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Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast

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SUMMARY

Analyses using amphibian embryos proposed that induction and anteroposterior patterning of the central nervous system is initiated by signals that are produced by the organizer and organizer-derived axial mesoderm. However, we show here that the initial anteroposterior pattern of the zebrafish central nervous system depends on the differential competence of the epiblast and is not imposed by organizer-derived signals. This anteroposterior information is present throughout the epiblast in ectodermal cells that normally give rise both to neural and non-neural derivatives. Because of this information, organizer tissues transplanted to the ventral side of the embryo induce neural tissue but the anteroposterior identity of the induced neural tissue is dependent upon the position of the induced tissue within the epiblast. Thus, *otx2*, an anterior neural marker, was only ever induced in anterior regions of the embryo, irrespective of the position of the grafts. Similarly, *hoxa-1*, a posterior neural marker was induced only in the posterior regions. Furthermore, the boundary of each ectopic expression domain on the ventral side was always at an equivalent latitude to that of the endogenous expression of the dorsal side of the embryo. The anteroposterior specification of the epiblast is independent of the dorsoventral specification of the embryo because neural tissues induced in the ventralized embryos also showed anteroposterior polarity. Cell transplantation and RNA injection experiments showed that non-axial marginal mesoderm and FGF signalling is required for anteroposterior specification of the epiblast. However, the requirement for FGF signalling is indirect in that cells with compromised ability to respond to FGF can still respond to anteroposterior positional information.

Key words: Neural induction, *otx2*, *hoxa-1*, Spemann's organizer, Embryonic shield, Mesoderm induction, Induction, Zebrafish

INTRODUCTION

Early in development, the vertebrate central nervous system (CNS) is regionalized along the anteroposterior (A-P) axis into forebrain, midbrain, hindbrain and spinal cord. Based upon analyses using amphibian embryos, it has been proposed that the A-P neural pattern is induced by the combined action of two signals produced by the organizer-derived dorsal mesoderm (two-step model; Nieuwkoop, 1950; Toivonen and Saxén, 1968). The first signal (activator) initiates neural development, inducing neural tissue of an anterior type and the second signal (transformer) converts the neural tissue induced by the first signal into progressively more posterior types of neural tissue (hindbrain and spinal cord) with increasing concentration. A number of candidates for the first signal have been identified recently, namely Noggin, Follistatin and Chordin (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994). Each of these proteins is expressed in the dorsal mesoderm during gastrulation, as appropriate for neural-inducing molecules, and each induces the formation of neuroectoderm with an anterior character. As for the second signal, retinoic acid (Conlon, 1995; Blumberg et al., 1997), FGFs (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995) and Wnts (McGrew et al., 1995) have been suggested as candidate molecules. In combination or alone, these factors on *Xenopus* gastrula ectoderm generate a wide range of neural tissues along the A-P axis (For review, see Doniach, 1995), supporting the notion that the nature of inducers determines the type of the neural tissues. This notion, however, can not fully explain the results obtained in classical experiments of organizer transplantation: whenever a secondary axis is induced, its A-P polarity is the same as that of the primary axis. Thus, its anterior end faces the host animal pole, suggesting that A-P positional values are present throughout the embryo including the ventral region. These observations have led us to examine the origin of A-P polarity in vertebrate CNS using the zebrafish embryo.

During embryogenesis in the zebrafish, the first morphological indicator of axial asymmetry appears at the beginning of gastrulation, with a thickening called the 'embryonic shield' on the dorsal side of the marginal zone. The shield is believed to be equivalent to the amphibian 'organizer' or amniote 'node'. This is supported by expression in the shield of genes also expressed in the *Xenopus* organizer and mouse

or chicken node, such as *goosecoid* (*gsc*; Blum et al., 1992; Cho et al., 1991; Izpisua-Belmonte et al., 1993; Stachel et al., 1993), *lim1* (Taira et al., 1993; Barnes et al., 1994; Toyama et al., 1995) and *chordin* (Sasai et al., 1994; Schulte-Merker et al., 1997) and by the ability of the shield to induce secondary axis when transplanted in the ventral region (Oppenheimer, 1936a; Shih and Fraser, 1996). Recently, Sagerström et al. (1996) analyzed shield function in vitro using an explant assay: the shield preferentially induced anterior neural markers when conjugated to animal caps, while the shield itself formed an axis with A-P neural pattern. However, the mechanisms that regulate zebrafish A-P axis formation in vivo are still unclear. For example, how much A-P information does the shield impart to the epiblast? Recent grafting experiments (Woo and Fraser, 1997) demonstrate that there is a signal from the germ ring that can impart anteroposterior pattern, but leave the nature of the signal unresolved. Does such an interaction impart a differential competence to the epiblast?

To investigate whether or not the response of zebrafish epiblast to inducing tissue depends on the position of the responding tissues along the A-P axis, we transplanted early organizer tissues into the ventral region of zebrafish embryos and related induced structures and gene expression patterns to the location of the grafts in the host. By performing this experiment, we were able to determine whether the epiblast cells had A-P information or whether such information could be imposed by the organizer. We present data showing that initial A-P patterning of zebrafish CNS depends on differential competence within the epiblast and that FGF-mediated signal produced by the non-axial marginal mesoderm is involved in the specification of the epiblast.

MATERIALS AND METHODS

Fish embryos

Zebrafish (*Danio rerio*) embryos were obtained from natural crosses of wild-type fish of the Oregon AB background. Collected embryos were maintained at 28.5°C and sorted into 1/3 Ringer (39 mM NaCl, 0.97 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM HEPES at pH 7.2) and staged according to hours postfertilization at 28.5°C and morphological criteria (Kimmel et al., 1995).

Transplantations

For Hensen's node (HN) transplantation, 50-100 μm fragments of chicken HN (stage 4), which included the most potent region, the medial sector of both the epiblast and mesenchyme (Storey et al., 1992), were manually removed and inserted into the ventral region of the zebrafish host during shield stage using a sharpened tungsten needle.

For transplantation of shield or ventral marginal tissues, fertilized eggs at the 2- to 8-cell stage were injected with a mixture of rhodamine- and biotin-dextran (Molecular Probes) (Miyagawa et al., 1996) into the yolk through a glass micropipette. The injected dye spreads through intercellular cytoplasmic connections to all cells of the blastoderm. When the embryonic shield became visible (6 h), shield or ventral marginal tissues were cut out with small scissors and then transplanted through a glass micropipette into the ventral region of a shield-stage host or animal pole of a sphere-stage host, respectively. Visualization of the labelled donor cells was conducted exactly as described previously (Westerfield, 1993; Miyagawa et al., 1996).

For transfection and transplantation of COS7 cells, we essentially followed the protocol described by Tonegawa et al. (1997). The plasmids that we used were pCDM8 (In Vitrogen) containing *Xenopus noggin* and *chordin* or *lacZ* cDNAs. The cell aggregate of approximately 50 μm diameter was used for transplantation. Transplantation of the yolk cell or blastomeres at the blastula stage was performed as described (Mizuno et al., 1996; Miyagawa et al., 1996). Grafted hosts were allowed to develop at 28.5°C for an appropriate period in 1/3 Ringer.

In some grafted zebrafish embryos, the graft was found dorsal in the vicinity of the original host axis and did not demonstrate its characteristic inductive activity. Therefore, we analyzed embryos only in which the graft remained in lateral or ventral regions.

Ventralized embryos

Ventralized zebrafish embryos were obtained by the removal of the vegetal yolk hemisphere as described (Mizuno et al., 1997) with some modifications. The vegetal yolk mass (about half of the total yolk mass) was squeezed out through a small hole made at the vegetal end by a glass needle. The operation was carried out between 5 and 20 minutes after fertilization, which produced *gsc*-negative embryos at a high frequency (more than 80%; T. Mizuno et al., unpublished data).

RNA injection

Capped sense RNAs were synthesized using the MEGAscript large-scale transcription kit (Ambion) from the plasmid containing full-length *Xenopus* dominant negative FGF receptor (XFD) or HAVnot cDNA (Griffin et al., 1995). HAVnot RNA used as a control encodes nonfunctional FGF-R in which a mutation is introduced in the ligand-binding domain. The mRNAs synthesized were purified by gel filtration (NICK Column, Pharmacia Biotech) and diluted to 0.4-0.6 μg/μl with distilled water and injected into 1-cell-stage embryos. Injected embryos were cultured in 1/3 Ringer until use.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as previously described (Takeda et al., 1994) with some modifications. Briefly, embryos fixed with 4% paraformaldehyde/PBS were dehydrated with methanol and rehydrated with PBS/0.1% Tween 20. They were then transferred to hybridization mixture (5× SSC, 50 μg/ml of heparin, 100 μg/ml of calf thymus DNA, 10 μg/ml of tRNA, 50% formamide, 0.1% Tween 20) and incubated for at least 1 hour. The hybridization mixture was then replaced with the mixture containing 100 ng/ml of DIG-labelled RNA probe (and fluorescein-labelled one for double in situ hybridization) and the embryos were incubated at 57°C overnight. After a rinse with 5× SSC, they were washed in 2× SSC/50% formamide at 65°C for 30 minutes, 2× SSC for 10 minutes and soaked in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl at pH 8.0) for 10 minutes. Following the treatment with 20 μg/ml of RNase A at 37°C for 1 hour, a series of washings (2× SSC for 10 minutes; 2× SSC/50% formamide at 65°C for 30 minutes; 0.2× SSC at 55°C for 15 minutes) was performed. Embryos were rinsed with MAB(150 mM NaCl, 100 mM maleic acid at pH 7.5)/0.1% Tween 20 and soaked in blocking solution (2% FCS, 0.2% Tween 20, 0.2% TritonX-100 in MAB) at 4°C for 1 hour. The embryos were then incubated with alkaline-phosphatase (AP)-conjugated anti-DIG Fab fragments diluted 1:8000 in blocking solution at 4°C overnight. After washing three times with blocking solution for 30 minutes, the embryos were rinsed three times with AP reaction buffer (50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 1 mM levamisole, 100 mM Tris-HCl at pH 9.0) for 10 minutes. Detection was performed with BM-purple (Boehringer Mannheim). After stopping the coloring reaction, we washed the specimens three times with AP reaction buffer excluding levamisole and stored then in 4% paraformaldehyde/PBS.

Double in situ hybridization was performed using ELF-97 mRNA In Situ Hybridization Kit (Molecular Probes) as described in Jowett and Yan (1996).

Immunohistochemistry

Following in situ hybridization, some embryos were immediately stained with anti-Ntl antibody (Schulte-Merker et al., 1992). The samples were washed three times with MAB for 10 minutes and twice with MABD (1% BSA, 1% DMSO in MAB) for 30 minutes. After blocking with MABDN (2% FCS in MABD) for 30 minutes, they were incubated with rabbit anti-Ntl polyclonal antibody diluted 1:1000 in MABDN at 4°C overnight. Following a series of washings with MABD (three times for 5 minutes, 4 times for 30 minutes), the samples were blocked with MABDN for 30 minutes and then incubated with biotin-conjugated goat anti-rabbit IgG diluted 1:500 in MABDN at 4°C overnight. A series of washings was carried out as follows: MABD 5 times for 5 minutes, 4 times for 30 minutes, rinse with MAB and PBS, PBSDT (1% DMSO, 0.1% TritonX-100 in PBS) three times for 5 minutes. The signals were detected with ABC staining Kit according to the manufacturer's instructions (Vector Laboratory Inc).

For histological analysis, the specimens were embedded in paraffin or Technovit 8100 (HERAEUS KULZER, Wehrheim) and cut at 8-10 μm.

RESULTS

Induction of secondary axes by three different inducing tissues

In addition to the fish organizer (embryonic shield) from a shield-stage embryo, we also used chicken Hensen's node (HN; stage 4, head organizer) and mammalian COS7 cells transfected with *Xenopus noggin* and *chordin* cDNA (Noggin/Chordin COS7; Lamb et al., 1993; Sasai et al., 1994) as a source of inducing tissue in the present study (Fig. 1A). In previous heterospecific organizer experiments between fish and salamander (Oppenheimer, 1936b), between mouse and *Xenopus* (Blum et al., 1992), between chick and *Xenopus* (Kintner and Dodd, 1991), and between chick and fish (Hatta and Takahashi, 1996), axial structures and/or neural-specific markers were ectopically induced, suggesting that signalling molecules from the organizer is conserved during vertebrate evolution.

As expected, we found that all inducing tissues examined were able to induce secondary axes when transplanted at midblastula to early gastrula stage into the ventral side of the fish embryo (Fig. 1B,C,F). However, they behaved differently in the secondary axes. Grafted embryonic shield contributed to the axial mesoderm and the ventral part of the neural tube (Fig. 1D,E), while grafted chicken HN and Noggin/Chordin COS7 showed no sign of self-differentiation presumably due to a different cellular environment, but were present in a cell mass within the neuroepithelium (Figs 1B, 2E) and under the neural tube (Fig. 1H) respectively. The secondary axes induced by HN or Noggin/Chordin COS7 tended to show cyclopean (one-eyed head), probably due to the lack of axial mesoderm: neither *gsc* (a marker for early organizer and prechordal plate) nor *no tail* (*ntl*; zebrafish orthologue of mouse *Brachyury*; a marker for the marginal mesoderm and notochord; Schulte-Merker et al., 1992) was detected in the secondary axis (Hatta and Takahashi, 1996; data not shown). Histological sections show that the neural tube, especially in the region anterior to the HN or Noggin/Chordin COS7 cell mass, did not exhibit dorsoventral (D-V) polarity (Fig. 1G). Thus, we had expected that, by use of chicken HN or Noggin/Chordin COS7, we could examine direct influences of the transplants, excluding interactions between axial mesoderm and the epiblast.

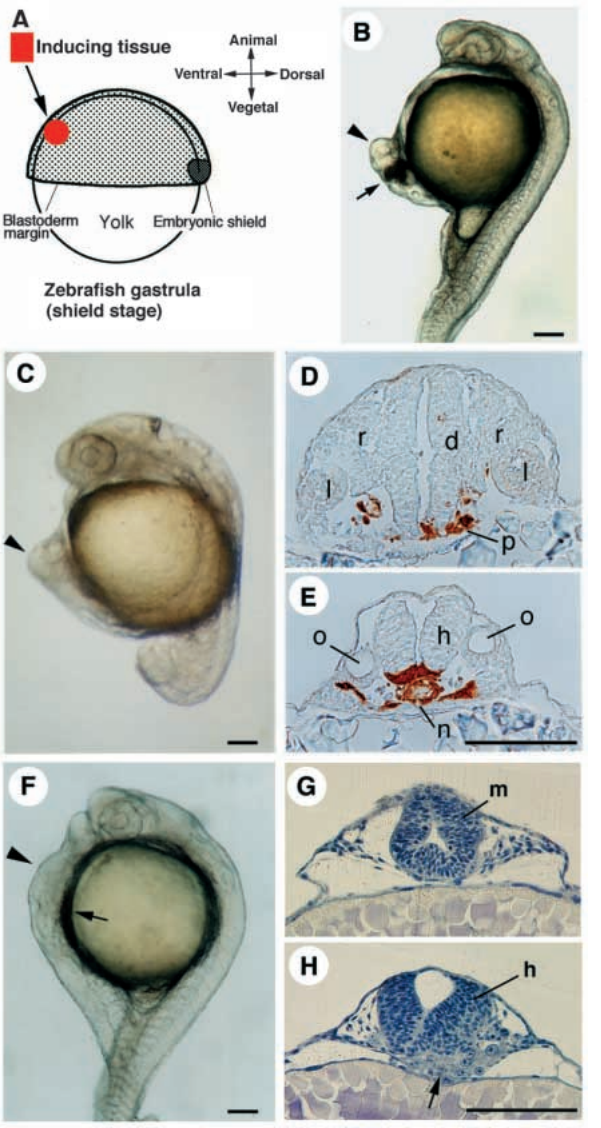

Fig. 1. Secondary axes induced by transplantation of organizer tissues. Arrowheads in B, C and F indicate the secondary axis. (A) Schematic representation of organizer transplantation. A small piece of avian Hensen's node (HN), fish organizer (embryonic shield) or Noggin/Chordin COS7 cell aggregate is transplanted into the host ventral region, mostly about halfway between the blastoderm margin and the animal pole, or near the margin in some cases. (B) Secondary axis induced by HN (stage 4) at 24 h. Arrow indicates the position of the transplanted HN. Chicken tissues appear dark because they contain yolk granules. (C-E) Secondary axis induced by the shield at 20 h. Cross sections at the level of the diencephalon (D) and the hindbrain (E) are shown. The donor cells are stained brown in the sections. Grafted shield differentiates into axial mesoderm and the ventral portion of the neural tube. (F-H) Secondary axis induced by Noggin/Chordin COS7 at 24 h. Cross sections at the level of the midbrain (G) and the hindbrain (H) are shown. Arrows indicate the position of the transplanted COS7 cells. COS7 cell mass is located under the induced neural tube (arrow in H). d, diencephalon; m, midbrain; h, hindbrain; l, lens; n, notochord; o, otic vesicle; p, prechordal plate; r, neural retina. Scale bars, 100 μm.

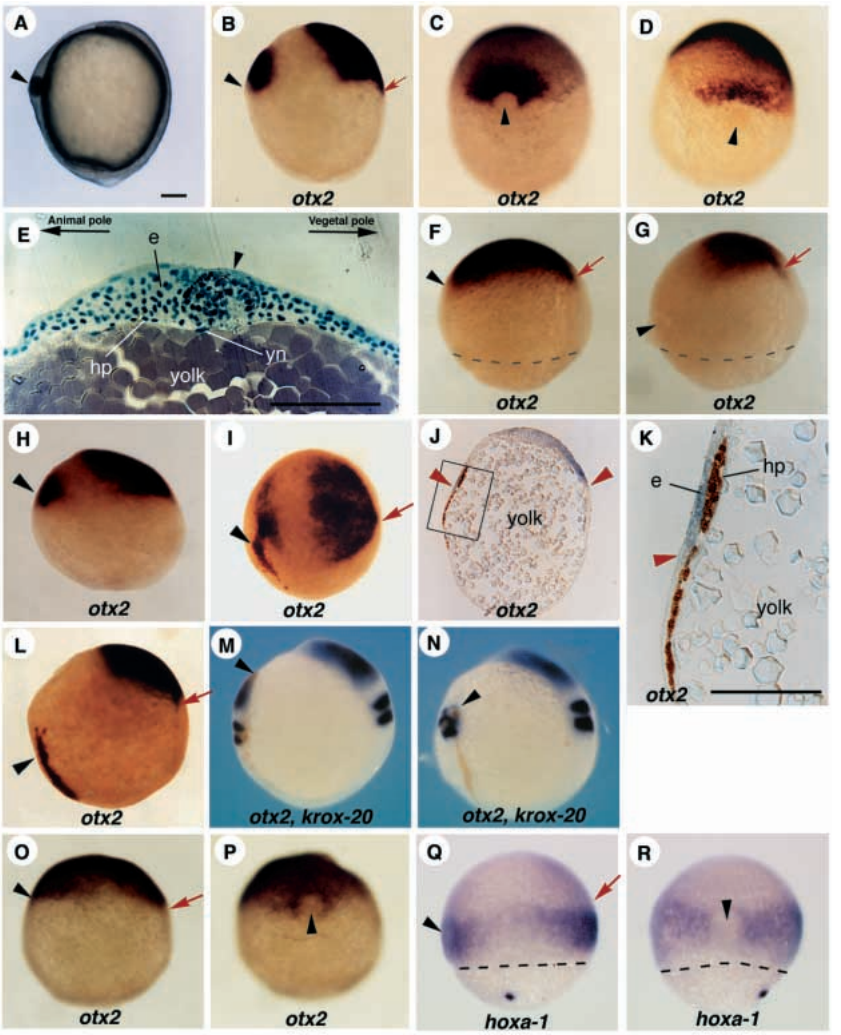

Fig. 2. Gene expression patterns around the grafts. Embryos are oriented with the animal pole to the top, except for E and I. Unless otherwise described, a black arrowhead, a red arrow and a dotted line indicate a grafted tissue, a dorsal midline of the host and the blastoderm margin, respectively. In all lateral views, dorsal is to the right. (A-E) Gastrula embryos grafted with the chicken Hensen's node (HN, stage 4) at the shield stage (6 h) in the ventral region. (A) Lateral view of the embryo 4 hours after transplantation (late gastrula). Lateral (B) and ventral views (C) of the same host hybridized with *otx2* probe. Ectopic expression of *otx2* appears around the grafted HN with a sharp posterior boundary at the same latitude as that of host endogenous expression. (D) Ventral view of the host hybridized with *otx2* probe, in which the graft is located more posteriorly (at the level of the hindbrain on the dorsal side) as compared with the sample shown in B and C. Ectopic expression is induced in the region just anterior to the graft but its posterior boundary remains unchanged with respect to the host endogenous expression. (E) Longitudinal section along the secondary axis induced by HN. The histological section shows neural induction as indicated by the thickened epithelium (e) around the grafted HN (arrowhead). The hypoblast (hp) and yolk syncytial nuclei (yn) are also seen in this section. (F,G) Lateral views of the host which received the HN at midblastula stage (4 h) and examined with *otx2* expression at the early gastrula stage (70% epiboly, 7 h). The ectopic expression domain, which is fused with the endogenous one at the animal pole, shares the same posterior boundary as the host domain (F). By contrast, when HN of the same stage was grafted near the blastoderm margin and located in the host trunk region, no ectopic expression of *otx2* is observed (G). (H-N) Host embryos that received biotin-labelled embryonic shields at early gastrula stage (6 h) in the ventral region. (H) Lateral view of the late gastrula (10 h) hybridized with *otx2* probe. Ectopic expression is induced with a sharp posterior boundary at the same level as that of host endogenous expression domain. (I) Oblique animal-pole view of the hybridized sample (shown in H) in which donor cells were visualized by biotin-peroxidase staining. Donor cells (brown, arrowhead) are widely distributed along the A-P axis but ectopic *otx2* expression is observed only in the anterior region. (J,K) Longitudinal section and high magnification view of the host shown in I. Red arrowheads indicate the posterior boundaries of *otx2* expression along both axes, showing that they are located at the same level (the secondary axis is to the left). The labelled donor cells are distributed along the A-P axis mainly in the underlying hypoblast. (L) Lateral view of the host stained with *otx2*, in which the donor cells (brown, arrowhead) are located in the trunk region by posterior transplantation. No induction of *otx2* expression is detected. (M,N) Lateral views of the embryos hybridized with *otx2* and *krox-20* probes (5-somite stage, 12 h). Arrowheads indicate the anterior limit of transplanted donor cells (brown). Both *otx2* and *krox-20* are induced when the donor cells are located in the anterior region (M), while only *krox-20* is induced when they are posteriorly located (N). (O-R) Gastrula embryos transplanted with Noggin/Chordin COS7 cells at the midblastula stage (sphere to dome stage). Lateral (O) and ventral views (P) of the host (10 h) hybridized with *otx2* probe, and lateral (Q) and ventral views (R) of the host (6.5 h) hybridized with *hoxa-1* probe are shown. Note that each ectopic expression domain shares the same boundary as the host domain. Scale bars, 100 μm.

Induction of region-specific neural markers by the organizer tissues

We examined gene expression patterns around the grafted tissues at the gastrula stage. We mainly focused on the anterior CNS and used the *otx2* gene as a marker for midbrain and forebrain. The expression of zebrafish *otx2* appears abruptly at high levels in a triangular patch at the animal pole of the early gastrula (about 6.5 hours postfertilization (h)), a dorsal region that contains cells fated to become midbrain and forebrain (Li et al., 1994; Mori et al., 1994; Fig. 6A). We also used the *hoxa-1* as a marker for the posterior neural tissues (hindbrain and spinal cord) (Alexandre et al., 1996; Fig. 6A). The anterior border of *hoxa-1* expression corresponds to the rhombomere 3/4 boundary. These genes are the earliest among known genes showing region-specific expression in CNS.

We first examined the expression pattern of *otx2* in host embryos with HN grafts (stage 4; head organizer; Storey et al., 1992). When host embryos received HN grafts at the early gastrula stage (shield, 6 h), a neural plate-like structure (thickened epiblast) was formed around the grafted HN (Fig.

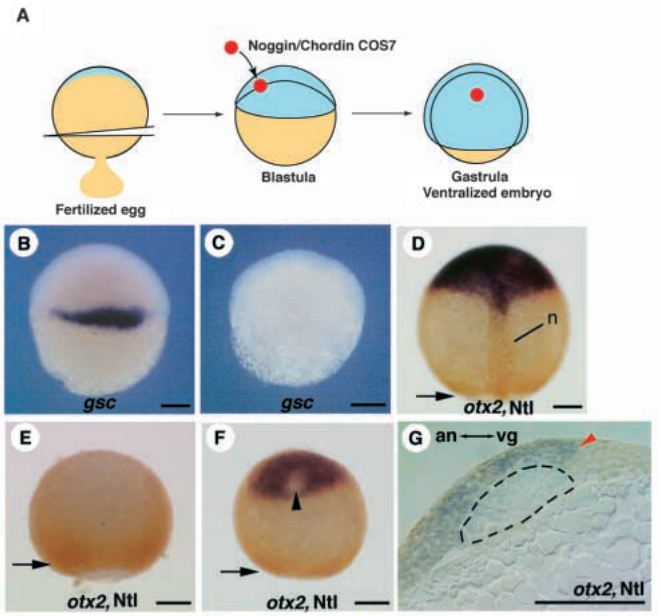

Fig. 3. Gene expression and neural induction in ventralized embryos. Embryos are oriented with the animal pole to the top, except for G. (A) Schematic representation of the experiment. Noggin/Chordin COS7 cells were transplanted into ventralized blastula (4 h) from which the vegetal yolk mass had been removed. (B,C) *gsc* expression in normal (B) and ventralized (C) embryos at the shield stage. No gsc expression is detected in a ventralized embryo. (D) Dorsal view of normal gastrula (90%-epiboly) stained with *otx2* probe and anti-Ntl antibody. The region fated to become forebrain and midbrain is positive for otx2 transcripts (dark blue), while the blastoderm margin (arrow) and the notochord (n) are positive for Ntl (light brown). (E) Lateral view of a ventralized gastrula (90%-epiboly) stained with *otx2* probe and anti-Ntl antibody. The blastoderm margin is positive for Ntl (arrow), while neither Ntl staining in the notochord nor *otx2* expression in the head region is detected. (F) The ventralized gastrula (90%-epiboly) stained with *otx2* probe and anti-Ntl antibody. The ventralized embryo was grafted with Noggin/Chordin COS7 cells at the blastula stage. *otx2* expression is induced in the anterior region around the graft (arrowheads) with a sharp posterior boundary. (G) Longitudinal section of the host shown in G along the transplanted Noggin/Chordin COS7 cells (dotted line). The posterior boundary of induced *otx2* expression domain is indicated by an arrowhead. The animal pole (an) is to the left and the vegetal pole (vg) to the right. Scale bars, 100 μm.

2A,E) at the late gastrula stage (bud stage, 10 h), and ectopic expression of *otx2* was detected around the grafts (30/37; 30 positive out of 37 successful operations in which the grafts are located in the ventroanimal region). No transcripts of *gsc* were detected in the secondary axis (0/10) induced by grafted HN, indicating that probably no axial mesoderm was formed. Thus, it was concluded that ectopic expression of *otx2* was directly induced by the grafted HN.

Ectopic expression of *otx2* was frequently induced around the anterior edge of the grafted HN. However, the induced expression domain always had a sharp posterior boundary which was at the same level (latitude) as the endogenous *otx2* expression domain on the dorsal side (Fig. 2B-D), suggesting that only the anterior epiblast is competent to express *otx2* in response to the grafted HN.

To examine the onset of competence to respond to organizer activity, we transplanted HN earlier before the formation of the endogenous shield. The transplantation was done at late blastula stage (sphere to dome stage, 4 h) and examined at mid gastrula stage (70-75%-epiboly, 8 h). The *otx2* expression domain in the ventral region was enlarged as compared with that in later transplantation, and sometimes fused with the endogenous one at the animal pole, forming a circumferential expression domain around the pole (18/22; Fig. 2F). Again, the ectopic expression domain shared the same posterior boundary as the host domain. In most experiments, the inducing tissue was grafted halfway between the blastoderm margin and the animal pole (Fig. 1A). When HNs of the same stage were grafted near the blastoderm margin, the grafts were located in the host trunk region and no ectopic expression of *otx2* was observed (21/21; Fig. 2G). These data suggested that the *otx2*-competent region is established by early gastrulation.

One possible explanation as to why HN is unable to specify A-P positional values within the ectoderm is that chicken cells are unable to contribute to any axial tissues, such as notochord and prechordal plate, that are known to have region-specific inducing properties (Hemmati-Brivanlou et al., 1990; Ang et al., 1994). We therefore examined whether endogenous organizer tissue (embryonic shield) could impart A-P positional information to the epiblast. In spite of the presence of the axial mesoderm derived from the transplanted shield (visualized by biotin-peroxidase staining), the posterior boundary of ectopic expression of *otx2* was always located at the same latitude as that of the host dorsal expression (26/30; Fig. 2H-K). By contrast, the donor cells, which were distributed in the host trunk region by posterior transplantation (transplantation near the blastoderm margin), never induced *otx2* expression (25/25; Fig. 2L) but induced posterior type CNS, which was shown by ectopic expression of *krox-20* (a marker gene of rhombomere 3 and 5; Oxtoby and Jowett, 1993) in later stages (Fig. 2N). *otx2* was induced only when the inducing tissues were present near the animal pole (compare Fig. 2M with Fig. 2N).

Our results suggest that the primary role of organizer tissues transplanted to the ventral side of the embryo is to convert non-neural to neural ectoderm. To investigate this issue further, instead of transplanting organizer tissue, which consists of different subpopulations of cells (Kimmel et al., 1990, zebrafish; Shih and Fraser, 1995, zebrafish; Storey et al., 1995, chick), we used COS7 cells secreting Noggin and Chordin, both potent neural-inducing molecules (Lamb et al., 1993; Sasai et al., 1994). In transplanted embryos, neither *gsc* nor *ntl* was ectopically induced in the hosts, indicating that no axial mesoderm was formed around the grafts (data not shown). The Noggin/Chordin COS7 exactly mimicked the organizer transplants. In response to the neural inducers, only the epiblast near the animal pole expressed *otx2* while the epiblast near the blastoderm margin expressed *hoxa-1*. Again, each expression boundary on the ventral side was shared by that of the host domain on the dorsal side, indicating that both anterior and posterior information are present in the epiblast (Fig. 2O-R).

Taken together, the transplantation of organizer tissues demonstrated that a differential competence of the epiblast existed in entire embryo along the A-P axis.

Induction of anterior neural marker with a clear A-P pattern in ventralized embryos

The above results did not rule out the possibility that the

endogenous early organizer specifies the A-P pattern in the epiblast and that our transplants are simply unable to override this initial specification. To test this possibility, we transplanted Noggin/Chordin COS7 into ventralized zebrafish embryos that lacked a shield and all axial derivatives (Mizuno et al., 1997; Fig. 3A), and determined whether the induced neural tissue had A-P polarity. We confirmed that, in ventralized embryos, *ntl* was normally expressed in the blastoderm margin but that neither *gsc* (a marker for early organizer) nor *otx2* expression was detected (Fig. 3B-E). As shown in Fig. 3F,G, *otx2* was induced by the graft only in the anterior region of the ventralized embryos and the expression domain had a clear posterior boundary (9/9), indicating that neural tissue induced in the absence of early organizer possessed an A-P pattern.

Roles of the yolk cell and the non-axial marginal mesoderm in A-P specification of the epiblast

We then asked which tissues and factors are involved in the specification of the epiblast. We first focused on the yolk cell which is known to be a source of mesoderm-inducing signals in fish (Mizuno et al., 1996). When transplanted in the animal pole region at the blastula stage (Fig. 4A), the yolk cell suppressed *otx2* (34/34) and induced *hoxa-1* (31/32) in the surrounding cells (Fig. 4B,C). Histological examination revealed ectopic *hoxa-1* expression in the epiblast close to the mesoderm, which was induced by the grafted yolk cell (data not shown). These results suggest that the yolk cell and/or mesoderm posteriorize the epiblast during normal development.

Given the potential role for fibroblast growth factors (FGFs) in mesoderm induction (Kimelman and Kirschner, 1987), we blocked the mesoderm formation by the injection of mRNA encoding dominant negative *Xenopus* FGF receptor (XFD) at the 1-cell stage. As reported (Amaya et al., 1991; Griffin et al., 1995), inhibition of FGF-receptor signalling by XFD suppressed the mesoderm formation, leading to complete loss of trunk and tail. Furthermore, we frequently observed that, in XFD-injected embryos, the posterior boundary of endogenous *otx2* expression domain shifted posteriorly near the margin (15/21), while expression of *hoxa-1* was greatly reduced (8/8) (Fig. 4D-G). In the injected embryos, the effect was sometimes limited probably due to an uneven distribution of injected RNA (Griffin et al., 1995). However, this kind of sample revealed a close correlation between a loss of the marginal mesoderm (indicated by a loss of Ntl staining) and a posterior shift in the *otx2*-expression domain (Fig. 4E), suggesting that the mesoderm in the margin is a primary tissue involved in the A-P specification of the epiblast. The transplantation experiments (Fig. 4H) confirmed that the *otx2*-competent region on the ventral side also expanded near the

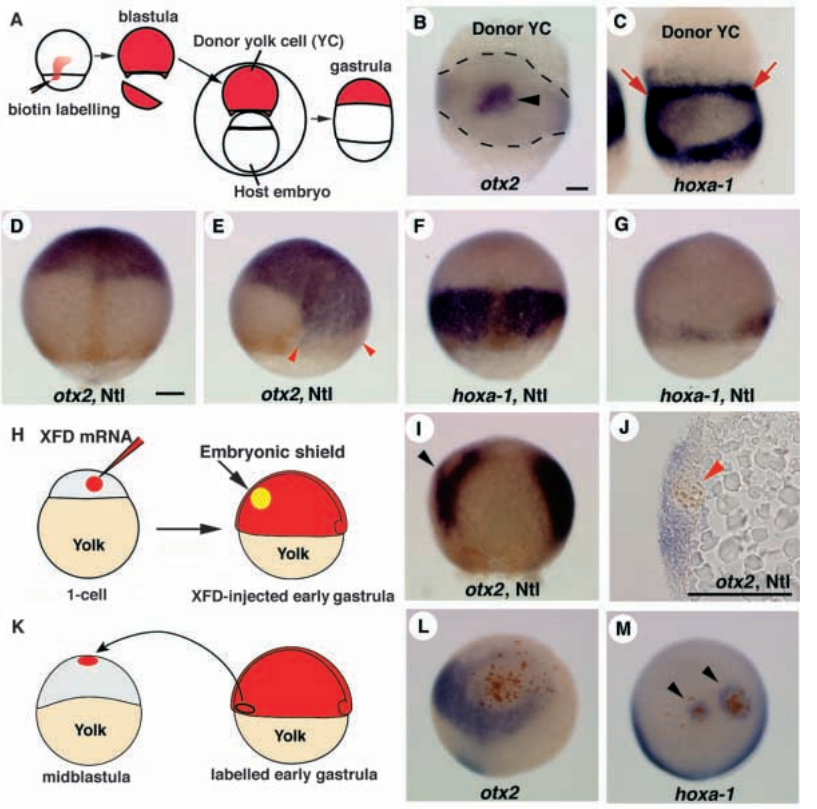

Fig. 4. Roles of the yolk cell, the marginal mesoderm and FGF-R signalling in A-P patterning of the epiblast. Embryos are oriented with the animal pole to the top. (A-C) Transplantation of the yolk cell (YC) in the animal pole region. (A) Schematic representation of the experiment. The recombinants were fixed when the sibling reached 80%-epiboly and examined for *otx2* (B) or *hoxa-1* (C) expression by in situ hybridization. In the recombinant, *otx2* expression domain is reduced into a small patch (arrowhead) in between both YC, while the donor YC induces *hoxa-1* expression (red arrows) in the animal pole region of the host. Dotted lines in B indicate the blastoderm margin on both sides. (D-G) Injection of mRNAs encoding dominant-negative FGF receptor (XFD) (E,G) or control (HAVnot) mRNAs (D,F). The gastrula embryos injected at the 1-cell stage were hybridized with *otx2* (D,E) or *hoxa-1* (F,G) probe, followed by anti-Ntl staining (light brown). Dorsal views are shown. The *otx2* expression domain shifts toward the blastoderm margin in XFD-injected embryos. Probably due to an uneven distribution of injected RNAs, the effects of RNA injection were sometimes limited. The posterior shift in the expression boundary is always correlated with the loss of the mesoderm in the blastoderm margin (a loss of the Ntl staining is indicated by a pair of red arrowheads in E). The XFD injection greatly reduces *hoxa-1* expression (G). Control RNA injection does not affect these gene expressions in the embryos (D,F). (H-J) Transplantation of the shield into XFD-injected early gastrula (6 h). (H) Schematic representation of the experiment. (I) Lateral view of the host embryo (late gastrula, 10 h) stained with *otx2* probe and anti-Ntl antibody. Ectopic (to the left, arrowhead) as well as endogenous (to the right) expression have no clear posterior boundary but extend near the margin. (J) Cross section of the ventral part of the host shown in I. Ectopic expression is induced around the donor-derived notochord (positive for Ntl staining, red arrowhead). (K-M) Transplantation of the ventral-marginal cells into the animal-pole region of the host blastula. (K) Schematic representation of the experiment. The transplanted embryos (late gastrula, 10 h) were stained with *otx2* (L, oblique animal-pole view) or *hoxa-1* probe (M, animal-pole view) and then the donor cells stained. *otx2* expression is suppressed while *hoxa-1* expression is induced (arrowheads) around the transplanted donor cells (brown). Scale bars, 100 μm.

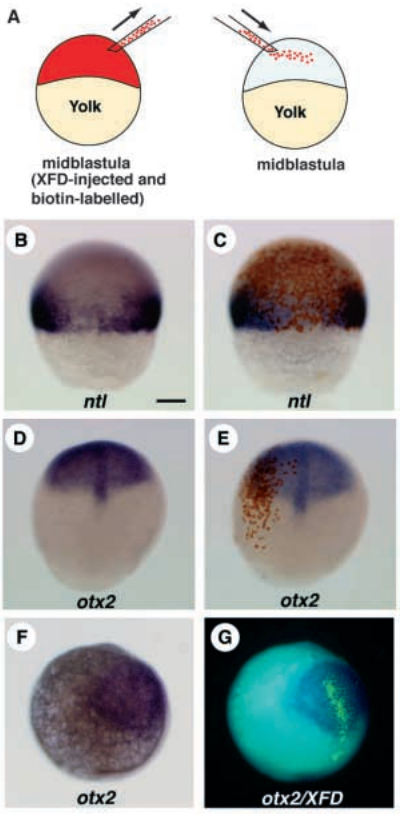

Fig. 5. Role of FGF-R signalling in defining the posterior boundary of the *otx2*-expression domain. Embryos are oriented with the animal pole to the top. (A) Schematic representation of the experiment. The normal host blastula was transplanted with XFD-injected blastomeres. (B,C) The transplanted early gastrula (shield) stained with *ntl* probe (B), followed by biotin-peroxidase. The donor cells do not express *ntl* transcripts even in the marginal region. Note that the host marginal cells surrounded by XFD-donor cells tended to be less positive for *ntl* transcripts. This is probably due to the community effect (Gurdon et al., 1993) or the absorption of ligands by XFD-overexpressed donor cells. (D,E) The transplanted embryos (late gastrula, 10 h) were first stained with *otx2* probe, followed by staining donor cells. Dorsal views of the same host before (D) and after (E) staining of the donor cells are shown. XFD-injected blastomeres (brown in E) strictly follow the host *otx2*-expression boundary. (F,G) The host embryo (late gastrula, 10 h) hybridized with DIG-labelled *otx2* probe and fluorescein-labelled *XFD* probe is seen under transmitted light (F) or under ultraviolet light (G). The blastomeres containing XFD mRNAs (light green in G) follow the host *otx2*-expression boundary. Scale bar, 100 μm.

margin in the XFD-injected embryos: ectopic expression domain did not have a clear posterior boundary, extending along the transplanted inducing tissue (Fig. 4I,J).

To directly examine the role of marginal mesoderm, the ventral marginal cells from early gastrula (shield stage, 6 h) were transplanted in the animal-pole region (future *otx2*-expression region) of the blastula embryo (sphere, 4 h) and examined for gene expression at the gastrula stage (Fig. 4K). As shown in Fig. 4L,M, the marginal cells suppressed *otx2* expression (7/7) and induced *hoxa-1* (11/16) in the surrounding host cells, implying that the cells in the margin possess posteriorizing activity.

FGF-R signalling is not directly involved in defining the posterior boundary of *otx2*-expression domain

Since XFD was overexpressed in whole embryos, RNA injection experiments did not rule out the possibility that FGF-R signalling is directly involved in restricting the expression of *otx2* in the anterior region. To test this possibility, we examined whether cells expressing XFD were still able to respond to A-P positional information by transplanting XFD-injected blastomeres into the normal host blastula (Fig. 5A). XFD-injected cells, when incorporated into the mesoderm region, did not express *ntl* (Fig. 5B,C). By contrast, XFD-injected cells strictly followed the normal *otx2*-expression pattern in the host (15/15; Fig. 5D-G): they expressed *otx2* when located within the host expression domain while they never expressed outside the region, implying that FGF-R signalling is not directly involved in defining the *otx2*-boundary. The presence of XFD mRNAs in transplanted cells was confirmed by double in situ hybridization with XFD and *otx2* probes (Fig. 5F,G).

DISCUSSION

Differential competence to neural inducers in zebrafish epiblast

In this paper, we present several lines of evidence that initial A-P pattern within neural tissues is determined by differential competence of the epiblast. Involvement of the marginal mesoderm in A-P specification of CNS has been shown by Woo and Fraser (1997). We further show that the signals from the marginal mesoderm impart a differential competence to the epiblast.

Although we use three different inducing tissues, embryonic shield, chicken Hensen's node and mammalian COS7-secreting organizer factors, we obtained essentially the same results. They induced neural tissue but the A-P identity of the induced neural tissue is dependent upon the position of the inducing tissues within the epiblast. Thus, *otx2*, an anterior neural marker, was only ever induced in anterior regions of the embryo, irrespective of the position of the grafts, and *hoxa-1*, a posterior neural marker, was induced only in the posterior regions. Furthermore, the boundary of each ectopic expression domain on the ventral side was always at an equivalent latitude to that of the endogenous expression of the dorsal side of the embryo. These results demonstrated that the competent region for each neural marker exists in a circumferential ring in the epiblast along the A-P axis.

It is worth emphasizing that differential competence to neural inducers is not intrinsic to the epiblast but that its establishment depends on the signals from the marginal mesoderm. The competence can be easily changed by transplantation or suppression of the marginal mesoderm. Thus, the differential competence of the epiblast is detected only in vivo in the presence of the marginal mesoderm. Furthermore, we do not know exactly when differential competence is established in the epiblast. However, our early transplantation experiments (Fig. 4L,M) suggest that the marginal mesoderm influences the epiblast at least by the onset of gastrulation.

Our finding, differential competence in the epiblast along the A-P axis, seems somewhat contradictory to a widely accepted model for neural induction in other vertebrates. In the model,

regional specification of the CNS depends on the nature of the inducers but not on differential competence of the ectoderm; for example, the early organizer or anterior axial mesoderm induces head structures, while the late organizer or posterior mesoderm induces the spinal cord (for example, Spemann, 1938). However, it is worth noting that recent experiments supporting this model have been carried out in vitro or without examining the influence of the host tissue (Hemmati-Brivanlou, 1990, *Xenopus*; Storey et al., 1992, chick; Ang and Rossant, 1993, mouse). Furthermore, we observed that, as development proceeded, the *otx2* expression domain in the secondary axis was posteriorly displaced with respect to that of the host (see Figs 1B, 2M), probably due to a difference in cell growth; the induced axis is usually smaller than that of the host. Thus, an examination at later stages like classical experiments would give the impression that the expression boundary of a secondary axis bears little relationship to that of a host axis.

Our data do not exclude the notion that the nature of inducing tissues determines the type of CNS. Sagerström et al. (1996) have shown that explanted zebrafish shield becomes into an axis with precise A-P neural pattern, suggesting that the shield itself has also A-P information. Probably, together with differential competence of the epiblast, the signals from the axial mesoderm are required for regional specification of CNS, especially in later development (Shimamura and Rubenstein, 1997).

In previous experiments by Hatta and Takahashi (1996) and Shih and Fraser (1996), secondary axes were frequently induced in zebrafish embryos by the organizer transplantation. However, those secondary axes lacked anterior head structures, which could be interpreted as the results of posterior transplantation: they grafted organizer tissues into the ventral marginal zone of zebrafish embryos. Thus, unlike in amphibians, the organizer must be grafted anteriorly near the animal pole to obtain a complete secondary axis in zebrafish.

A-P specification in the epiblast is independent of D-V axis formation

The experiments with ventralized zebrafish embryos have shown that the A-P pattern in the epiblast is not imposed by early organizer. Since ultraviolet (UV) irradiation does not work well in fish embryos (Strähle and Jesuthasan, 1993), ventralized zebrafish embryos were obtained by the removal of the vegetal yolk hemisphere soon after fertilization. As in goldfish (Mizuno et al., 1997), zebrafish embryos from which the vegetal yolk mass had been removed showed a typical ventralized phenotype: no dorsal structures such as embryonic shield, notochord and neural tube were formed, leading to a bilaterally symmetrical morphology (T. Mizuno et al., unpublished data). In situ hybridization analysis confirmed that neither *gsc* nor *otx2* was expressed in the embryos while *ntl* was normally expressed in the margin, indicating that mesoderm induction proceeds normally without D-V specification (Fig. 3). This phenotype is similar to that of *Xenopus* ventralized embryos generated by UV irradiation (Cook and Smith, 1987). Surprisingly, *otx2* was induced by Noggin/Chordin COS7 with a clear A-P pattern in the ventralized embryo, indicating that differential competence in the epiblast appears independent of D-V axis formation.

Signals from the non-axial marginal mesoderm posteriorize the epiblast

Our transplantation experiments show that the yolk cell that is known as a mesoderm inducer (Mizuno et al., 1996) up-regulated the posterior neural marker and suppressed the anterior marker in the surrounding cells. Since the mesodermal cells in the margin, when transplanted in the animal-pole region, mimicked the effect of the yolk cell on A-P neural patterning, it is reasonable to conclude that the marginal mesoderm induced by the yolk cell posteriorizes the epiblast during normal development. This is consistent with recent studies showing the posteriorizing activity of the non-axial mesoderm in zebrafish and chicken embryos (Sagerström et al., 1996; Bang et al., 1997; Woo and Fraser, 1997). Especially, Woo and Fraser (1997) have shown in zebrafish that the marginal cells posteriorized forebrain progenitors when grafted nearby, resulting in an ectopic hindbrain-like structure. In addition to transplantation experiments, we show here a close correlation between the loss of the marginal mesoderm and a posterior shift in the *otx2*-expression domain in XFD-injected embryos, confirming that the marginal mesoderm is a source of posteriorizing signals. Since A-P information also exists on the ventral side where no neural induction takes place, it is

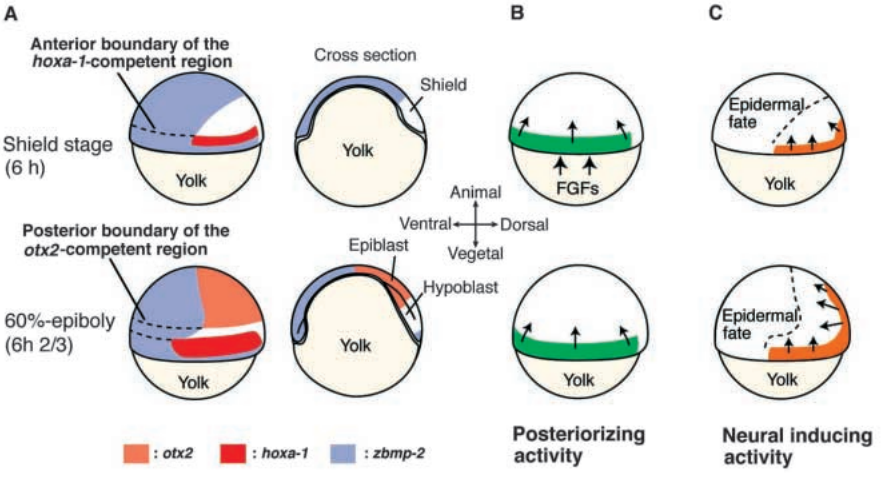

Fig. 6. Proposed model for zebrafish neural induction and A-P specification. (A) Normal expression patterns of zebrafish *otx2* (Li et al., 1994; Mori et al., 1994), *hoxa-1* (Alexandre et al., 1996) and *bmp-2* (Nikaido et al., 1997) in early gastrula stages. The competent region for *otx2* and *hoxa-1* expression on the ventral side is established by the time each gene starts to be activated on the dorsal side. (B) Organizer-independent A-P specification by the posteriorizing signals produced by the mesoderm (green) in the blastoderm margin. The production of the signals is mediated by FGF signalling. Since the grafted shield can not respecify the competence in the epiblast, the signal must be absent in the organizer region. (C) Neural induction on the dorsal side. The neural inducers (probably, zebrafish Chordino; Schulte-Merker et al., 1997) produced by the organizer region neuralize the dorsal epiblast by antagonizing BMP signalling (Sasai et al., 1994).

likely that the posteriorizing signals have no neural-inducing activity but they affect the competence of the epiblast.

What molecules mediate posteriorization of the epiblast?

There is accumulating evidence in *Xenopus* that FGFs or FGF-R signalling function in specifying posterior identity in the developing neural tube (Kengaku and Okamoto, 1993 and 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995; Doniach, 1995). The present study showed that, in XFD-injected zebrafish embryos, the posterior boundary of both endogenous and ectopic *otx2*-expression domains shifted near the margin. These results demonstrate that all the epiblast remains competent to express *otx2* if the FGF-R signalling is blocked. However, the requirement of FGF-R signalling seems indirect because XFD-injected cells, transplanted in the posterior region of the host, still responded to A-P positional information. Woo and Fraser (1997) also demonstrated that FGF beads, implanted in the animal pole of zebrafish gastrula, caused severe perturbation of the host's forebrain but did not induce the posterior neural marker, suggesting that FGFs may act in concert with other factors to be responsible for the posteriorizing activity. Consistent with these results in zebrafish, the conclusions of recent transgenic and in vitro tissue recombination experiments using *Xenopus* indicate that FGF-R signalling may not be solely responsible for the neural posteriorizing activity (Kroll and Amaya, 1996; Bang et al., 1997).

Retinoic acid (RA) is an attractive candidate (Conlon, 1995; Blumberg et al., 1997). However, RA may not be an endogenous posteriorizing factor in zebrafish early embryos, because it has been reported that, although the amount of expression was increased, the anterior boundary of *hoxa-1* expression remained unchanged in RA-treated zebrafish gastrula (Alexandre et al., 1996). Therefore, the nature of the endogenous posteriorizing signal(s) remains unclear.

A model for induction and A-P patterning of the zebrafish neural tissue

From our present data, we present a model for neural induction and A-P specification in zebrafish (Fig. 6B, C), which is similar to that of Woo and Scott (1997). There exists two distinct signals, posteriorizing signals involved in A-P specification of the epiblast, and organizer factors (Schulte-Merker et al., 1997) involved in neural induction. This model is different from the classical amphibian two-step model (Nieuwkoop, 1950; Toivonen and Saxén, 1968) in that posteriorizing signals (may be equivalent to transformers) are secreted by non-axial mesoderm and that the two signals are independently regulated by the yolk cell which is responsible for induction and dorsal specification of the mesoderm (Mizuno et al., 1997).

Before and/or during neural induction, the epiblast near the blastoderm margin, including on the ventral side, loses the competence to express anterior neural markers under the influence of posteriorizing signals produced by the marginal mesoderm. When the epiblast on the dorsal side is exposed to neural inducers produced by the organizer region, the posteriorized epiblast near the margin expresses posterior neural markers, while the anterior epiblast (probably the equivalent of the amphibian animal cap), which escapes from posteriorization, expresses anterior neural markers. Thus, A-P positional values are present in the entire embryo as differential competence of the epiblast, which imposes an initial A-P pattern on zebrafish CNS. Probably due to this A-P information present in the entire epiblast, the secondary axis induced on the ventral side always forms in the same direction as the primary axis, irrespective of the orientation of the grafted organizer.

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