Induction in Xenopus

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We previously showed that FGF was capable of inducing Xenopus gastrula ectoderm cells in culture to express position-specific neural markers along the anteroposterior axis in a dose-dependent manner. However, conflicting results have been obtained concerning involvement of FGF signaling in the anterior neural induction in vivo using the same dominant-negative construct of Xenopus FGF receptor type-1 (ΔXFGFR-1 or XFD). We explored this issue by employing a similar construct of receptor type-4a (XFGFR-4a) in addition, since expression of XFGFR-4a was seen to peak between gastrula and neurula stages, when the neural induction and patterning take place, whereas expression of XFGFR-1 had not a distinct peak during that period. Further, these two FGFRs are most distantly related in amino acid sequence in the Xenopus FGFR family. When we injected mRNA of a dominant-negative version of XFGFR-4a (ΔXFGFR-4a) into eight animal pole blastomeres at 32-cell stage, anterior defects including loss of normal structure in telencephalon and eye regions became prominent as examined morphologically or by in situ hybridization. Overexpression of ΔXFGFR-1 appeared far less effective than that of ΔXFGFR-4a. Requirement of FGF signaling in ectoderm for anterior neural development was further confirmed in culture: when ectoderm cells that were overexpressing ΔXFGFR-4a were cocultured with intact organizer cells from either early or late gastrula embryos, expression of anterior and posterior neural markers was inhibited, respectively.

We also showed that autonomous neuralization of the anterior-type observed in ectoderm cells that were subjected to prolonged dissociation was strongly suppressed by ΔXFGFR-4a, but not as much by ΔXFGFR-1. It is thus indicated that FGF signaling in ectoderm, mainly through XFGFR-4a, is required for the anterior neural induction by organizer. We may reconcile our data to the current “neural default model,” which features the central roles of BMP4 signaling in ectoderm and BMP4 antagonists from organizer, simply postulating that the neural default pathway in ectoderm includes constitutive FGF signaling step.

Key Words: neural induction; organizer; FGF receptor; dominant-negative receptor; Xenopus.

INTRODUCTION

Development of the vertebrate nervous system is initiated at the gastrula stage by the inductive action of the presumptive dorsal mesoderm (dorsal marginal zone or Spemann's organizer in amphibians; Spemann and Mangold, 1924) on neighboring dorsal ectoderm. During the subsequent neurula stage, the affected dorsal ectoderm begins to differentiate into neural tissue that is well patterned along the anteroposterior axis under the inductive influence of the dorsal mesoderm derivatives. It has long been believed that the Spemann's organizer and its derivatives secrete neural inducing molecules during gastrula and subsequent neurula stages, but the chemical nature of these molecules has not long been clear until quite recently.

Recent work using Xenopus, however, has identified a number of promising candidates for the neural inducers (reviewed by Wilson and Hemmati-Brivanlou, 1997). These include at least six proteins: noggin (Lamb et al., 1993), FGF (Kengaku and Okamoto, 1993), follistatin (Hemmati-Brivanlou et al., 1994), chordin (Sasai et al., 1995), cerberus (Bouwmeester et al., 1996), and Xnr3 (Hansen et al., 1997).
At least three of them, noggin, chordin, and cerberus, are shown to directly bind BMP4, preventing it from activating its receptor (Piccolo et al., 1996, 1999; Zimmerman et al., 1996). Since local BMP4 signaling appears to induce ectoderm cells to form epidermis and suppress neuralization, noggin and chordin might exert their effect by antagonizing the neural-inhibitory action of BMP4 (Wilson and Hemmati-Brivanlou, 1995, 1997).

Neural tissue induced from ectoderm by all the candidate molecules except for FGF appears to have solely the anterior characteristics, but not posterior characteristics. The mode of action of FGF seems to be unique in this respect. FGF can induce ectoderm cells to express not only the anterior but also posterior neural character, and it does so in a dose-dependent manner; with lower doses eliciting more anterior neural marker genes and higher doses more posterior neural marker genes (Kengaku and Okamoto, 1995). There are some other lines of evidence indicating that FGF is a potent posteriorizing factor (Amaya et al., 1993; Isaacs et al., 1994; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Pownall et al., 1996; Holowacz and Sokol, 1999). However, conflicting results have been obtained so far concerning the contribution of FGF signaling to the anterior neural induction. Overexpression of a dominant-negative version of FGF receptor in the ectoderm with synthetic mRNA for the mutant receptor blocked neural induction by noggin and organizer tissues (Launay et al., 1996; Zimmerman et al., 1996). Since local BMP4 signaling appears to induce ectoderm cells to form epidermis and suppress neuralization, noggin and chordin might exert their effect by antagonizing the neural-inhibitory action of BMP4 (Wilson and Hemmati-Brivanlou, 1995, 1997).

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In the present studies, we have studied the spatiotemporal expression pattern of transcripts of five members of XFGFR to explore which type of FGF receptor is working at the right time and place during development to participate in neural induction and patterning. These members include two novel members (XFGFR-3 and XFGFR-4b) in addition to the three previously reported ones (XFGFR-1, -2, and -4a).

It is found that the transcripts of XFGFR-2, -4a, and -4b peak between the gastrula and neurula stages and these transcripts are relatively abundant in the ectoderm compared to the marginal zone at the gastrula stage. When we microinjected animal pole blastomeres of early embryos with mRNA encoding a dominant-negative form of XFGFR-4a that is most divergent from XFGFR-1 in amino acid sequence, development of anterior neural tissue was disrupted in vivo and in vitro. Autonomous neuralization in dissociated ectoderm cells was also suppressed by overexpression of the dominant-negative XFGFR-4a (ΔXFGFR-4a). XFD (ΔXFGFR-1) appeared to far less effectively block the anterior neural induction and autonomous neuralization than ΔXFGFR-4a.

MATERIALS AND METHODS

Animal Care

Methods for keeping frogs and for obtaining embryos have been described previously (Mitani and Okamoto, 1989).

Cloning of XFGFR cDNAs from Xenopus Library

An oligo(dT)-primed Xenopus gastrula stage cDNA library was constructed in λgt 10 (Promega). Probes for FGF receptors in screening the library were prepared using polymerase chain reaction (PCR) that was performed on Xenopus gastrula stage cDNA. A pair of degenerate deoxynucleotidetriphosphor primers were designed in conserved regions of the intracellular tyrosine kinase domains (I and II) of previously reported XFGFR-1 (Musci et al., 1990; Friesel and Dawid, 1991) and XFGFR-2 (Gillespie et al., 1989; Friesel and Brown, 1992); U, 5'-GA(A/G)G(T/G)GTG(CT)TT(T/C)GG(AGCT)CA(AG)GT-3' and D, 5'-GGIGCCATCCA(T/C)TT(AG)ACATGC(G)3'. Cloning of the PCR products yielded four types of closely related cDNAs that were used for screening of about 5 × 10^6 recombinant phage.

Sequence Analysis

DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) with Sequenase Version 2 (United States Biochemicals Corp.) according to the manufacturer's recommendations. The sequence homology was analyzed by consulting the GenBank/EMBL/DDJ database.

Whole-Mount in Situ Hybridization

In situ hybridization experiments were performed following the methods described by Harland (1991) and Sive et al. (1995). Digoxigenin-labeled sense and antisense RNAs were prepared by in vitro transcription of respective plasmids that were linearized. Hybridized digoxigenin-containing RNAs were visualized with anti-digoxigenin antibodies conjugated to alkaline phosphatase. To detect XFGFR transcripts, albino embryos were used and hybridization probes were prepared from the extracellular and transmembrane domains. To detect marker transcripts in embryos overexpressing ΔXFGFR-4a, pigmented embryos were used for the ease of...
injection and staging. The markers used in this experiment were Nrp-1 (Richter et al., 1990), BF-1 (Bourguignon et al., 1998), Rx-1 (Mathers et al., 1997), and En-2 (Hemmati-Brivanlou et al., 1991).

**RNase Protection Analysis**

Total RNA was extracted from embryos and larvae at various developmental stages by the proteinase K method (Sambrook et al., 1989). RNase protection assay was performed using RPAII (Ambion). Hybridization probes were prepared from the divergent 5’ ends of XFGFR cDNAs (Fig. 4). The ornithine decarboxylase (ODC) sequence was used as probe for the internal standard (Isaacs et al., 1992). Each cDNA fragment from XFGFRs was subcloned into pBluescriptII SK+ to direct the transcription of antisense transcripts using T3 or T7 RNA polymerase (Stratagene) in the presence of [α-32P]UTP (800 Ci/mmol, ICN Biomedicals Inc., Costa Mesa, CA). Hybridization were carried out at 47°C for 16 h according to RPAII protocol. The samples were digested by 0.5 mg/ml RNase A and 10,000 units/ml RNase T1 at 30°C for 1h. Following RNase inactivation and ethanol precipitation, the protected fragments were separated on a 4% polyacrylamide gel and radioactivity of each of the protected fragments were estimated using a laser image analyzer (Fujix BAS 2000, Fuji Film).

**Quantitative RT-PCR Assay**

Total RNA was extracted from each of four dissected pieces (40 fragments each) from ectoderm, dorsal marginal zone, ventral marginal zone, and endoderm as shown in Fig. 6A) of gastrula at stage 10.5 (Nieuwkoop and Faber, 1967) by the proteinase K method. These RNA samples were subjected to reverse transcription with oligo(dT) 12-18 as primers. For quantitative analysis, PCR was performed as described (Kinosita et al., 1992; Kengaku and Okamoto, 1995) with slight modifications. In brief, 1-100 to 1/10 of the reverse transcribed mixture was used as template DNA, which was amplified in a reaction volume of 15 μl with 3 μl of [α-32P]CTP (ICN Biomedicals Inc.) and 1.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification was initiated with the particular primers for each XFGFR sequence of interest (Table 1). The primers for the internal control, elongation factor 1α (EF1α; Krieg et al., 1989) sequence, were added after the first six to eight cycles to avoid the possible interference of large amounts of its PCR product with the amplification efficiency of the sequence of interest. For quantitative assessment, the PCR products were analyzed within the exponential phase of amplification and were standardized against a coamplified internal control, the EF1α. After the completion of the amplification, PCR products were separated on a 4% polyacrylamide gel and radioactivity of each of the PCR products were estimated using a laser image analyzer (Fujix BAS 2000, Fuji Film).

**Microinjection of mRNA of Truncated XFGFRs**

A truncated form of XFGFR-4a cDNA (ΔXFGFR-4a) or XFGFR-1 (ΔXFGFR-1) was constructed in a similar manner employed for the construction of a truncated human FGFR1 cDNA (Ueno et al., 1992). Each cDNA fragment, that encodes the full extracellular and transmembrane regions and 21 or 22 amino acids in the cytoplasmic region was finally subcloned into pSP64T (Krieg and Melton, 1984). Capped synthetic mRNA for microinjections was made in vitro using the Megascript message Machine (Ambion). For template preparation, ΔXFGFR-4a-pSP64T and ΔXFGFR-1-pSP64T were linearized with Sall and BamHI, respectively. Synthesized mRNA was purified with Dynabeads oligo(dT)25 (Dynal) according to the manufacturer’s recommendations, and its quantity was determined by comparison with a mRNA of known concentration on a denaturing 1% agarose gel. The quality of mRNAs was analyzed by in vitro translation using Flexi rabbit reticulocyte lysate system (Promega). Each mRNA yielded a protein of expected size with the same translation efficiency, when determined by SDS-PAGE.

ΔXFGFR-4a mRNA was injected into the four animal blastomeres at the 8-cell stage or eight animal blastomeres at the 32-cell stage in 0.5× modified Barth solution (MBS; Gurdon, 1977) plus 3% Ficol. Each blastomere received 4 nl of a solution containing 80 to 300 pg of mRNA. Injected embryos were maintained in the same medium for 2 h at 15°C and then transferred to 0.05× MBS plus 3% Ficol. After an additional 1 h at 15°C, the embryos were transferred to fresh 0.05× MBS plus 3% Ficol and the temperature of incubation was gradually increased to 23°C. Embryos were cultured to the appropriate stage and then operated for further microculture, fixed in 4% formaldehyde/10% methanol solution, or processed for in situ hybridization.

**Microculture of Xenopus Embryonic Cells**

Gastrula embryos of Xenopus laevis (stage 10–11.5; Nieuwkoop and Faber, 1967) that were injected or uninjected with ΔXFGFR-4a mRNA at the eight-cell stage were used. Methods for culturing early gastrula cells were essentially as described previously (Mitani and Okamoto, 1989, 1991). Animal cap fragments were dissected from injected or uninjected embryos (stage 10), whereas dorsal marginal zone (DMZ or organizer) fragments were dissected solely from uninjected embryos (stage 10/4 or 11.5). These fragments were dissociated by incubating in Ca2+-, Mg2+-deficient MBS containing 1% BSA at room temperature. The dispersed cells were then suspended in standard MBS containing 1% BSA and the desired number of cells from each gastrula regions were inoculated separately or in combination (coculture) into plastic culture wells of Terasaki plates (Nunc) (160 ectoderm cells/well and 0 to 80 DMZ cells/well). After completion of reaggregation by brief centrifugation, cells were incubated at 22.5°C in humidified air until control embryos reached tailbud stage (stage 25; Nieuwkoop and Faber, 1967). In some experiments, the dispersed cells were further subjected to prolonged dissociation before inoculation and reaggregation. Some ectoderm cell cultures were done in the presence of recombinant bovine bFGF (Progen Biotechnik, Heidelberg, Germany). RNA was extracted from 20 cultures for each experimental set and subjected to quantitative RT-PCR assay as described above. Amplification was initiated with the primers for the marker (BF-1, Rx-1, En-2, Xenk2, Krox-20, Xihbox1, Xhhbox6, Xcad-3, NCAM, Nrp-1, epidermal keratin, and XAG-1) listed in Table 1, expect for the case of XAG-1, where XAG-1 primers were added after the first cycle of amplification of EF1α sequence.

**RESULTS**

**Sequence Analysis of Five XFGFRs**

PCRs were performed on cDNA synthesized from Xenopus gastrula stage mRNA using primers corresponding to conserved regions in the intracellular kinase domains (I and II) of previously reported XFGFR-1 (Musci et al., 1990;
TABLE 1
Oligonucleotide Primers used for Quantitative RT-PCR Assay

<table>
<thead>
<tr>
<th>Markers (Refs.)</th>
<th>Sequences</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XFGFR-1 (Friesel and Dawid, 1991)</td>
<td>F 5'-AAATGGAGCCACATTATCCGCTC-3'</td>
<td>286</td>
</tr>
<tr>
<td>XFGFR-2 (Friesel and Brown, 1992)</td>
<td>F 5'-TGGATGGAGCTGCTGACAGC-3'</td>
<td>295</td>
</tr>
<tr>
<td>XFGFR-3 (this report)</td>
<td>F 5'-AGAAGGGAGATCTTTCAGTTCC-3'</td>
<td>308</td>
</tr>
<tr>
<td>XFGFR-4α (this report)</td>
<td>F 5'-CAAAGTGAAGGACCTGTTC-3'</td>
<td>303</td>
</tr>
<tr>
<td>XFGFR-4β (this report)</td>
<td>F 5'-CTACCACACCTCCTGACCTC-3'</td>
<td>326</td>
</tr>
<tr>
<td>BF-1 (Bourguignon et al., 1998)</td>
<td>F 5'-TACGGGGCCATCCTCATC-3'</td>
<td>269</td>
</tr>
<tr>
<td>Rx-1 (Mathers et al., 1997)</td>
<td>F 5'-AGAAGGGAGATCTTTCAGTTCC-3'</td>
<td>260</td>
</tr>
<tr>
<td>XeNK-2 (Saha et al., 1993)</td>
<td>F 5'-AGAAACAGCAACGAACTC-3'</td>
<td>301</td>
</tr>
<tr>
<td>En-2 (Hemmati-Brivanlou et al., 1991)</td>
<td>F 5'-ATAACAGGGAAGTTGGAACCC-3'</td>
<td>272</td>
</tr>
<tr>
<td>Krox-20 (Hemmati-Brivanlou et al., 1994)</td>
<td>F 5'-GACTTCTGGAAGGACCTCTTG-3'</td>
<td>448</td>
</tr>
<tr>
<td>Xcad-3 (Nothrop and Kimelman, 1994)</td>
<td>F 5'-GACTTCTGGAAGGACCTCTTG-3'</td>
<td>269</td>
</tr>
<tr>
<td>XHbox1 (Oliver et al., 1988)</td>
<td>F 5'-GCCAGCTTCCAATGCTGAAATGC-3'</td>
<td>291</td>
</tr>
<tr>
<td>XHbox6 (Wright et al., 1990)</td>
<td>F 5'-CCAATTCCTCCTGAGCTTC-3'</td>
<td>274</td>
</tr>
<tr>
<td>NCAM (Hemmati-Brivanlou and Melton, 1994)</td>
<td>F 5'-CAGAGGACACACGAACTC-3'</td>
<td>344</td>
</tr>
<tr>
<td>Nrp-1 (Richter et al., 1990)</td>
<td>F 5'-TTGAGATGCTGCTGAAATGC-3'</td>
<td>287 (Nrp-1A)</td>
</tr>
<tr>
<td>Epidermal keratin (Jonas et al., 1989)</td>
<td>R 5'-TTTGGCTGCTGCTGAAATGC-3'</td>
<td>291</td>
</tr>
<tr>
<td>XAG-1 (Blitz and Cho, 1995)</td>
<td>F 5'-CTACTGGACAGTGCATG-3'</td>
<td>208</td>
</tr>
</tbody>
</table>
| EF1α (Krieg et al., 1989; Hemmati-Brivanlou and Melton, 1994) | F 5'-GAGACTGCTGCTGAAATGC-3' | 269

a For XAG-1 coamplification.

b For other genes coamplification.

Friesel and Dawid, 1991) and XFGFR-2 (Gillespie et al., 1989; Friesel and Brown, 1992). Cloning of the PCR products yielded four types of closely related cDNAs. Nucleotide sequence analysis indicated that three of them were derived from previously reported Xenopus FGF receptors, XFGFR-1, -2, and -4 (Shiozaki et al., 1995), but the other one had a novel sequence. Using these four types of cDNAs as probes, we screened the cDNA library constructed from Xenopus gastrula stage mRNA and obtained five types of closely related cDNA clones. Three of them had already been reported but the other two were not known. The deduced amino acid sequences of these two novel cDNA clones (XFGFR-3 and -4b, respectively, for designation see below) are aligned with XFGFR-1, -2, and -4a (originally XFGFR-4 in Shiozaki et al., 1995) in Fig. 1. The XFGFR-3 and -4b have several features in common with previously reported members of the Xenopus FGFR family. They appear to have a signal peptide sequence and an extracellular domain that consists of three immunoglobulin-like domains and includes a region called the acid box (a stretch of 7 to 12 acidic amino acids) between first and second immunoglobulin-like domains. A hydrophobic transmembrane region joins the extracellular domain to the intracellular tyrosine kinase domains I and II that are highly homologous to the corresponding domains of XFGFR-1, -2, and -4a.
The deduced amino acid sequences of five cDNA clones differ considerably in the extracellular domain, especially near the N-terminal region that includes the signal peptide sequence, immunoglobulin-like domain I, and acid box. The only exceptions are XFGFR-4a and XFGFR-4b, which differ just 6% both in the whole nucleotide sequence and amino acid sequence. It seems likely that these two cDNAs represent divergent copies of different FGFR-4 genes present in the pseudo-tetraploid genome of X. laevis (Graf and Kobel, 1991). The main difference between XFGFR-4a and -4b lies in the acid box region. The acid box of the XFGFR-4a consists of four aspartic acids plus three glutamic acids (DEEDDED), whereas that of the XFGFR-4b consists of five aspartic acids plus seven glutamic acids (DEEEEEEDDED).

The deduced amino acid sequence of XFGFR-3 has the highest homology to human FGFR-3 among the four types of FGFR defined in this species (Fig. 2A; Johnson et al., 1990). It is also most homologous to FGFR-3 of Pleurodeles waltl (PFR-3), when compared with the other three FGFR members: 64% amino acid identity to PFR-3 versus 60% to PFR-1, 59% PFR-2, and 57% to PFR-4 (Shi et al., 1992, 1994a,b). We conclude that XFGFR-3 encodes a Xenopus homologue of FGFR-3 cloned from other species. In Fig. 2B, the match between amino acid sequences of five types of FGFRs of X. laevis is displayed as a dendrogram to show the evolutionary relatedness between them. It should be noted that the divergence is most prominent between XFGFR-1/2 and XFGFR-4a/4b.

Differential Spatiotemporal Expression Patterns of XFGFR Transcripts during Embryogenesis

Expression patterns of XFGFR transcripts were examined by in situ hybridization to whole embryos with digoxigenin-labeled RNA probe. In Fig. 3, spatial distributions of XFGFR transcripts are compared at stage 19, when differential expression patterns of these transcripts became evident. Expression of XFGFR-1 is seen broadly in the dorsal region all along the anteroposterior axis, which appears to include the whole neural and some mesodermal tissues (Fig. 3A). Other types of XFGFR mRNA are expressed in more discrete areas of neural tissue, each characteristically: XFGFR-2 mRNA in the forebrain and the midbrain–hindbrain boundary (Fig. 3B), as previously reported (Friesel and Brown 1992); XFGFR-3 mRNA in the forebrain and the hindbrain (Fig. 3C); and XFGFR-4a (and -4b) mRNA in the forebrain through the spinal cord and the head neural crest (Fig. 3D). Similar hybridization analysis with sense strand RNAs were negative for all XFGFRs (not shown).

We next explored which type of XFGFR is expressed at the right time and place during development for the neural induction and patterning. At gastrula stages, XFGFR transcripts were detected only in the ectoderm region (not shown). Since the in situ procedure fails to show up the presence of transcripts in vegetal cells of gastrulae or endodermal cells of neurulae (Frank and Harland, 1992), the spatiotemporal expression patterns of each XFGFR transcript were examined using more sensitive biochemical methods such as RNase protection assay or RT-PCR assay.

Total RNA was extracted from embryos of increasing age and subject to RNase protection assay with specific probes that hybridize to the divergent N-terminal region of XFGFRs (Fig. 4). Figure 5 shows that each of the five types of XFGFR mRNA was expressed as early as in the two-cell stage. These transcripts are presumably of maternal origin. When the results were quantified using a laser image analyzer with ODC mRNA expression as internal standard (Isaacs et al., 1992), there seems to be a general tendency for early stage transcripts to decrease rapidly down to blastula stage except for the transcript of XFGFR-2, which appears to stay at a low level. Then, the transcripts of XFGFR-2, -4a, and -4b start to increase and peak between the gastrula and early neurula stages, whereas those of XFGFR-1 and -3 appear not to have such a peak during that period. The increased fraction of transcripts may represent those of zygotic origin. The expression patterns of mRNAs of XFGFR-1, -2, and -4a observed in these studies are fairly consistent with those in the previous ones (Musci et al., 1990; Friesel and Dawid, 1991; Gillespie et al., 1989; Friesel and Brown, 1992; Shiozaki et al., 1995). Degradation of maternal transcripts during early developmental stages has been observed for Xenopus-myc (Taylor et al., 1986) and β-amyloid precursor protein (Okado and Okamoto, 1992). The temporal expression patterns of XFGFR-2, -4a, and -4b mRNA shown in Fig. 4 raise the possibility that these receptors are involved in the neural induction and patterning that occur during gastrula and subsequent neurula stages.

Early gastrula stage embryos were dissected into four pieces as depicted in Fig. 6A. The expression of each mRNA species of XFGFRs in these pieces was examined by RT-PCR using specific primers designed in the divergent N-terminal region (Table 1). Figures 6B and 6C show the spatial pattern of expression of each transcript, as quantified using a laser image analyzer with the expression of EF1α transcript as internal standard. Rather unexpectedly, the relative abundance of all the five transcripts examined to the control EF1α transcript is highest in endodermal piece. This finding is, however, in agreement with the previous observation that FGF signaling is required for the endodermal development in Xenopus (Henry et al., 1996). The ectoderm region, which gives rise to the neural tissue by responding to signals from the DMZ (or organizer), also expresses each of the five types of transcripts. With close inspection, however, the relative abundance of XFGFR-2, -4a, and -4b transcripts are high in the ectoderm compared to MZs. These results support the idea that these receptors contribute to neural induction and patterning.
FGF Signaling and Anterior Neural Induction

Requirement of FGF Signaling in Ectoderm for Anterior Neural Induction in Vivo

Contradictory results have been obtained concerning the requirement of FGF signaling for the anterior neural induction (Launay et al., 1996; Sasai et al., 1996; Barnett et al., 1998; versus Kroll and Amaya, 1996; Pownall et al., 1998; Holowacz and Sokol, 1999). All these studies used a truncated form of XFGFR-4a that is most divergent from XFD. Thus, although we have also taken a dominant-negative approach, we have used a truncated form of XFGFR-4a that is most divergent from XFGFR-1 among Xenopus FGFRs in addition to XFD.

We first ascertained whether a truncated XFGFR-4a ($\Delta XFGFR-4a$) was capable of blocking the FGF signaling in ectoderm cells. In the previous studies, it was shown that low doses of bFGF induced gastrula ectoderm cells in culture to express the anterior neural marker genes such as XeNK-2 and En-2 (Kengaku and Okamoto, 1995). When ectoderm cells were cultured without bFGF, they differentiated into epidermal cells expressing the epidermal marker such as epidermal keratin (Jonas et al., 1989), but the expression of keratin was suppressed in the presence of bFGF. We used this system to measure FGF signaling in ectoderm cells.

$\Delta XFGFR-4a$ mRNA was injected into the four animal blastomeres at the eight-cell stage (150 or 300 pg/blastomere). Animal cap fragments were dissected from injected or uninjected embryos at early gastrula stage and cells from these fragments were cultured in the absence or presence of a low dose (0.25 ng/ml) or high dose (5.0 ng/ml) of bFGF. The expression of anterior neural marker and epidermal marker genes in these cultures was examined by RT-PCR assay as described under Materials and Methods (Fig. 7A).

Quantitative analysis shows that when gastrula ectoderm cells from the injected embryos were cultured in the presence of a low dose of bFGF (0.25 ng/ml), the expression of the two anterior neural marker genes, XeNK-2 and En-2, was substantially inhibited compared to the expression of these genes in cultured cells from uninjected embryos (Fig. 7B, top and middle graphs, 0.25 bFGF). In contrast, the expression of the epidermal marker Keratin, which was suppressed by bFGF in uninjected series of cultures, was largely restored in injected series of cultures (Fig. 7B, bottom graph, 0.25 bFGF). The extent of keratin expression in injected series of cultures in the absence of bFGF was at the same level as observed in uninjected series of cultures (Fig. 7B, bottom graph, 0 bFGF), indicating that $\Delta XFGFR-4a$ mRNA injected in amounts up to 300 pg/blastomere is not toxic by itself. When Prolactin mRNA was injected instead of $\Delta XFGFR-4a$ (300 pg/blastomere) as a control, the response of injected gastrula ectoderm cells to bFGF did not differ from that obtained for uninjected series of cells (data not shown).

It should be noted that, when $\Delta XFGFR-4a$ mRNA-injected cells were treated with a high dose of bFGF (5.0 ng/ml), the two anterior neural markers were expressed to a considerable extent (Fig. 7B, top and middle graphs, 5.0 bFGF) and the epidermal marker was still largely suppressed (Fig. 7B, bottom graph, 5.0 bFGF). Particularly, the expression of En-2 that was substantially inhibited by a high dose of bFGF in control cultures was largely restored in...
injected series of cultures (Fig. 7B, middle graph, 5.0 bFGF). All these may be brought about by a low level of FGF signaling through unblocked FGFRs in injected cells. Similar sets of experiments were repeated several times and we obtained essentially the same results. We conclude that ΔXFGFR-4a is capable of effectively, though not completely, blocking the FGF signaling in ectoderm cells.

When embryos injected at the 8-cell stage as described above were reared until later developmental stages, they exhibited a wide range of anterior defects that include lack

![Fig. 3. Whole-mount in situ hybridization analysis of expression of XFGFR-1 (A), XFGFR-2 (B), XFGFR-3 (C), and XFGFR-4 (D) in stage 19 embryos. (Top panels) Anterior view; (bottom panels) dorsal view with the anterior downward.](image)

![Fig. 4. Probes for RNase protection assay. The NotI site in XFGFR-1 is derived from a cloning site in a truncated XFGFR-1 cDNA. The PvuII site in XFGFR-2 and the BamHI site in XFGFR-4b are in the untranslated regions of the respective cDNAs.](image)
The maximum value of the ratio is presented in each profile. The ratio of the intensity of the probe for each probe (an internal standard). (A) RNase protections of the five XFGFRs. The intensity of photostimulated luminescence (PSL) of each probe was calculated from the respective PSL value and plotted against developmental stages. The percentage of intensity of the protected probe in (A) was measured with a laser image analyzer. (B) A quantitative comparison of the temporal expression profiles for transcript levels of five probes and ODC probe. The ratio of the intensity of the probe for each XFGFR to the intensity of the ODC probe was calculated from the respective PSL value and plotted against developmental stages. The percentage of the maximum value of the ratio is presented in each profile.

FIG. 5. Expression of XFGFR transcripts at different developmental stages. (A) RNase protections of the five XFGFR probes and ODC probe (an internal standard). (B) A quantitative comparison of the temporal expression profiles for transcript levels of five XFGFRs. The intensity of photostimulated luminescence (PSL) of each protected probe in (A) was measured with a laser image analyzer. The ratio of the intensity of the probe for each XFGFR to the intensity of the ODC probe was calculated from the respective PSL value and plotted against developmental stages. The percentage of the maximum value of the ratio is presented in each profile.

of a part of telencephalon to considerable reduction of anterior head mass with fused eyes. However, more than half of the affected embryos contained trunk defects that seemed to be caused by the defects in gastrulation process. Although gastrulation defect does not necessarily produce anterior deficiency, as shown in case of XFD mRNA-injected embryos (Musci et al., 1990; Amaya et al., 1993), there are such cases in which retarded gastrulation is accompanied with anterior defects, for instance, in embryos that receive an injection of heparin (Mitani, 1989) or synthetic mRNA encoding a dominant-negative PDGF receptor (Ataliotis et al., 1995). To circumvent this problem, we may need spatially more restricted expression of a dominant-negative construct. For this purpose we injected △XFGFR-4a or △XFGFR-1 mRNA into eight animal blastomeres around the animal pole at the 32-cell stage and examined resulting morphological changes at the stage 35/36. Typical results of such an experiment are shown in Fig. 8. As the amount of injected mRNA of △XFGFR-4a was increased (80 to 300 pg/blastomere), anterior defects including loss of normal structure in telencephalon and eye regions became more and more prominent, whereas gastrulation process seemed little affected (Fig. 8B, 300 pg/blastomere; Fig. 8D, 120 pg/blastomere; Fig. 8F, 80 pg/blastomere). In embryos that were injected with the highest amount of mRNA, the cement gland tissue appeared to be expanded to a considerable extent (Fig. 8B, arrows). As will be described later, overexpression of △XFGFR-4a in dissociated ectoderm cells enhanced the expression of a cement gland marker at the expense of expression of neural markers (Fig. 12B). It is thus highly likely that some progeny of injected animal pole blastomeres differentiate into cement gland cells instead of anterior neural cells. In contrast to △XFGFR-4a mRNA, injection of △XFGFR-1 mRNA into eight animal blastomeres caused little, if any, defects in the anterior structure with the same dose range (Fig. 8C, 300 pg/blastomere; Fig. 8E, 120 pg/blastomere; Fig. 8G, 80 pg/blastomere). Although some anterior defects were seen occasionally with the highest amount of mRNA injected (Fig. 8C, lower two embryos), the extent of defects was much smaller than that with △XFGFR-4a mRNA and the incidence was low (20/77, i.e., 20 of 77 injected embryos were affected). This compares with a high incidence of severe defects in embryos injected with △XFGFR-4a mRNA (85/90). When these two types of mRNA were injected into the same dose range (data not shown). It thus appears that the development of anterior structure is more sensitive to the blockade of FGF signaling by △XFGFR-4a than that by △XFGFR-1.

To confirm the requirement of FGF signaling for the anterior neural induction, we examined whether overexpression of △XFGFR-4a affects the expression of embryonic neural marker genes by in situ hybridization to whole embryos. These neural markers include a panneural marker, Nrp-1, and three anterior neural markers: BF-1 for probing the telencephalon (Bourguignon et al., 1998), Rx-1 for probing the eye primordium (Mathers et al., 1997), and En-2 for probing the midbrain–hindbrain boundary (Hemmati-Brivanlou et al., 1991). △XFGFR-4a mRNA were injected into eight animal blastomeres at the 32-cell stage. They caused typical gastrulation defects such as the open blastopore within the same dose range (data not shown). It thus appears that the development of anterior structure is more sensitive to the blockade of FGF signaling by △XFGFR-4a than that by △XFGFR-1.

FIG. 6. Expression of XFGFR transcripts at different developmental stages. (A) RNase protections of the five XFGFR probes and ODC probe (an internal standard). (B) A quantitative comparison of the temporal expression profiles for transcript levels of five XFGFRs. The intensity of photostimulated luminescence (PSL) of each protected probe in (A) was measured with a laser image analyzer. The ratio of the intensity of the probe for each XFGFR to the intensity of the ODC probe was calculated from the respective PSL value and plotted against developmental stages. The percentage of the maximum value of the ratio is presented in each profile.

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BF-1; Fig. 9C for Rx-1; Fig. 9D for En-2). A gross morphological difference was not evident between uninjected and injected embryos at this stage.

**Requirement of FGF Signaling in Ectoderm Cells for the Neural-Inducing Action of Organizer Cells**

To further confirm that FGF signaling is required in ectoderm cells for the anterior neural induction, we used a microculture system for early gastrula cells in which the inductive differentiation of neuronal cells can be analyzed quantitatively using molecular probes (Mitani and Okamoto, 1991; Kengaku and Okamoto, 1993, 1995). In this system, ectoderm cells from early gastrula are induced to differentiate into neurons of the central nervous system by the action of cocultured DMZ (organizer) cells.

ΔXFGFR-4a mRNA was injected into the four animal blastomeres at the eight-cell stage (150 pg/blastomere). Ectodermal cells were prepared at the early gastrula stage (stage 10) from the injected or uninjected embryos. A fixed number of these cells (160 cells/well) were cocultured in microculture wells with increasing number of DMZ cells (0 to 80 cells) that are prepared from intact early gastrula embryos (stage 101/4, see Fig. 10A for experimental design). These DMZ cells were prepared from the involuted part of DMZ, which mainly gives rise to the axial mesoderm. It was shown previously that they did not express neural markers by themselves in the microculture system, as judged by the expression of neuron-specific N1 antigen (Mitani and Okamoto, 1991). The expression of anterior neural marker and epidermal marker genes in these cultures was examined by RT-PCR assay as described under Materials and Methods (Fig. 10B).

Quantitative analysis shows that all the four anterior neural marker genes examined, BF-1, Rx-1, XeNK-2, and En-2, are expressed in the uninjected series of cells depending on the numbers of cocultured intact DMZ cells (Fig.

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**Fig. 6.** Regional distribution of XFGFR transcripts. (A) Schematic illustration of dissected regions from ectoderm (Ec), dorsal marginal zone (DMZ), ventral marginal zone (VMZ), and endoderm (En) at an early gastrula stage. (B) Autoradiographs of RT-PCR products of XFGFR transcripts in the four regions defined in (A) coamplified with EF 1α transcript (an internal standard). (C) A quantitative comparison of the spatial expression profiles for transcript levels of five XFGFRs. The intensity of PSL of each RT-PCR product in (B) was measured with a laser image analyzer. The ratio of the intensity of the RT-PCR product for each XFGFR to the intensity of the RT-PCR product for EF1α was calculated from the respective PSL value and illustrated as histograms. The percentage of the maximum values of the ratio is presented in each histogram.
10C). The expression of these markers is substantially suppressed in the injected series of cells. DMZ cells alone do not appear to express these markers as expected. In contrast, the expression of an epidermal marker, Keratin, is strongly suppressed in the uninjected cells by increasing number of cocultured DMZ cells, but the suppression is weakened in the injected cells. These results indicate that FGF signaling in ectoderm cells is required for the anterior neural-inducing and epidermal-inhibiting action of DMZ (organizer) cells. It should be noted that BF-1 is expressed in the uninjected series of cells in the absence of DMZ cells. This may be due to the autonomous neuralization of ectoderm cells by endogenous FGF signaling (see next section for detail). BF-1 expression in uninjected cells is suppressed with a larger number of cocultured DMZ cells. Similar suppression with larger number of DMZ cells is also seen for En-2 expression in uninjected cells. These observations appear to correspond to our previous one that the expression of En-2, which is induced in ectoderm cells in culture with lower doses of FGF, is suppressed in a higher dose range (Kengaku and Okamoto, 1995).

In the coculture system using DMZ cells from stage 101/4 embryos described above, we could barely detect the expression of posterior neural marker genes such as XlH-box1 and XlH-box6 (not shown). Classical transplant experiments showed that DMZ region from early gastrula induced the anterior head structure, whereas DMZ region from late gastrula induced the posterior trunk and tail structures (Spemann and Mangold, 1924). We then examined the expression of posterior neural marker genes in cocultures of ectoderm cells with DMZ cells from late gastrula (stage 11.5) and asked whether the expression of posterior marker

**FIG. 7.** Suppression of FGF signaling in ectoderm cells by a truncated XFGFR-4a (D XFGFR-4a). (A) Effects of overexpression of ΔXFGFR-4a in ectoderm cells on neural induction and epidermal inhibition by bFGF. ΔXFGFR-4a mRNA was injected into four animal blastomeres at the eight-cell stage (150 or 300 pg/blastomere). The injected or uninjected embryos were incubated until stage 10 when ectoderm cells were isolated and cultured in the presence of a low (0.25 ng/ml) or high (5.0 ng/ml) dose of bFGF in microculture wells. The transcription levels of two anterior neural markers, XeNK-2 and En-2, and an epidermal marker, Keratin, in these cultures were analyzed by quantitative RT-PCR assay as described (Kengaku and Okamoto, 1995). Autoradiographs are shown of RT-PCR products of the marker transcripts coamplified with EF1α transcript (an internal standard) in uninjected ectoderm cells (left panels) and injected ectoderm cells (middle and right panels). (B) Quantitative assessment of effects of injected ΔXFGFR-4a mRNA. Each RT-PCR product shown in (A) was quantified as in Fig. 5B. Values were normalized to EF1α expression as in Fig. 6B and presented as percentages of the maximum values of the ratio in each histogram for XeNK-2 (top), En-2 (middle), and Keratin (bottom).
FIG. 8. Suppression of anterior neural development in vivo by blocking FGF signaling in animal blastomeres with ΔXFGFR-4a. Increasing amounts of ΔXFGFR-4a or ΔXFGFR-1 mRNA (80 (F, G), 120 (D, E), and 300 (B, C) pg/blastomere, respectively) were injected into eight animal pole blastomeres at the 32-cell stage as illustrated in the top left schema. The injected (B to G) and uninjected (A) embryos were reared until stage 35/36 and photographed. The expanded region of cement gland is indicated by white arrows in (D).
genes, if any, is suppressed by overexpression of $\Delta$XFGFR-4a in ectoderm cells. The experimental design was the same as described for cocultures with DMZ cells from early gastrula. Typical results are shown in Fig. 11. All four marker genes examined (Krox-20, XlHbox1, XlHbox6, and Xcad-3) are expressed in the uninjected series of cells depending on the numbers of cocultured late DMZ cells as expected. The expression of these markers is substantially suppressed in

FIG. 9. Suppression of early neural markers in embryos injected with $\Delta$XFGFR-4a mRNA. In each panel, a pair of uninjected (left) and injected (right) embryos at stage 20 is shown. (A) Expression of Nrp-1. (B) Expression of BF-1. (C) Expression of Rx-1. (D) Expression of En-2. White arrows in (B) and (D) point to the respective site of marker expression in uninjected embryos.
the injected series of cells. Late DMZ cells alone (80 cells/well) do not express detectable amount of these markers. In contrast, the expression of Keratin is strongly suppressed in the uninjected cells by increasing number of cocultured late DMZ cells, whereas the suppression is weakened in the injected cells as in case of cocultures with early DMZ cells. These results are consistent with previous observations that FGF signaling is required for the expression of posterior marker genes (Pownall et al., 1996; Hallowacz and Sokol, 1999).

Taking into account that Keratin is expressed in the injected cells to the same or even larger extent compared to the expression in the uninjected cells (Figs. 10 and 11, Keratin, 0 DMZ cells), the suppression of both anterior and posterior markers was not due to a toxic effect of the injected ΔXFGFR-4a mRNA but rather a specific effect on the FGF signaling. Similar sets of experiments were repeated several times and we obtained essentially the same results, except for about half the cases in which suppression of En-2 expression in the uninjected series of cells with
larger number of cocultured DMZ cells was not so complete as observed in Fig. 10. In these cases as well, however, the suppressive effect of injected ΔXFGFR-4a mRNA was obvious (data not shown). From these results, we strongly suggest that FGF signaling in ectoderm cells is required for the anterior neural-inducing action of early DMZ cells as well as for the posterior neural-inducing action of late DMZ cells.

**Requirement of FGF Signaling for Autonomous Neuralization in Dissociated Ectoderm Cells**

It has been shown that ectoderm cells in culture express neural, specifically anterior neural, markers by themselves when they are subjected to prolonged dissociation before reaggregation and culture, instead of being subjected to immediate reaggregation and culture as in our experiments described in Figs. 7, 10, or 11 (Grunz and Tacke, 1989; Wilson and Hemmati-Brivanlou, 1995). We asked whether FGF signaling is required for this autonomous neuralization in dissociated ectoderm cells. ΔXFGFR-4a or ΔXFGFR-1 (XFD) mRNA was injected into the four animal blastomeres at the eight-cell stage. Animal caps from injected and uninjected embryos were dissected and dissociated at the early gastrula stage (stage 10). The dissociated cells were subjected to dissociation for 30 or 90 min before they were inoculated into microculture wells and reaggregated as described under Materials and Methods. The expression of two panneural markers NCAM and Nrp-1, two anterior neural markers BF-1 and Rx-1, an epidermal marker Keratin, and a cement gland marker XAG-1 was examined by RT-PCR assay (Fig. 12A).

Quantitative analysis shows that when gastrula ectoderm cells from the embryos injected with ΔXFGFR-4a mRNA were cultured after prolonged dissociation, the expression of all the four neural marker genes, two panneural genes (NCAM and Nrp-1), and two anterior neural genes (BF-1 and Rx-1) was substantially inhibited compared to the expression of these genes in cultured cells from uninjected embryos (Fig. 12B, left and middle graphs). Injection of ΔXFGFR-1mRNA caused the inhibition less effectively than that of ΔXFGFR-4a mRNA. In contrast to these neural marker genes, the expression of the epidermal marker Keratin, which was low in uninjected series of cultures, became prominent in injected series of cultures (Fig. 12B, top right graph). The effect of injection of ΔXFGFR-1 mRNA was again less profound than that of ΔXFGFR-4a mRNA. These results indicate that constitutive FGF signaling in dissociated ectoderm cells is required for their autonomous neuralization. The signal required appears to be mediated preferentially through XFGFR-4.

When we examined the expression of XAG-1, a marker gene of cement gland that develops adjacent to the anterior border of the neural tissue, the XAG-1 expression was seen to be largely enhanced in injected, especially ΔXFGFR-4a mRNA-injected, series of cultures compared to the expression of this gene in uninjected series of cultures (Fig. 12B, bottom right graph). Interestingly, the corresponding phenotype in vivo, that is, expansion of the cement gland tissue at the expense of anterior neural tissues was observed in injected embryos at stage 35/36 (Fig. 8B).

**DISCUSSION**

**FGF Signaling and Anterior Neural Induction**

In the present studies, we have shown that overexpression of a dominant-negative form of XFGFR-4a (ΔXFGFR-4a) effectively blocks FGF signaling in ectoderm cells, which leads to disruption of anterior neural development both in vivo and in vitro. Our data are in agreement with the previous observation that overexpression of a dominant-negative form of XFGFR-1 (XFD) in ectodermal explants with synthetic XFD mRNA blocked anterior neural induction by noggin and organizer tissues (Launay et al., 1996), by chordin (Sasai et al., 1996), and by notochord (Barnett et al., 1998). However, using the same dominant-negative construct, contradictory results were also obtained: In embryos or explants that received XFD mRNA, expression of anterior neural marker genes was largely restored (Pownall et al., 1996; Kroll and Amaya, 1996; Holowacz and Sokol, 1999), and transgenic embryos expressing XFD gene contained well-patterned nervous system, though the gastrulation process was largely disrupted in these embryos (Kroll and Amaya, 1996).

One of possible explanations for the apparent conflict is that FGF signaling required for the anterior neural induction is mediated mainly through member(s) of FGF receptor family other than XFGFR-1, the function of which is not effectively blocked by XFD. Indeed, the present studies show that overexpression of ΔXFGFR-1 causes much smaller defects in the anterior structure compared to those by overexpression of ΔXFGFR-4a (Fig. 8). Further, autonomous neuralization of dissociated ectoderm cells is more effectively blocked by ΔXFGFR-4a than by ΔXFGFR-1. In accord with these observations the transcripts of XFGFR-2, -4a, and -4b but not of XFGFR-1 peak between the gastrula and neurula stages, when the neural induction and patterning take place, and these transcripts are relatively abundant in the ectoderm compared to the marginal zone at the gastrula stage. Since a truncated form of FGR-1 is shown to effectively block FGF signaling through FGR-2 as well (Ueno et al., 1992) and XFGFR-4a and -4b are most divergent from XFGFR-1 (Fig. 2B), XFGFR-4s are the most promising candidates of the signal mediator for the anterior neural induction. It is interesting in this context that a physiological response to FGF signaling, such as membrane ruffling in some mammalian cells, is mediated not by FGR-1, -2, or -3, but solely by FGR-4 (Johnston et al., 1995). However, it should also be noted that we do not know relative abundance of ΔXFGFR-4a and ΔXFGFR-1 proteins expressed in outer surface of ectoderm cells that have been injected with the same amount of respective mRNA, although we confirmed they possessed the same translation efficiency in in vitro translation system (not shown). Thus, we could not
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exclude the possibility that the observed difference between the potency to block anterior neural development of \( \Delta XFGFR-4a \) and \( \Delta XFGFR-1 \) overexpressed in ectoderm is not qualitative but just quantitative, that is, \( \Delta XFGFR-4a \) is expressed in ectoderm cell surface somehow with much higher efficiency than \( \Delta XFGFR-1 \) and member(s) of FGF receptor family other than XFGFR-4s also contribute to signal mediation for the anterior neural induction.

FGF signaling appears to be involved in mesoderm induction (Amaya et al., 1993) and gastrulation (Kroll and Amaya, 1996) in Xenopus. Defects in these processes can affect the neural induction secondarily and this seems to bring about some difficulties in interpreting the results obtained with dominant-negative versions of FGF receptor. It is plausible that a certain threshold of FGF signaling strength is required for mesoderm induction or gastrulation and this threshold is higher than that required for the anterior neural induction. Indeed, we have previously shown that the dose of bFGF required for reproducible differentiation of myocytes, a mesodermal derivative, from blastula animal cap cells in culture is as high as 10 ng/ml, which is 40-fold higher than the dose (0.25 ng/ml) required for the expression of anterior neural marker genes in gastrula animal cap cells (Kengaku and Okamoto, 1995). In these circumstances, anterior neural induction would be less sensitive to a dominant-negative construct of FGF receptor than mesoderm induction or gastrulation. Thus, blocking the mesoderm induction or gastrulation by a dominant-negative FGF receptor at a relatively lower level of its expression would not necessarily be accompanied with defects in anterior neural induction, whereas blocking the anterior neural induction at a relatively higher level of expression would inevitably cause defects in the mesoderm induction or gastrulation that may affect the anterior neural induction secondarily. In the latter case, a straightforward interpretation of results would be difficult. To fully confirm the requirement of FGF signaling for the anterior neural induction, we may need spatially and/or temporally more restricted expression of a dominant-negative construct. Indeed, when we injected \( \Delta XFGFR-4a \) mRNA into the eight animal pole blastomeres at the 32-cell stage, anterior neural development was severely impaired with little damage to mesoderm induction and gastrulation (Figs. 8 and 9).

FGF Signaling and the “Neural Default Model”

Our present results seem, at first glance, inconsistent with the neural default model that was recently proposed for the molecular mechanism of neural induction. This model features central roles for BMP signaling within ectoderm and for BMP antagonists such as noggin and chordin secreted by the organizer (Wilson and Hemmati-Brivanlou, 1997). BMP signaling alone induces ectoderm to form epidermis and suppress its neuralization (Wilson and Hemmati-Brivanlou, 1995), whereas noggin and chordin work by locally antagonizing the BMP signaling through directly binding BMP4 to prevent it from activating its receptor (Piccolo et al., 1996; Zimmerman et al., 1996), allowing dorsal ectoderm to follow its default neural fate. It is argued that neural fate, specifically anterior neural fate, is the default fate of gastrula ectoderm in the sense that the neural induction, at least its initial step, requires only the absence of epidermal-inducing signals. However, our present and some of previous observation by others indicate that the presence of FGF signaling in ectoderm is also required for neural induction.

One of the basis for the neural default model is the finding that ectodermal cells, subjected to prolonged dissociated culture during gastrula stages, formed histologically recognizable neural tissue after reaggregation (Grunz and Tacke, 1989). It was also found that ectodermal explants from gastrula embryos were neuralized by expression of a dominant-negative version of a BMP receptor (Hemmati-Brivanlou and Melton, 1994). In both cases, ectoderm adopted an anterior neural fate in the absence of neural inducing signals from the organizer. It was thus postulated that deprivation of endogenous neural-inhibiting signaling such as BMP signaling in ectoderm cells by prolonged dissociation or overexpression of a dominant-negative receptor is enough to cause their neuralization. However, we showed in the present studies that autonomous neuralization in dissociated ectodermal cells required FGF signaling (Fig. 12). Interestingly, Xenopus gastrula ectodermal cells have been shown to express several members of FGF family in addition to BMPs, though the level of their expression is considerably lower than that in the organizer region (Kimelman et al., 1988; Isaacs et al., 1992; Tannahill et al., 1992; Song and Slack, 1996). It should be noted that these FGF family members have the common property of binding to...
components of extracellular matrix such as heparin, the characteristics that would make them not readily released from the cell surface compared to BMPs even in prolonged dissociated culture. More interestingly, gastrula ectoderm cells contain novel types of ligands for the FGF receptor, FRL1 and FRL2, which have a N-terminal signal sequence and can be anchored to the cell membrane by their C-termini (Kinoshita et al., 1995). It is possible that some of ligands of FGF receptor listed above support constitutive FGF signaling in ectoderm cells, contributing their neuralization without signals from the organizer. Indeed, there is strong evidence that FGF signaling is working in blastula animal cap, an immediate precursor of gastrula ectoderm, to complement activin signaling for the mesoderm induction (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; LaBonne et al., 1995).

We may reconcile the neural default model and our present data by simply postulating that the default state of ectoderm is endowed with constitutive FGF signaling. According to this idea, both BMP and FGF signaling are working constitutively in intact ectoderm in either autocrine or paracrine manner. When ectoderm is isolated from...
contact with the organizer, BMP signaling in it somehow overrides FGF signaling in due course, allowing the ectoderm to follow epidermal fate. In normal development, however, a dorsal part of ectoderm receives signals from the organizer, specifically from the organizer of early gastrula at first that antagonize the BMP signaling. This is achieved by noggin and/or chordin, which directly bind BMP4 and prevent it from activating its receptor. In ectoderm cells that receive antagonizing signals of sufficient strength from the early organizer before BMP signaling completely overrides FGF signaling in them, the dominance of the two signaling system is reversed and the FGF signaling allows these cells to follow anterior neural fate. In contrast, ectoderm cells that do not receive antagonizing signals of sufficient strength to reverse the signaling dominance follow epidermal fate by BMP signaling. When the two signaling system is balanced somehow in ectoderm cells, these cells may adopt cement gland fate as suggested by our present results (Fig. 8B and 128). The question remains, however, whether only relative strength of the two signaling system in ectoderm cells is crucial for their fate determination, or absolute strength of each system is also important. The data so far obtained appear to support the latter idea. Overexpression of FGF in whole embryos or explants does not increase the amount of neural tissue, but converts the anterior neural tissue to the posterior one (Pownall et al., 1996; Holowacz and Sokol, 1999). It is also shown, on the other hand, that overexpression of a dominant-negative BMP receptor increase the amount of anterior neural tissue (Hemmati-Brivanlou and Melton, 1994). Taken together with our present data, it seems likely that lowering of BMP signaling in ectoderm cells to a certain strength makes them competent to adopt neural fate, thus demarcating the future neural area, while FGF signaling disclosed in the competent ectoderm cells enforces them to follow anterior neural fate with a relatively low constitutive level of strength.

The subsequent reinforcement of FGF signaling in the anterior neurally fated cells by some members of FGF family from the organizer, specifically from the organizer of late gastrula which gives rise to posterior mesoderm derivatives, would make these cells adopt more posterior neural fates (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). It is indeed shown that blocking FGF signaling in ectoderm cells in cocultures with organizer cells of late gastrula (Fig. 11) or in recombinants with posterior mesoderm (Holowacz and Sokol, 1999) inhibit the expression of posterior neural markers. Since organizer cells of early gastrula are not capable of inducing posterior neural markers, but just inducing anterior ones (Fig. 10), we suggest that these early organizer cells mainly emanate BMP antagonists such as chordin and noggin, whereas late organizer cells emanate, in addition, larger amount of member(s) of FGF family such as eFGF (Isaacs et al., 1992).

The idea of a graded manner of action of FGF signaling in the anteroposterior neural patterning first arises from the observation that FGF is capable of inducing Xenopus ectoderm cells in culture to express position-specific neural markers along the anteroposterior axis in a dose-dependent manner; with lower doses eliciting more anterior markers and higher doses more posterior markers (Kengaku and Okamoto, 1995). Recent observation by Holowacz and Sokol (1999) appears to be in agreement with the idea, which indicates that overexpression of XFD in Keller explants results in a shift of anterior neural markers toward the posterior region, possibly due to incomplete block of FGF signaling by XFD in the explants in our view. Addition of FGF to Keller explants, on the other hand, results in a shift of a more posterior neural marker toward the anterior region. They also show that overexpression of XFD in dorsal ectoderm in recombinants with dorsal mesoderm causes an apparent overall shift from posterior to more anterior neural tissue, which also seems due to incomplete block of FGF signaling by XFD in the dorsal ectoderm.

The model reformulated as described above is consistent with the concept of competence, the classical but central idea in embryology that states that the capacity to respond to an inducing signal is limited in space and time during embryogenesis (Gurdon, 1987). It should also be noted that the reformulated model does not necessarily exclude possible involvement of other signaling system in the anterior neural induction than FGF or BMP signaling. There is some evidence suggesting that animal cap cells of late blastula receive a planar signal from vegetal blastomeres, and gastrula animal cap cells are accordingly predisposed to form anterior neural tissue from these cells. Even if it were the case, however, the simplest scenario seems that the prepatterned planar signal works also by somehow suppressing BMP signaling in animal cap cells.

ACKNOWLEDGMENTS

We thank Professors Tomokazu Oshima and Kunitaro Takanashi for advice and discussions and Dr. David W. Saffen for critical reading of the manuscript. This work was supported in part by a grant of the COE project (H.O.) from the Agency of Science and Technology of Japan.

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Received for publication August 5, 1999
Revised September 13, 1999
Accepted September 13, 1999