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Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm

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SUMMARY

Early neural patterning in vertebrates involves signals that inhibit anterior (A) and promote posterior (P) positional values within the nascent neural plate. In this study, we have investigated the contributions of, and interactions between, retinoic acid (RA), Fgf and Wnt signals in the promotion of posterior fates in the ectoderm. We analyze expression and function of *cyp26/P450RAI*, a gene that encodes retinoic acid 4-hydroxylase, as a tool for investigating these events. *Cyp26* is first expressed in the presumptive anterior neural ectoderm and the blastoderm margin at the late blastula. When the posterior neural gene *hoxb1b* is expressed during gastrulation, it shows a strikingly complementary pattern to *cyp26*. Using these two genes, as well as *otx2* and *meis3* as anterior and posterior markers, we show that Fgf and Wnt signals suppress expression of anterior genes, including *cyp26*. Overexpression of *cyp26* suppresses posterior genes, suggesting that the anterior expression of *cyp26* is important for restricting the expression of posterior genes. Consistent with this, knock-down of *cyp26* by morpholino oligonucleotides leads to the anterior expansion of posterior genes. We further show that Fgf- and Wnt-dependent activation of posterior genes is mediated by RA, whereas suppression of anterior genes does not depend on RA signaling. Fgf and Wnt signals suppress *cyp26* expression, while Cyp26 suppresses the RA signal. Thus, *cyp26* has an important role in linking the Fgf, Wnt and RA signals to regulate AP patterning of the neural ectoderm in the late blastula to gastrula embryo in zebrafish.

Key words: Fgf, Wnt, Retinoic acid, Posteriorization, Cyp26, Raldh2, Zebrafish

INTRODUCTION

Studies of axis formation in *Xenopus laevis* have led to models in which two signaling events that are initiated by localized maternal determinants cause mesendoderm induction in the marginal zone, and organizer formation on its dorsal side at blastula-to-gastrula stages. Mesendoderm induction divides the embryo into three germ layers along the vegetal-to-animal axis, while signals from the organizer induce neural tissue at the dorsal side of the ectoderm. The neural ectoderm is patterned along its anteroposterior (AP) axis, coincident with, and subsequent to, its initial specification; this patterning is initiated by posteriorizing signals derived from prospective or definitive mesendodermal tissues (Nieuwkoop, 1950; Toivonen, 1968). Three candidate posteriorizing signals have been suggested: retinoic acid (RA) (Blumberg et al., 1997; Conlon, 1995; Durston et al., 1989; Sive et al., 1990); fibroblast growth factors (Fgfs) (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1993; Kengaku and Okamoto, 1995; Koshida et al., 1998; Lamb and Harland, 1995) and Wnts (Fekany-Lee et al., 2000; Kazanskaya et al., 2000; Kelly et al., 1995; Kiecker and Niehrs, 2001; McGrew et al., 1995; Yamaguchi, 2001). Although it is likely that factors in all three families participate in this process, the precise role of each in the temporal and spatial aspects of neural patterning as well as the molecular consequences of their action have not been fully clarified.

In zebrafish, a region called the yolk syncytial layer (YSL), which is located beneath the blastoderm, may have a role in both mesendoderm and organizer induction (Mizuno et al., 1996). As a consequence of inductive signals that emanate from the YSL, the blastoderm margin forms the mesendoderm, one side of which develops into the organizer (which, in turn, induces neural specification within the dorsal ectoderm). As in *Xenopus*, there is evidence for signals emanating from the prospective mesendodermal layer that transform early neural ectoderm from an anterior to a posterior fate (Koshida et al., 1998).

One feature that adds complexity to the mechanisms of AP patterning is the repeated use of the same type of signal in different stages and regions of the embryo, with context-dependent consequences. Fgfs and Wnts are both expressed in undifferentiated mesendoderm from the blastula stage onwards, and in the area of the presumptive midbrain-

hindbrain region, beginning at the late gastrula stage (Furthauer et al., 1997; Kelly et al., 1995; Phillips et al., 2001). Signals mediated by members of these two major classes of secreted factors are involved in early AP patterning in the neural ectoderm, as well as subsequent regional patterning processes within the developing brain (Houart et al., 2002; Kim et al., 2000; Reifers et al., 1998). To avoid having to consider a large range of these complexities, we have focused on the earliest manifestation of AP specification that is evident from the late blastula through gastrula stages.

We have searched for genes that may have an early role in axis formation as part of a random in situ screen for regionally expressed genes in zebrafish embryos (Kudoh et al., 2001). In this screen, we noted the anterior neural ectodermal expression of the gene *cyp26/P450RAI*, which encodes all trans retinoic acid 4-hydroxylase, an enzyme that degrades and inactivates RA. Zebrafish *cyp26* has originally been cloned from regenerating fin tissue as an RA-responsive gene (White et al., 1996). We find that *cyp26* is specifically expressed in the presumptive anterior neural ectoderm from a surprisingly early stage (from the late blastula onwards). At the early gastrula stage, the earliest known marker of posterior neural ectoderm, *hoxb1b* (Alexandre et al., 1996), is expressed in a complementary pattern to *cyp26*. We focused on the regulation of this earliest subdivision along the AP axis in the neural ectoderm, using *cyp26* and *hoxb1b* as our primary tools of analysis. We show that posteriorization of the neural ectoderm has two separable steps: suppression of anterior gene expression and activation of posterior gene expression. Suppression of anterior gene expression in the posterior region is the first step of AP differentiation and is caused by Fgfs/Wnts in an RA-independent pathway. The activation of posterior gene expression is the second step, and this event is RA dependent. These two posteriorizing steps are linked through the regulation and function of the *cyp26* gene.

MATERIALS AND METHODS

Constructs, mRNA synthesis and embryo injections

The coding region in *cyp26* was amplified by PCR and subcloned into the pCS2+ expression vector. Cyp26-GFP fusion construct was made using PCR primers forward (F; ccggatcctcgaggtcgacccacgcg) and reverse (R; ccgaattcggtgtcagagcccaggatgg), which amplifies 82 bp of 5′ non-coding region with 375 bp of coding region. This PCR product was inserted to pCS2+ and subsequently EGFP cDNA was inserted into the 3′ end of cyp26 cDNA. mRNA was synthesized by mMESSAGE mMACHINE (Ambion). The following mRNAs were injected into zebrafish embryos: *cyp26* (500pg), *cyp26*-EGFP (300pg), *fgf3* (50pg) (Koshida et al., 2002), *XFD* (500pg) (Amaya et al., 1991) and *dkk1* (50pg) (Hashimoto et al., 2000).

Retinoic acid (RA) and LiCl treatments

RA was stored at 10^{-3} M in ethanol and diluted to 10^{-6} M in fish water before use. Embryos were treated with RA from the 40% epiboly stage onward for 80 minutes, followed by thorough washing. For LiCl treatment, 50% epiboly stage embryos were exposed to 300 mM LiCl for about 8 minutes and washed immediately with fish water several times. These embryos were fixed at late gastrula stage and stained by in situ hybridization.

Injection of morpholino oligonucleotides

A sequence complementary to the region of *cyp26* cDNA around the start codon was used to synthesize a morpholino antisense oligonucleotide, mCYP1, by Gene Tools (Philomath, USA). The sequence of mCYP1 is 5′-cgcaactgatcgccaaaacgaaaaa-3′. Five nanograms of morpholino were injected at the one- to two-cell stage into the yolk. For comparison, the same amount of standard control morpholino (Gene Tools) was injected.

Whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, manually dechorionated and stored in methanol up to several weeks. In situ hybridization was performed essentially as described previously (Kudoh and Dawid, 2001). Probes for the following genes were used: *cyp26*, *iro1*, *meis3*, *hoxb1b*, *otx2* and *ntl*. These probes were obtained from our in situ-based screen (Kudoh et al., 2001). For injected and drug-treated embryos, 15 to 30 embryos were used in each experiment.

RESULTS

Early expression pattern of *cyp26*

cyp26 is first expressed at the late blastula stage in two distinct regions, at the animal side of the dorsal ectoderm (Fig. 1A,A′, closed arrowhead) and at the blastoderm margin (Fig. 1A,A′, open arrowhead). These two domains continue to express *cyp26* up to the bud stage at the end of gastrulation (Fig. 1A-D). At later gastrula and somitogenesis stages, *ntl* expression also marks the notochord (Fig. 1C,F). *cyp26* expression in the anterior neural ectoderm decreases during gastrulation so that only weak expression in a region surrounding the neural plate is seen by the three-somite stage (Fig. 1D-F). By contrast, *cyp26* expression in the blastoderm margin persists as this domain extends towards the posterior, contributing to the tail. This expression pattern suggests that *cyp26* is important for establishing the anterior character of neural ectoderm during gastrula stages, as well as for the formation of the tail during later development.

Complementary expression of *cyp26* and *hoxb1b*, an early marker of posterior neural ectoderm

The expression of *cyp26* was compared with the posterior neural ectoderm expression of *hoxb1b* (originally named *hoxa1*) (Alexandre et al., 1996). At the late blastula stage, there is a small gap between the animal and marginal domain of *cyp26* expression (Fig. 2A, asterisk); this gap is expanded subsequently through convergence-extension movements during gastrulation (Fig. 2B-E). At about the 60% epiboly stage, *hoxb1b* expression is initiated within this gap. When *cyp26* and *hoxb1b* are doubly stained by single-color in situ hybridization, they show complementary expression patterns from the onset of *hoxb1b* expression to the end of gastrulation (Fig. 2K-M); a narrow gap persists between the *cyp26* and *hoxb1b* domains, emphasizing the exclusiveness of these domains. In these double-labeled embryos, cells completely double negative for *cyp26/hoxb1b* are not observed at high magnification, suggesting that this gap is not a distinct domain but represents the border of the anterior and posterior domains where the expression of the two markers decreases. This complementarity suggests that initial AP patterning in the neural ectoderm is reflected by the anterior expression of *cyp26* in the late blastula, while the expression of *hoxb1b*, the earliest posterior marker, constitutes a subsequent step in the evolving process.

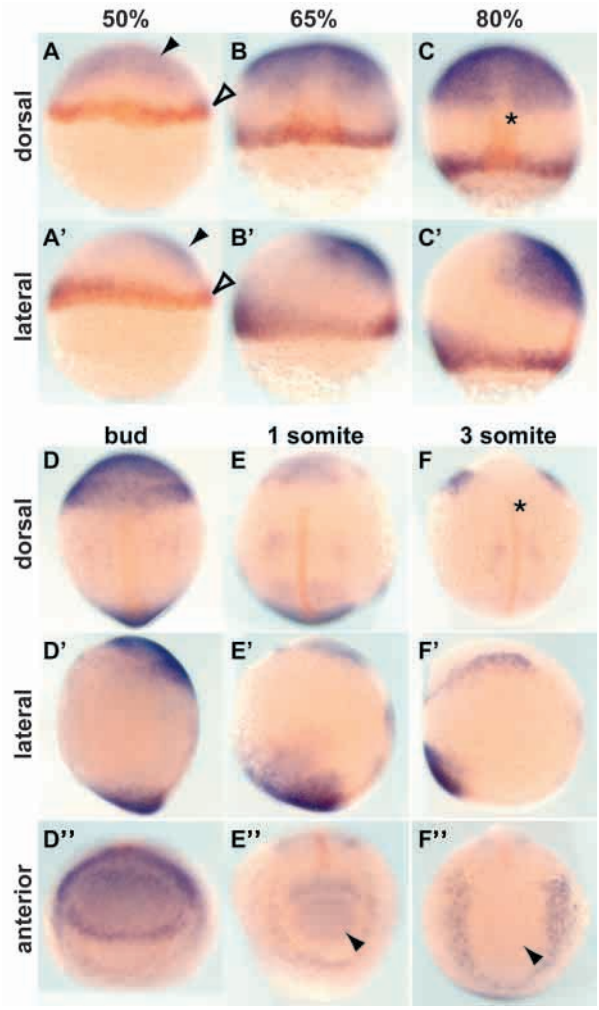

Fig. 1. Expression of *cyp26* at gastrula to early somitogenesis stages. Views of whole embryos (orientation indicated to the left of each row) at the stages indicated on top of each column. *cyp26* was stained by in situ hybridization (purple), while *ntl* (red) was used to mark the blastoderm margin (A, A′, open arrowhead) and developing notochord (C,F, asterisk). *cyp26* is expressed in the presumptive anterior neural ectoderm (A,A′ filled arrowhead) and at the blastoderm margin (A,A′ open arrowhead) throughout gastrulation (B-D). Subsequently, expression in the anterior neural ectoderm decreases rapidly (E″, F″ filled arrowhead), while expression continues in the tail bud.

To further resolve and confirm expression domains, *cyp26* was also analyzed in combination with *iro1*, *krox20* and *raldh2*. *iro1* is expressed as two pairs of bands at late gastrula stage; the anterior bands contain the presumptive midbrain-hindbrain boundary and possibly rhombomere 1 (Itoh et al., 2002). The anterior domain of *iro1* and the *cyp26* expression domain share a common posterior border of expression, suggesting that at this stage, the posterior end of *cyp26* expression is around prospective rhombomere 1 (Fig. 2P). Double staining of *cyp26* with *krox20*, which marks rhombomeres 3 and 5 at bud stage, revealed a gap between the posterior edge of *cyp26* expression and rhombomere 3 (Fig. 2Q). This gap presumably represents the future rhombomere 2. *raldh2*, which encodes a major RA synthesis enzyme, is expressed in the posterior paraxial mesoderm in a pattern complementary to *cyp26* along the AP axis, with a small but widening gap between the two expression domains (Fig. 2N,O). The complementarity of RA synthesis (*raldh2*) and degradation (*cyp26*) domains supports the idea that refinement of RA-positive and -negative domains of the ectoderm is achieved by the action of these opposing gene products (Chen et al., 2001; Swindell et al., 1999).

The expression of *cyp26* and of other AP-marker genes is regulated by Fgf signals

As *cyp26* exhibits extremely early anterior neuroectodermal expression, we tested the regulation of its expression by Fgf, a candidate posteriorizing factor; the behavior of *cyp26* was compared with another anterior neural gene, *otx2*, and to the posterior gene *hoxb1b*. To generate conditions that correspond to both gain and reduction of function of Fgf signaling, *fgf3* and dominant-negative Fgf-receptor (*XFD*) (Amaya et al., 1991) mRNAs were injected into one- to two-cell stage embryos. *fgf3* mRNA injection led to suppression of anterior expression of *cyp26* and *otx2* (Fig. 3B,J), whereas expression of the posterior gene *hoxb1b* was expanded anteriorly (Fig. 3F). *XFD* mRNA injected embryos showed the opposite phenotype in that *cyp26* and *otx2* domains were expanded posteriorly (Fig. 3D,L) (Koshida et al., 1998) and *hoxb1b* was suppressed (Fig. 3H arrowhead). These results indicate that Fgf signaling is necessary and sufficient for the suppression of the anterior genes *cyp26* and *otx2*, as well as for the activation of the posterior gene *hoxb1b* during gastrulation.

RA modulates the expression of AP-marker genes

RA has been shown to affect the specification of AP values in the developing nervous system of different vertebrates and is a candidate posteriorizing signal (Blumberg et al., 1997; Conlon, 1995; Durston et al., 1989; Niederreither et al., 1999; Sive et al., 1990). RA is also the substrate of the *cyp26* gene product. To examine the role of RA in early AP pattern specification in the neural ectoderm, we examined the expression of the anterior genes *cyp26* and *otx2* and the posterior genes *hoxb1b* and *meis3* in RA-treated embryos. Consistent with the designation of RA as a posteriorizing factor, the expression of *hoxb1b* and *meis3* was expanded (Fig. 4D,F) and that of *otx2* was suppressed (Fig. 4H). However, *cyp26* expression was expanded in RA-treated embryos, in spite of the anterior character of its ectodermal expression domain (Fig. 4B). This outcome is most likely to be due to the fact that *cyp26* is a direct target of RA and acts as an RA-responsive gene in different stages of embryogenesis such as fin formation (Loudig et al., 2000; White et al., 1996). As it encodes an RA-metabolizing enzyme, *cyp26* may be part of a negative feedback loop that limits RA signaling in different biological contexts.

Overexpression of *cyp26* suppresses posterior neural genes

To analyze the function of *cyp26* and its substrate, RA, in early AP patterning, *cyp26* mRNA was injected into one blastomere of two-cell embryos, and the expression of AP marker genes was examined during gastrulation (Fig. 5). Consistent with the activity of its gene product as an RA-degrading enzyme, *cyp26* injection showed the opposite effect to application of RA on the expression of the posterior genes *hoxb1b* and *meis3*, both

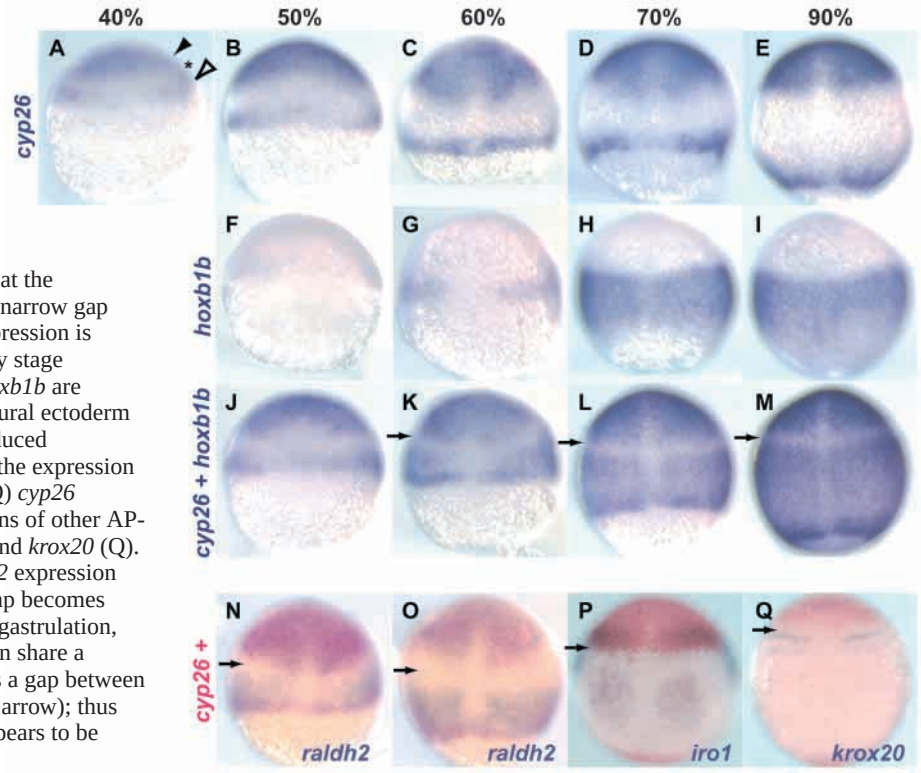

Fig. 2. *cyp26* and *hoxb1b* expression domains define an early AP boundary within the prospective neuroectoderm. Dorsal views of whole embryos at the stages indicated on top of. Genes analyzed are indicated at the left of the column or in the figure, with text color matching the in situ stain. *cyp26* expression starts at the 30 to 40% epiboly stage (A) in presumptive anterior neural ectoderm (filled arrowhead) and at the blastoderm margin (open arrowhead), leaving a narrow gap (asterisk). As gastrulation proceeds, *hoxb1b* expression is initiated within the gap at about the 60% epiboly stage (C,G,K). From this time onwards, *cyp26* and *hoxb1b* are expressed in a complementary manner in the neural ectoderm throughout gastrulation; a narrow domain of reduced expression is observed at the interface between the expression domains of these two genes (K-M, arrow). (N-Q) *cyp26* expression (red) in relation to expression domains of other AP-specific genes (purple), *raldh2* (N,O), *iro1* (P) and *krox20* (Q). There is a narrow gap between *cyp26* and *raldh2* expression domains at early gastrula (N, arrow), and this gap becomes wider by late gastrula (O, arrow). At the end of gastrulation, *cyp26* and the anterior domain of *iro1* expression share a common posterior boundary (P, arrow). There is a gap between *cyp26* and rhombomere3 staining of *krox20* (Q, arrow); thus the posterior boundary of *cyp26* at this stage appears to be positioned around rhombomere 1.

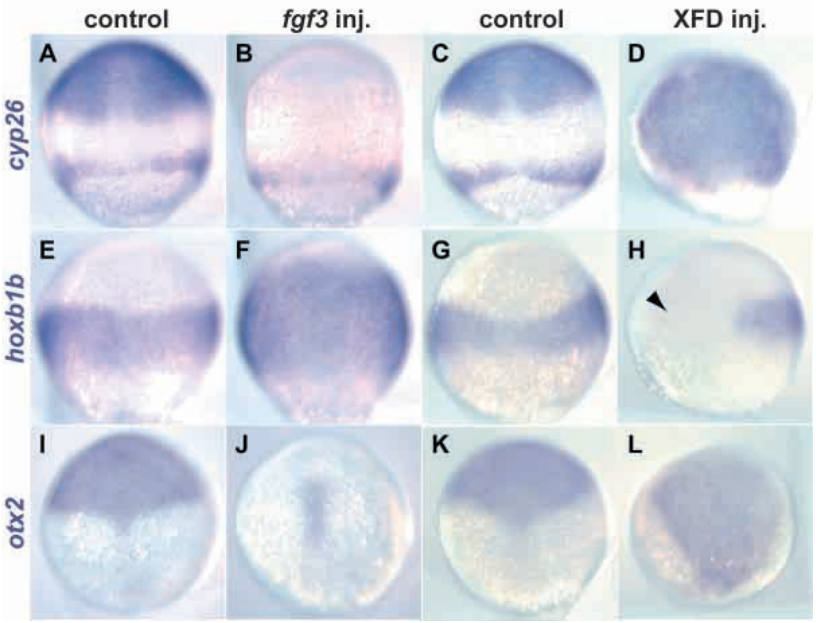

Fig. 3. Fgf signaling alters the expression of early AP-specific genes. Dorsal views of whole embryos at late gastrula stage. One- to two-cell embryos were injected with mRNAs for *fgf3* (B,F,J) or RNA encoding the dominant negative Fgf receptor, XFD (D,H,L). Embryos were stained either with *cyp26* (A-D), *hoxb1b* (E-H) or *otx2* (I-L). The anterior expression of *cyp26* and *otx2* was suppressed by Fgf3 (B,J) and expanded in a posterior direction by XFD (D,L). Expression of the posterior gene *hoxb1b* was expanded by Fgf3 (F) and suppressed by XFD (H, arrowhead). The entire embryo is affected by injection at the one-cell stage (B), while in some cases only half the embryo is affected when one of two cells is injected (H).

of which were suppressed (Fig. 5B,D). We tested an additional gene, *iro1*, which has two expression domains, an anterior domain that overlaps with *cyp26* and a posterior domain that overlaps with *hoxb1b* (Fig. 2P). Consistent with the results above, only the posterior domain of *iro1* was suppressed by *cyp26* (Fig. 5F, arrowhead).

By contrast, expression of the anterior gene *otx2* was not affected by *cyp26* (Fig. 5H). To confirm the differential effect of *cyp26* on anterior and posterior neural markers, control and injected embryos were double stained with *otx2* and *hoxb1b*, showing that *hoxb1b* expression was decreased, whereas that of *otx2* was neither suppressed nor expanded (Fig. 5J). However, injection of *cyp26* could at least partially restore *otx2* expression in the presence of RA (Fig. 5L).

The effects of *cyp26* on gene expression illustrated in Fig. 5 for mid-to-late gastrula stages were also seen at early gastrulation (data not shown). These results suggest that RA is necessary for the induction of posterior gene expression, but not for the suppression of anterior gene expression. This lack of an effect of *cyp26* in promoting anterior gene expression in the posterior domain represents a difference between RA and Fgf in their action as posteriorizing agents (compare Fig. 3L with Fig. 5H,J).

Distinct roles of Fgf and RA in patterning the neural ectoderm

To investigate the relationship of the

posteriorizing signals delivered by Fgf and RA, we applied agonists and antagonists of these two signals to the same embryos. When *fgf3* and *cyp26* mRNAs were co-injected, *fgf3*-mediated activation of *hoxb1b* expression was strongly suppressed (Fig. 6B,C). This result suggests that Fgf3-dependent induction of *hoxb1b* is mediated by RA. By contrast, Fgf3-mediated suppression of *otx2* expression was not affected by co-injection of *cyp26* (Fig. 6E,F). Thus, the effect of Fgf in suppressing *otx2* does not require RA, and suppression of the RA signal is not sufficient for the induction of *otx2*.

In complementary experiments, embryos injected with *XFD* mRNA were treated with RA. XFD blocks Fgf signaling, and consequently inhibits *hoxb1b* expression (Fig. 6H). Consistent with the results above, this XFD-induced suppression of *hoxb1b* was overcome by the addition of RA (Fig. 6I). Furthermore, XFD led to an expansion of *otx2* towards the posterior (Fig. 6K), and both the expanded and normal expression of *otx2* were suppressed in the presence of RA (Fig. 6L). Thus, RA can repress *otx2* expression in either the anterior or posterior compartment, but reduction of RA alone is not sufficient to induce ectopic *otx2* expression (Fig. 5H, Fig. 6F). These results support the view that RA acts downstream of Fgf in inducing posterior genes in the neural ectoderm.

Distinct roles of Fgfs and Wnts in patterning the neural ectoderm

Next, we examined the involvement of Wnt signaling in the regulation of expression of these early marker genes. LiCl inhibits the kinase activity of GSK3 and consequently activates β-catenin, a downstream effector of the canonical Wnt pathway (Klein and Melton, 1996). Activation of the Wnt pathway in the early gastrula by treatment with LiCl at the 50% epiboly stage led to suppression of the anterior markers, *cyp26* and *otx2*(Fig. 7A,B,F,G). However, the posterior marker *hoxb1b* was not induced in the anterior-most area of the embryo, although some anterior expansion was observed (Fig. 7K,L). To reduce canonical Wnt signaling, *dkk1* mRNA was injected. Dkk1 slightly expanded *otx2* and *cyp26* expression, and reduced that of *hoxb1b*, but there was always residual posterior character near the margin (Fig. 7C,M).

In order to elucidate the relationship between Wnt and Fgf signals in regulating AP polarity, we concomitantly altered activity of both pathways. XFD injected embryos were

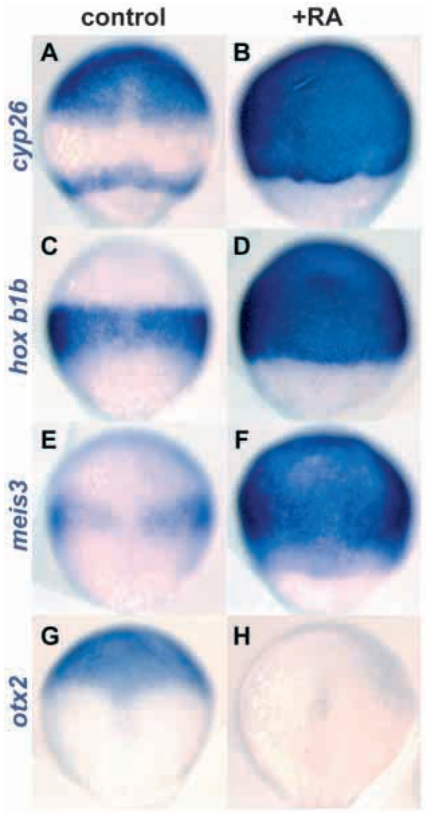

Fig. 4. RA alters the expression of early AP-specific genes. Dorsal views of whole embryos at late gastrula stage. Embryos at the 40% epiboly stage were treated with 10^{-6} M RA for 80 minutes. The embryos were fixed at the 80 to 90% epiboly stage, and stained with *cyp26* (A,B), *hoxb1b* (C,D), *meis3* (E,F) or *otx2* (G,H). The posterior genes *hoxb1b* and *meis3* were ectopically induced in the anterior region by RA (B,D), while the anterior gene *otx2* was suppressed (H). Although *cyp26* is expressed in the anterior region, its expression was activated by RA(B).

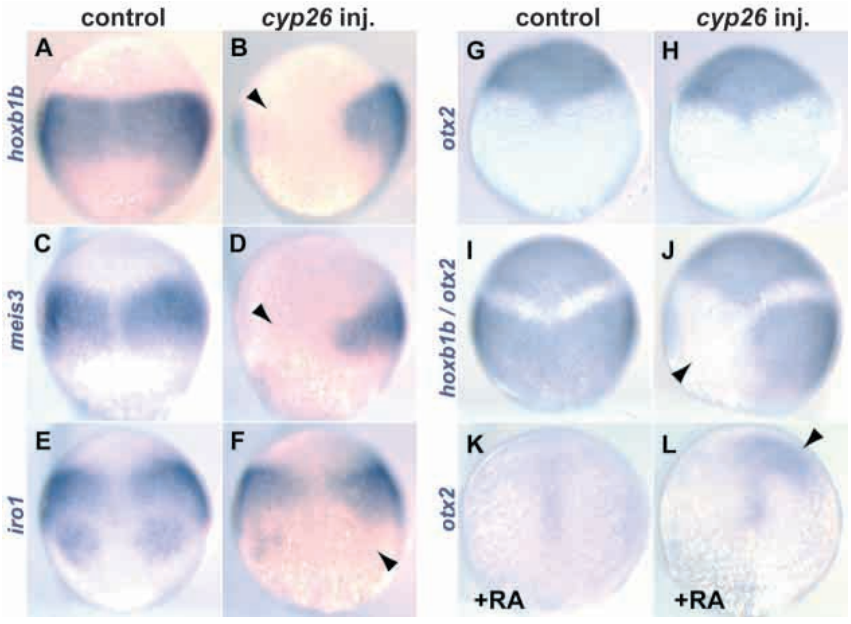

Fig. 5. Cyp26 can suppress posterior genes but does not induce anterior genes in the posterior region. Dorsal views of whole embryos at late gastrula stage. *cyp26* mRNA was injected at the two-cell stage into one blastomere. Some of the embryos were treated with RA (K,L). Embryos were stained for *hoxb1b* (A,B), *meis3* (C,D), *iro1* (E,F), *otx2* (G,H,K,L) and *otx2/hoxb1b* together (I,J). *hoxb1b*, *meis3* and the posterior domain of *iro1* were suppressed by *cyp26* injection (B,D,F,J, arrowhead), but *otx2* and the anterior domain of *iro1* expression were neither suppressed nor expanded (F,H,J). The suppression of *otx2* by RA was partially rescued by *cyp26* (L, arrowhead).

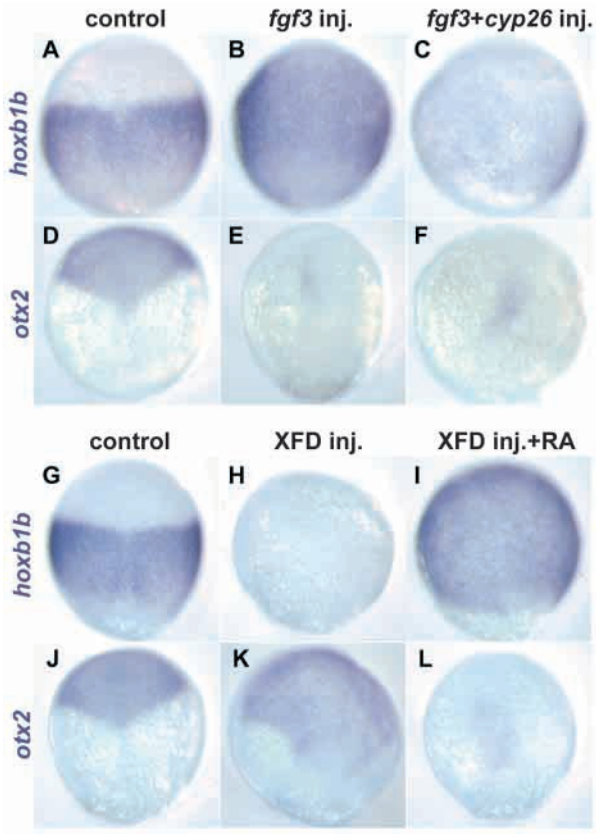

Fig. 6. Epistatic analysis of the function of Fgf and RA in patterning the neural ectoderm. Dorsal views of whole embryos at late gastrula stage. RNA for *fgf3*, alone or together with *cyp26* RNA, was injected at the one-cell stage. The embryos were stained for *hoxb1b* (A-C) or *otx2* (D-F). XFD mRNA was injected at the one-to-two cell stage, and half of the embryos were treated with RA. Embryos were stained for *hoxb1b* (G-I) or *otx2* (J-L).

treated with LiCl to suppress Fgf activity, while enhancing Wnt signaling. In these embryos, *cyp26* and *otx2* as well as *hoxb1b* were suppressed (Fig. 7D,I,N). This suggests that activation of Wnt signaling can suppress anterior markers in the absence of Fgf signaling but that activation of posterior markers requires Fgf.

As a complementary experiment, *fgf3* and *dkk1* were co-expressed to enhance Fgf while suppressing Wnt signaling. In these embryos, *cyp26* and *otx2* were suppressed, indicating that Dkk1 is not sufficient to promote anterior markers in the presence of exogenous Fgf signaling (Fig. 7E,J). Dkk1 did, however, inhibit the ability of Fgf to expand *hoxb1b* expression into anterior regions (Fig. 7O).

Distinct roles of RA and Wnt in patterning the neural ectoderm

In the next set of experiments, we examined the relationship between the RA and Wnt signaling pathways. RA activity was suppressed by overexpression of *cyp26*, and subsequently LiCl immersion at 50% epiboly stage was used to activate Wnt signaling. In these embryos both *otx2* and *hoxb1b* were suppressed, indicating that Wnt signals inhibit anterior development in the absence of RA (Fig. 8B,E). As a complementary experiment, *dkk1* mRNA-injected embryos were treated with RA. In these embryos, *otx2* was largely suppressed and *hoxb1b* was ectopically expressed up to the animal pole (Fig. 8C,F). Although there is *otx2* expression remaining in the dorsal-most area (Fig. 8C, arrowhead), because these cells are hypoblast they are possibly the expanded axial mesoderm. These results suggest that Wnts and RA both can suppress *otx2* in the anterior neural ectoderm but that activation of *hoxb1b* depends on RA.

One possibility is that Wnts promote *hoxb1b* expression through regulating the production of RA. To assess if this might be the case, expression of the gene encoding an RA synthesis enzyme, *raldh2*, was examined in conditions in

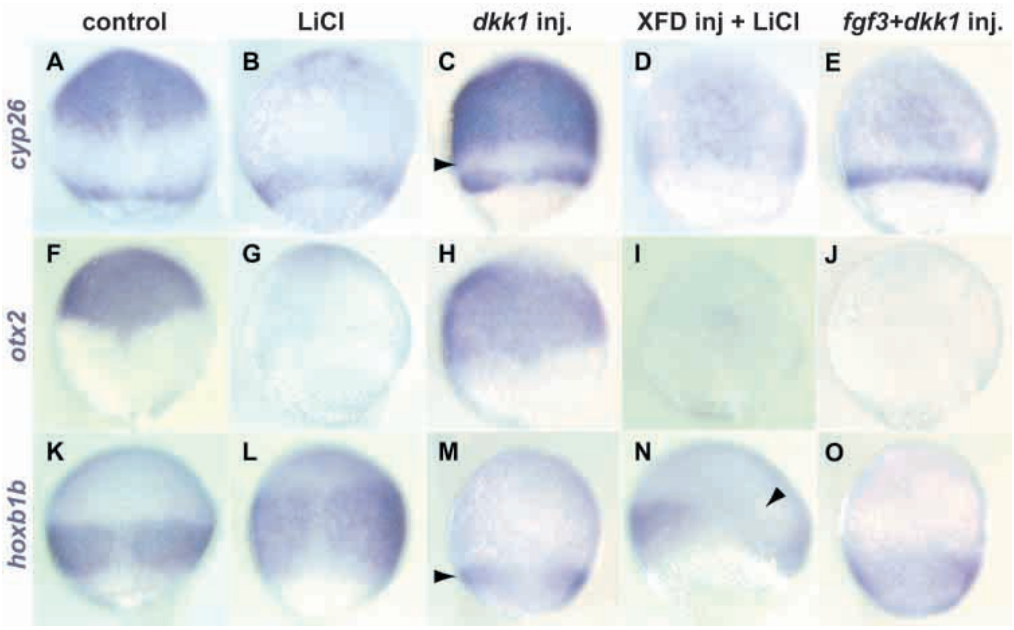

Fig. 7. Epistatic analysis of the function of Wnt and Fgf in patterning the neural ectoderm. Dorsal views of whole embryos at late gastrula stage. Gain and loss of function of Wnt activity was achieved by LiCl treatment (B,G,L) and *dkk1* injection (C,H,M), respectively. Epistasis with Fgf was examined by *XFD* injection followed by LiCl treatment (D,I,N) and by *fgf3 +dkk1* co-injection (E,J,O). Embryos were fixed at late gastrula and stained with *cyp26* (A-E), *otx2* (F-J) and *hoxb1b* (K-O). Arrowheads in C and M indicate the limit of caudal expansion of *cyp26* expression (C) and remaining *hoxb1b* expression (M). In some cases, only half the embryo is affected when one of two cells is injected (N, arrowhead).

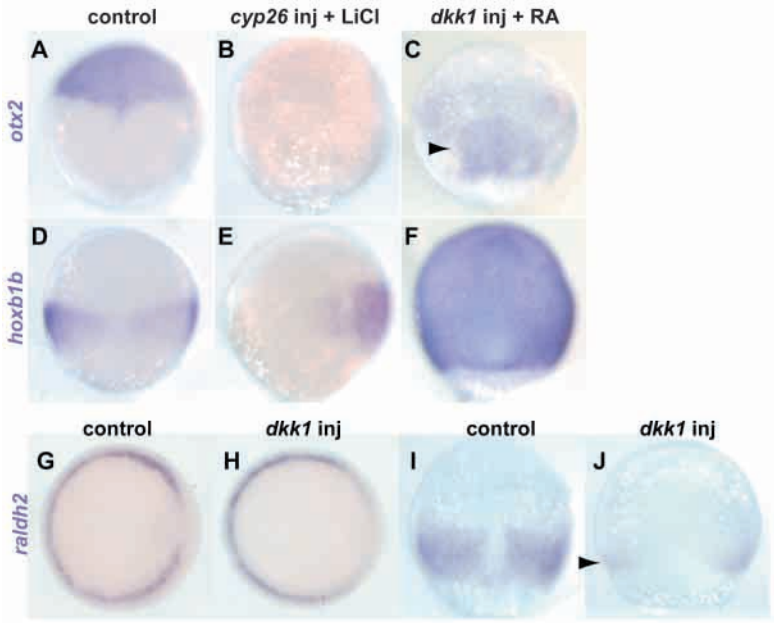

Fig. 8. Epistatic analysis of the function of Wnt and RA in patterning the neural ectoderm. Dorsal views of whole embryos at late gastrula stage (A-F,I,J), and animal views of 55% epiboly stage embryos (G,H). *cyp26*-injected embryos were treated with LiCl at the 50% epiboly stage (B,E), and *dkk1*-injected embryos were treated with RA at 40% epiboly stage (C,F). Embryos were fixed at late gastrula and stained with *otx2* (A-C) and *hoxb1b* (D-F). Regulation of the expression of *raldh2* was examined in *dkk1*-injected embryos (H,J). At 55% epiboly stage, *raldh2* expression is not suppressed at the blastoderm margin by *dkk1* injection though the *raldh2* negative area in the dorsal-most margin is slightly expanded (H). At late gastrula stage, *raldh2* expression remains restricted to the blastoderm margin in *dkk1*-injected embryos (J, arrowhead).

which Wnt signaling was increased or decreased. At the 50% epiboly stage, *raldh2* is expressed in nascent paraxial mesoderm at the blastoderm margin. In embryos in which the Wnt antagonist *dkk1* was injected, *raldh2* expression was not suppressed although the dorsal *raldh2*-negative domain was slightly expanded, most probably because Dkk1 activity is known to enhance axial mesodermal fates (Hashimoto et al., 2000). This suggests that Wnt signaling is not necessary for the initiation of *raldh2* expression. However, by late gastrula stage, *raldh2* expression remained restricted to the blastoderm margin in *dkk1*-injected embryos, whereas in control embryos, *raldh2* was highly expressed in the involuted mesoderm as it migrated anteriorly (Fig. 8I,J). This limitation of *raldh2* expression could account for the similar restriction of *hoxb1b* expression to the marginal zone of *dkk1*-injected embryos. *raldh2* expression was also examined in LiCl-treated embryos and shown to retain normal expression (data not shown). Together, these results suggest that Wnt signals indirectly influence *hoxb1b* expression through the modulation of RA activity.

Cyp26 morpholino, mCYP1, causes partial posteriorization

To examine the physiological role of *cyp26* in early AP patterning, morpholino antisense oligonucleotide (mCYP1) was injected into zebrafish embryos. In mCYP1-injected embryos, *otx2* expression was decreased (Fig. 9B) and *hoxb1b* expression was expanded anteriorly (Fig. 9D). Complementing this, the expression domains of *meis3* and *iro3* were both shifted in an anterior direction (Fig. 9F,H). These results suggest that the activity of endogenous Cyp26 is required to restrict the expression of posterior genes at their anterior border and to protect anterior genes from repression by RA. Cyp26 is likely to carry out these functions by degrading RA molecules that encroach into the anterior ectodermal compartment, thereby limiting their presence to the posterior compartment.

The efficacy of mCYP1 was confirmed in experiments using a cyp26-GFP fusion construct. GFP fluorescence was strongly suppressed by the co-injection of mCYP1 but not by the control morpholino (Fig. 9I,J); thus mCYP1 at this concentration efficiently suppressed the translation of Cyp26 fusion protein. In addition to the changes in expression of AP marker genes, morpholino injected embryos were more ovoid in shape by late gastrulation (Fig. 9B,D,F,H). A similar shape change was observed in RA-treated embryos at the end of gastrulation (not shown) supporting the notion that endogenous RA activity is increased by the mCyp1.

Summary of experiments

The consequences of altering Fgf, RA and Wnt signals upon early AP patterning of the ectoderm are summarized as a cartoon in Fig. 10. In general, all anterior and all posterior markers behaved similarly in each set of experiments, with the exception of the *cyp26* gene, which was induced by RA even though it is normally expressed in the anterior region of the neural ectoderm (see Discussion). In the cartoon, we summarize the results obtained from analysis of the expression of other markers, primarily *otx2* and *hoxb1b*. From this, it is evident that anterior gene and posterior gene expression domains never overlap. However, although in some cases, the entire embryo acquired anterior or posterior identity (i.e. *fgf3* or *XFD* injection), in other cases, anterior and posterior genes retained complementarity of expression but with a shifted boundary (i.e. *dkk1* injection). Finally, in some situations we observed that anterior or posterior character could be specifically lost in one region (i.e. after *cyp26* injection or LiCl treatment) or throughout the entire embryo (i.e. after *fgf3+cyp26* injection or *cyp26* injection plus LiCl treatment). The latter case is particularly instructive: excessive signaling by either Fgf or Wnt eliminates anterior character, but establishing posterior character depends on RA. Thus, the combination of ectopic Fgf or Wnt signals and suppression of RA leaves the presumptive neuroectoderm without any AP identity.

DISCUSSION

cyp26 modulates the action of RA in AP patterning of the neural ectoderm

Our study has revealed that AP regionalization of the prospective neural ectoderm occurs very early and that the *cyp26* gene appears to be involved in this early patterning. We

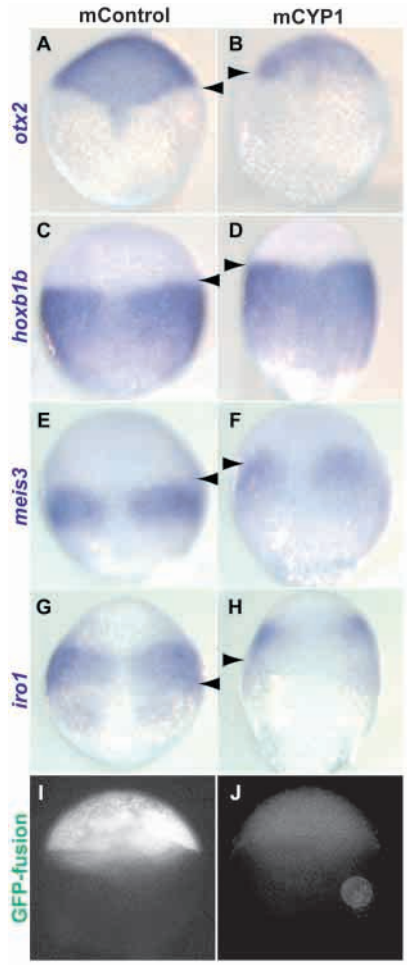

Fig. 9. Abrogation of Cyp26 activity causes moderate posteriorization. Dorsal views of whole embryos at late gastrula stage (A-H) and lateral views of late blastula stage embryos (I,J). A control morpholino or mCYP1, which is complementary to *cyp26* mRNA sequence, was injected at 5 ng per embryo. Embryos were stained with *otx2* (A,B), *hoxb1b* (C,D), *meis3* (E,F) and *iro1* (G,H). In addition, cyp26-GFP fusion construct was injected with either morpholino, and fluorescence was examined. (I,J). (A,C,E,G,I) Control morpholino; (B,D,F,H,J) mCYP1-injected embryos.

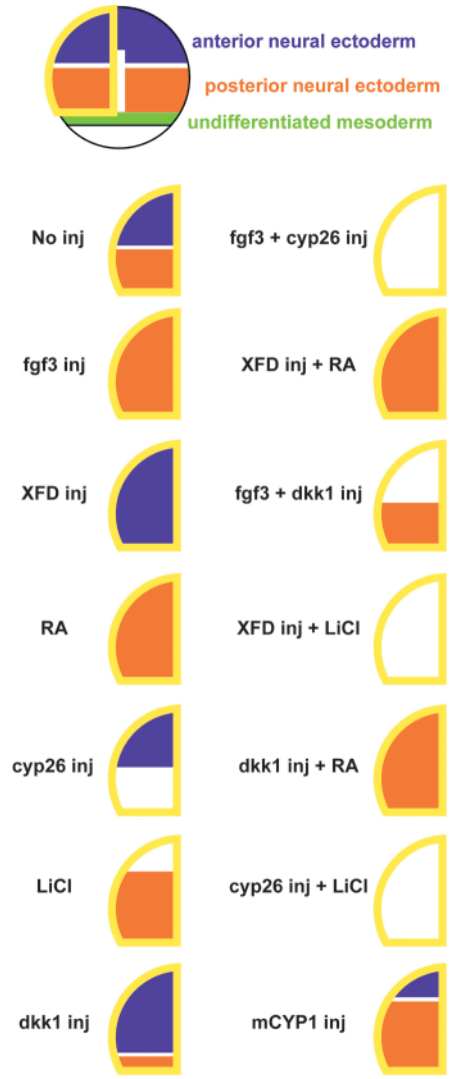

Fig. 10. Summary of experiments. The experimental results are summarized as a cartoon. *otx2* and *hob1b* are used as representative markers of anterior and posterior neural ectoderm, respectively, as these genes were examined in all of experiments. The top picture is a dorsal view of a late gastrula embryo divided into anterior (purple) and posterior (red) neural ectodermal regions and undifferentiated mesendoderm at the blastoderm margin (green). One half of the dorsal ectoderm is surrounded by yellow line and experimental results are summarized in this area as marker genes for mesoderm and axial area were not analyzed in any detail in this study.

have analyzed the expression pattern of zebrafish *cyp26* and found that its dorsoanterior expression begins around 30-40% epiboly in the late blastula. This observation is of interest as it suggests that AP patterning of the neural ectoderm is initiated well before the onset of gastrulation. Expression in the anterior neural domain persists through gastrulation but disappears at early somitogenesis stages, suggesting that *cyp26* affects AP patterning mainly from the blastula through the gastrula stages. *cyp26* has been identified in *Xenopus* and in the mouse, and is also expressed in the anterior neural ectoderm in these species (de Roos et al., 1999; Fujii et al., 1997; Hollemann et al., 1998).

As Cyp26 degrades RA it is expected to limit the range of RA-mediated posteriorization in the embryo. Our Cyp26 overexpression and loss-of-function studies support this idea. RA is known to be involved in the expression of posterior genes including Hox genes (Kolm et al., 1997; Kolm and Sive, 1995; Niederreither et al., 1999; Simeone et al., 1990). In our experiments, RA induced posterior genes such as *hoxb1b* and *meis3*, while *cyp26* injection suppressed expression of these genes, presumably by reducing the endogenous RA concentration. However, the expression domain of the anterior gene *otx2* was not expanded by *cyp26* mRNA injection, suggesting that RA-independent pathways suppress *otx2* in the posterior neural ectoderm. We believe that limiting the range of RA action is the in vivo function of Cyp26 as morpholino antisense oligonucleotide injection caused anterior expansion of posterior genes such as *hoxb1b*, and a shrinking of the expression domain of the anterior gene, *otx2*. Cyp26 thus has an important role in defining the border between anterior and posterior neural ectoderm.

cyp26 is also expressed at the blastoderm margin with a gap

between its two domains of expression. The blastoderm margin expression persists into the developing tail region during somitogenesis, suggesting that suppression of RA signals is also required in tail development. Consistent with this view, RA treatment in *Xenopus* and zebrafish causes truncation of tail as well as head structures (Durston et al., 1989) (data not shown). Recently, mice carrying targeted mutations in *cyp26* were generated and these animals showed loss of tail and adjacent posterior structures, stressing the importance of RA signal suppression by *cyp26* for tail development (Abu-Abed et al., 2001; Sakai et al., 2001).

Overexpression experiments using *cyp26* in *Xenopus* showed *otx2* expression was slightly expanded in a posterior direction (Hollemann et al., 1998). This is somewhat different from our observation that *otx2* was not expanded by *cyp26* overexpression. The differences may be due to the fact that phenotypes in frog were examined at later stages, when many other CNS patterning events are likely to have occurred and modified early effects of exogenous Cyp26 activity. Our observation that RA caused the expansion of *hoxb1b* expression in anterior neural ectoderm differs from results reported previously by Alexandre et al., who described more subtle phenotypes following RA treatment (Alexandre et al., 1996). This difference can be explained by the different concentration of RA in the two studies. When we applied 10^{-7} M RA to zebrafish embryos, *hoxb1b* was induced only in axial mesoderm, as reported by Alexandre et al. (Alexandre et al., 1996). However we used 10^{-6} M RA because this or higher doses generate anterior truncations in *Xenopus* (Durston et al., 1989; Sive et al., 1990). Furthermore, the internal concentration of RA under these conditions is not known. Recently, the zebrafish mutants *neckless* and *nofin*, which have a decrease in hindbrain size, have been shown to carry mutations in the RA synthesis enzyme, Raldh2 (Begemann et al., 2001; Grandel et al., 2002). Begemann et al. have shown that the mutant phenotype in the hindbrain could be rescued by 5×10^{-7} M or 10^{-6} M RA, but not by lower concentrations. This suggests that when applied externally, 10^{-6} M RA may mimic physiological conditions that are achieved by normal RA synthesis in the wild-type embryo (Begemann et al., 2001).

Fgf and Wnt signals regulate early AP polarity

With the aim of investigating the nature of the signals that initiate differential expression of *cyp26* and *hoxb1b*, we examined the role of Fgfs in this process, as Fgfs have been implicated in posteriorization of the neural ectoderm (Griffin et al., 1995; Koshida et al., 1998). Experiments that involved injection of mRNAs for Fgf3 and the truncated receptor XFD into zebrafish embryos showed that Fgf signaling is both necessary and sufficient, directly or indirectly, for the suppression of the anterior genes *cyp26* and *otx2* and the induction of the posterior gene *hoxb1b*.

Like Fgf signaling, Wnt activity is known to affect the early specification of AP polarity in the neural ectoderm (Fekany-Lee et al., 2000; Kelly et al., 1995). We found that activation of Wnt signaling suppressed *otx2* and *cyp26*, while the expression of *hoxb1* was partly expanded by Wnt activation and largely suppressed by inhibition of Wnt signaling.

Interactions between the posteriorizing pathways were studied by combined activation of one signal and inhibition of another, as summarized in Fig. 10. These experiments indicate that either an Fgf or a Wnt signal can suppress anterior genes such as *cyp26* and *otx2* even when the other pathway is inhibited. Therefore, Wnts and Fgfs can act independently of each other in the suppression of anterior genes; this suppression was also independent of the activity of RA, but RA can suppress anterior genes even in the presence of Fgf or Wnt pathway inhibitors. By contrast, activation of posterior gene expression could only be initiated by Fgf or Wnt signals in the presence of an intact RA signaling pathway. It is likely that RA acts directly on the *hoxb1b* promoter, as RA-responsive elements are present in the mouse *Hoxb1* gene and have been shown to have a crucial role in regulating its expression (Marshall et al., 1994; Ogura et al., 1996a; Ogura et al., 1996b). The expansion of *hoxb1b* expression after ectopic Wnt activation may in part be explained by the suppression of *cyp26*, which results in expansion of the range of RA activity in an anterior direction. This interpretation is in agreement with the similar expansion of the *hoxb1b* domain after injection of a *cyp26* morpholino antisense oligonucleotide. By contrast, the more extensive expansion of *hoxb1b* expression by Fgf overactivation is unlikely to simply be due to abrogation of Cyp26 activity. Instead we suggest that other factors must contribute to the ectopic production of an RA signal in anterior regions of Fgf injected embryos.

A model for the interactions between Fgf, Wnt and RA in early AP patterning

A model for the mechanism of early AP patterning, based on the observations described in this paper, is presented in Fig. 11. A key feature of this model is that promotion of posterior fates and suppression of anterior fates are treated as separable events.

Fgfs and Wnts can suppress anterior genes in an RA-independent pathway. The set of genes sensitive to this suppression includes *cyp26*, which encodes an enzyme that degrades RA. While suppression of RA signaling is not necessary/sufficient for activation of anterior genes, we suggest that it is needed to prevent activation of posterior gene expression within the prospective anterior neural plate. The factors important for activation of anterior gene expression remain unknown, but many studies have suggested that 'anterior' is a default fate for induced neural tissue (Nieuwkoop, 1950; Toivonen, 1968).

Within prospective posterior neural tissue, RA is necessary and sufficient for the activation of at least some posterior genes. Furthermore, the ability of Fgfs and Wnts to promote expression of these posterior genes depends upon RA. In part, this is likely to be due to the ability of Fgfs and Wnts to promote RA activity through suppression of *cyp26* expression, but is also likely to be due to additional regulatory events, such as promotion of *raldh2* expression.

In Fig. 11B, a spatial and temporal model underlying these AP patterning events is outlined. At the late blastula stage, genes in the Fgf and Wnt families are expressed in the prospective mesoderm at the blastoderm margin; *fgf3*, *fgf8* and *wnt8* are known to be expressed in this pattern (Furthauer et al., 1997; Kelly et al., 1995; Koshida et al., 2002; Phillips et al., 2001). We suggest that an early role for these margin-derived signals is to suppress, directly or indirectly, the expression of *cyp26* and possibly other anterior genes in dorsal ectoderm adjacent to the margin, thereby initiating the specification of this region as presumptive posterior neural ectoderm at the late blastula stage (Fig. 11B, part i). The initial

suppression of these genes is likely to be achieved through a planar signal because, at the 30-40% epiboly stage when localized *cyp26* expression is first seen, the mesendodermal layer has not yet involuted below the ectodermal layer. This view is consistent with the report that posterior neural specification in dorsal ectoderm is observable at the shield stage (50-55% epiboly) but not in the blastula at the 30% epiboly stage (Grinblat et al., 1998).

Our model further proposes that the widening Cyp26-free area allows the accumulation of RA and the consequent induction of RA-dependent posterior genes, including *hoxb1b*, at early-to-mid gastrula stages (Fig. 11B, part ii). Subsequently, ongoing convergence-extension movements move lateral posterior ectoderm cells in a dorsal direction, causing further anteroposterior expansion of the Cyp26-negative, RA-positive area, thereby maintaining and enhancing the expression of *hoxb1b* and other posterior genes. Cyp26 maintains its expression anteriorly, thereby defining the rostral limit of expression of the posterior genes (Fig. 11Biii).

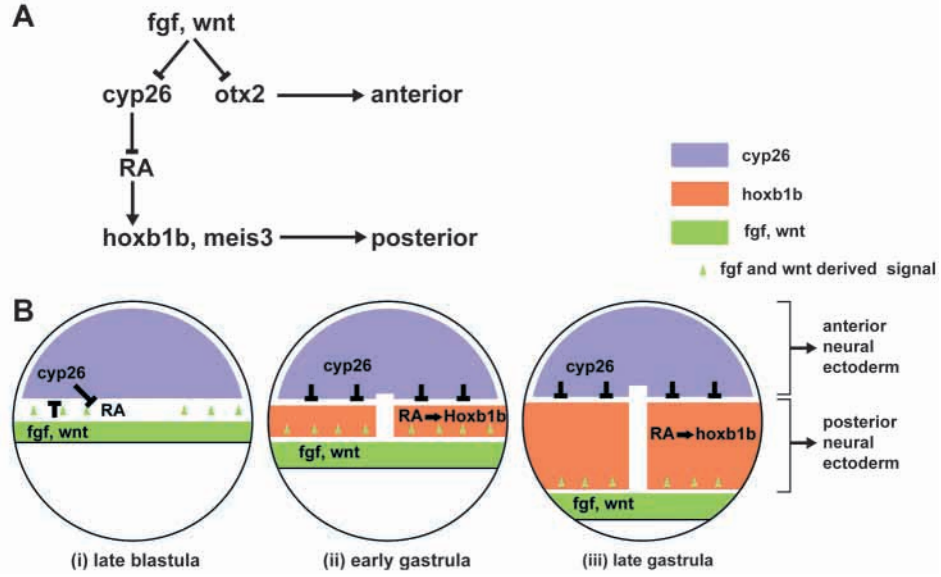

Fig. 11. A model for interactions between Fgf, Wnt and RA signaling in the neural ectoderm during gastrulation. (A) Sequence of posteriorization signals. Fgf and/or Wnt signals initiate the first step of posteriorization by suppressing expression of anterior genes, represented here by *cyp26* and *otx2*. This process is not mediated by RA. In the posterior domain, where *cyp26* expression is suppressed by Fgfs/Wnts, RA accumulates at least in part due to the activity of Raldh2, and activates posterior genes such as *hoxb1b* and *meis3*. (B) At the late blastula stage, Fgfs/Wnts are expressed in the mesoderm at the blastoderm margin. Fgf/Wnt signals from the margin block the expression of *cyp26* in the adjacent ectoderm, which will give rise to the posterior neural plate. *cyp26* is expressed in the anterior domain, at a distance from the source of the Fgf/Wnt signals; as a consequence, RA is degraded and the expression of posterior genes is prevented. After the beginning of gastrulation, convergence-extension movements lead to a widening of the *cyp26*-negative area, allowing RA to accumulate to a level where it can activate posterior genes such as *hoxb1b*. Subsequent cell movements expand the domain that will give rise to the posterior neural ectoderm.

The RA-synthesizing enzyme, Raldh2, is likely to be involved in these early patterning events. In several species, including zebrafish, Raldh2 is expressed in the posterior mesoderm (Begemann et al., 2001; Berggren et al., 1999; Chen et al., 2001; Grandel et al., 2002; Niederreither et al., 1997; Swindell et al., 1999). *Raldh2* mutations in mouse and zebrafish reduce posterior neural ectoderm with concomitant downregulation of Hox genes (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 1999). In a complementary manner, increased Raldh2 levels posteriorize the nervous system of *Xenopus* embryos (Chen et al., 2001). Therefore, two mechanisms might regulate RA accumulation, one mediated by the synthetic enzyme Raldh2, which augments RA in the posterior neural ectoderm, the other mediated by Cyp26, which degrades RA in the anterior neural ectoderm (Chen et al., 2001; Swindell et al., 1999). Mutations in the *Cyp26* gene in mouse do indeed lead to a moderate anterior expansion of the *Hoxa1* expression domain (Abu-Abed et al., 2001; Sakai et al., 2001), a result similar to that obtained by *cyp26* morpholino injection in fish. As Raldh2 is expressed only in the posterior region, RA may not significantly accumulate in the anterior-most region, even in the absence of Cyp26, possibly explaining the relatively mild phenotypes after abrogation of Cyp26 activity in mouse and zebrafish. Therefore, although *cyp26* is widely expressed in the anterior neural ectoderm, its major role may be the definition of the boundary in the presumptive hindbrain beyond which expression of posterior genes such as *hoxb1b* and *meis3* does not expand.

In summary, we show in the present paper that AP patterning is initiated in the presumptive neural ectoderm in the late blastula at 30-40% epiboly stage. We can distinguish two posteriorization steps in this process. The earliest step involves the Wnt/Fgf-dependent, RA-independent suppression of anterior genes, including *cyp26* in the presumptive posterior domain. The next step involves the activation of genes such as *hoxb1b* and *meis3* in the posterior domain; this step is mediated by RA signaling. The antagonism between cyp26 activity and RA signaling links the initial and the subsequent steps of AP patterning, thereby contributing to the establishment of the earliest known border between anterior and posterior neural ectoderm.

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