterior CNS in frogs and fish (but neither mice nor chicks).

While we expected that *Xenopus* and the newt *Pleurodeles* would show similar FGFR-4 expression patterns, there are differences even between these amphibians. FGFR-4 mRNA is expressed maternally in *Xenopus*, while *Pleurodeles* PFR4 is not expressed until the midblastula stage (Shi et al. 1992). This suggests that PFR-4 is not involved in mesoderm induction in *Pleurodeles*, and, by extension, XFGFR-4 may not be involved in this process in *Xenopus* embryos either.

The distinct patterns of expression of FGFR-4 mRNAs in different vertebrate embryos, together with the relatively high divergence of FGFR-4 sequences (compared to the other FGFRs), argue that FGFR-4 genes may play different roles in development in different taxa. In particular, in frogs and fish, FGFR-4 may act in signaling between rhombomeres, and in induction of the neural crest. Because FGFR-4 expression is different in mice and chicks, FGFR-4 is unlikely to serve similar roles in these vertebrates. The generation of a specific FGFR-4 knockout in *Xenopus* or in zebrafish is likely to shed light on these processes.

FGFR-1

During gastrulation, XFGFR-1 mRNA is expressed predominantly in future paraxial mesoderm; after gastrulation, XFGFR-1 is also expressed in the anterior neural plate. A comparison of FGFR-1 mRNA expression in

mouse (Orr-Urtreger et al. 1991; Yamaguchi et al. 1992) and *Xenopus* embryos reveals a striking similarity between the two species.

In the mouse, at headfold stages, FGFR-1 is expressed in dorsal mesoderm, in a medial to lateral gradient, but is absent from midline cells; this pattern is remarkably similar to the pattern that we observe in late gastrula *Xenopus* embryos (compare our Fig. 5d with Fig. 3d of Yamaguchi et al. 1992). During neurula stages, XFGFR-1 begins to be expressed in the anterior neural plate. Mouse FGFR-1 is also expressed in the future neural plate during headfold stages. As development progresses, mouse FGFR-1 is found at the highest levels in the forebrain (Yamaguchi et al. 1992), as in *Xenopus*. Interestingly, mouse FGFR-1 is expressed strongly in the kidney; we observe FGFR-1 expression in the pronephros.

Finally, in *Xenopus* we observe FGFR-1 mRNA expression in the paraxial mesoderm, just caudal to the formed somites (see Fig. 5c). Mouse FGFR-1 is also expressed in paraxial mesoderm just posterior to the last-forming somite, marking paraxial mesoderm that is condensing into definitive somites (Orr-Urtreger et al. 1991; Yamaguchi et al. 1992). If FGFR-1 is expressed in paraxial mesoderm just prior to the demarcation of somites, there should be a wave of FGFR-1 expression in *Xenopus* embryos, from anterior to posterior, corresponding to the wave of somite formation (Hamilton 1969). While our observations are consistent with such a wave of expression, because *Xenopus* embryos undergo considerable morphogenetic movement during this period, more extensive analysis is needed to verify whether XFGFR-1 is expressed in an anterior to posterior wave.

In summary, the early expression patterns of FGFR-1 mRNA in *Xenopus* and mouse embryos share many common features. The concordance of these patterns argues strongly that FGFR-1 has retained similar functions during the development of these two vertebrates.

FGFR-2

The patterns of XFGFR-2 mRNA expression described here extend the observations of Friesel and Brown (1992) in *Xenopus*, and Shi et al. (1994) in the newt *Pleurodeles*. In *Pleurodeles* FGFR-2 expression was not detected at neurula stages, perhaps because short exon-specific probes were used; in *Xenopus* we and Friesel and Brown (1992) clearly observe expression at neurula stages. In both organisms, tailbud embryos show strong staining in the head. In *Xenopus* this staining appears to be confined to neural tissue, the eye, and otic vesicle; in *Pleurodeles* expression is also observed in the pharynx and dorsal dermis (Shi et al. 1994).

In mouse embryos many regions of FGFR-2 expression correspond to those observed in *Xenopus*. Mouse FGFR-2 mRNA is expressed at various sites, notably in anterior neural tissue: at 6–7 days in primitive ectoderm/neural plate, at 8 days in the cranial folds

(anterior neural plate), and at 9 days in the cranial and dorsal neural tube (Orr-Urtreger et al. 1991). In mice FGFR-2 mRNA is also expressed in the developing eye and otic vesicle (Orr-Urtreger et al. 1991). We observe XFGFR-2 expression in these domains – anterior neural plate, eye, and otic vesicle – in *Xenopus*. However, additional sites of expression are observed in mouse embryos, including, at various stages, surface ectoderm, anterior body wall, somites, heart primordium, lateral and splanchnic mesoderm, and nephrogenic cords. At later stages mouse FGFR-2 is expressed in various epithelia and skin derivatives (Orr-Urtreger et al. 1991, 1993). We have not observed FGFR-2 expression in *Xenopus* at these sites.

These observations argue that FGFR-2 is expressed more broadly in the mouse than in *Xenopus*. There are common domains of expression (anterior neural tissue, the developing eye and ear), and there are domains in which FGFR-2 is expressed in mice, but not in *Xenopus*.

Conclusions

Our data show that FGFR-1, FGFR-2, and FGFR-4 mRNAs are expressed in distinct patterns in the *Xenopus* embryo. These data extend earlier observations that FGFR subtypes are differentially expressed in chicken, mouse, and newt embryos. The expression patterns suggest, among other things, that XFGFR-4 plays roles in neural crest cell development and rhombomere formation, and that XFGFR-1 is involved in somitogenesis. By comparing the expression patterns of FGFR subtypes among different vertebrate embryos we conclude that the expression pattern of FGFR-1 is highly conserved between mouse and *Xenopus* embryos; FGFR-2 expression also shows significant conservation, though it is more widely expressed in mice than in frogs. In contrast, FGFR-4 expression is quite divergent among vertebrate embryos. These conclusions bear on the roles played by FGFR subtypes during vertebrate evolution, and suggest that FGFR-1 and FGFR-2 play similar roles in different taxa, while FGFR-4 plays different roles in different taxa.

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