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xCOUP-TF-B regulates *xCyp26* transcription and modulates retinoic acid signaling for anterior neural patterning in *Xenopus*

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ABSTRACT Early embryogenesis in *Xenopus* development depends on correct anterior-posterior (A-P) neural patterning during gastrulation. It is known that high levels of retinoic acid (RA), a major intracellular signaling molecule, determine posterior cell fate, also reflecting an involvement in A-P neural patterning. Here we show that the known RA effector, xCOUP-TF, plays important roles in head development of *Xenopus* embryo. xCOUP-TF-B injection into the dorsal region of embryos induced formation of an abnormal head with small eyes. Analysis of brain marker gene expression revealed that xCOUP-TF-B injection induced slight anteriorization in embryos and attenuated the effects of RA treatment. This anteriorization effect was enhanced when xCOUP-TF-B was co-injected with xCyp26A or xCyp26C, which are known RA metabolizing factors. Furthermore, xCOUP-TF-B injection enhanced xCyp26A/C transcription. Together, these results suggest that xCOUP-TF and xCyp26 are both regulated by Wnt signaling, and cooperatively function in RA signaling to affect A-P neural patterning.

KEY WORDS: *Xenopus, retinoic acid, Wnt signaling, A-P patterning*

Introduction

Axis determination is one of the most important events in embryonic patterning. During gastrulation, neural tissues are induced in ectodermal cells and, at the same time, anterior-posterior (A-P) neural patterning is determined. Several intracellular signaling pathways including Wnt and FGF play crucial roles in the determination of A-P neural patterning (Niehrs, 1999; Sasai and DeRobertis, 1997).

Retinoic acid (RA) signaling is also important for precise A-P patterning, with an RA concentration gradient formed in early neurula (Chen *et al*., 1994). Further, RA treatment blocks head formation in *Xenopus* embryo, reflecting posteriorization (Durston *et al*., 1989; Ruiz i Altaba and Jessell, 1991), and alters hindbrain patterning (Papalopulu *et al*., 1991). Two RA nuclear receptors, RAR and RXR, are expressed in *Xenopus* embryos; these receptors associate with the regulatory region of target genes to promote target gene transcription (Ellinger-Ziegelbauer and Dreyer 1991: Brumburg *et al*., 1992).

Two factors are critical for adjusting the level of RA concentration along the A-P axis during patterning. The first is the RalDH family of genes, which function in RA synthesis. In *Xenopus,* RalDH2 is expressed in the dorsal blastopore lip of the embryonic gastrula where it functions in brain development (Chen *et al*., 2001). Second, the Cyp26 genes are important for RA metabolism (White *et al*., 1996), with xCyp26A, B, and C implicated in *Xenopus* embryogenesis (Hollemann *et al*., 1998; de Roos *et al*., 1999; Kudoh *et al*., 2002).

In a previous DNA microarray screen for novel genes that affect A-P neural patterning and that are regulated by canonical Wnt signaling (Tanibe *et al*., 2008; Michiue *et al*., 2007), we identified chicken ovalbumin upstream promoter transcription factor (COUP-TF) as a candidate gene (reviewed in Pereira *et al.,* 1995). COUP-TF was first identified as a DNA-binding protein that interacts directly with the ovalbumin promoter (Pastorcic *et al*., 1986). COUP-TF encodes a steroid/thyroid hormone receptor that contains a DNA-binding domain including two zinc finger domains and one ligand-binding domain (LBD) (Evans, 1988). In *Drosophila*, COUP-TF is a causative gene of the seven-up mutation (Mlodzik *et al*., 1990), while two homologues of COUP-TF, xCOUP-TF-A and xCOUP-TF-B, have been identified in *Xenopus*. In this study, it is shown

Abbreviations used in this paper: A-P, anterior-posterior; DA, dorso-animal; MO, morpholino oligo; RA, retinoic acid.

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that xCOUP-TF-B is similar with mammalian COUP-TFII, and that xCOUP-TFA is distinct with xCOUP-TFI (Qiu *et al.*, 1994; van der Wees *et al.*, 1996). These genes are differentially expressed (van der Wees *et al.*, 1996). For example, both genes are expressed in anterior neuroectoderm, but only COUP-TF-A is expressed in somite (van der Wees *et al.,* 1996). The function of COUP-TF has been extensively studied, with some studies revealing an inhibitory role in RA signaling (Tran *et al.*, 1992; Kliewer *et al.*, 1992), and *Xenopus* COUP-TF implicated as a potential regulator of RA signaling in neural development (Schuh and Kimelman, 1995).

This study of xCOUP-TF function revealed important cooperative roles with xCyp26 in A-P neural patterning. xCOUP-TF-B is expressed in restricted sets of rhombencephalon at the neurula stage and affects the expression of several brain markers. The xCOUP-TF-B expression was dependent on Wnt signaling and enhanced anteriorization in cooperation with xCyp26. Moreover, xCOUP-TF was found to promote xCyp26 transcription. These data suggests that a novel mechanism of A-P neural patterning could exist via the regulation of RA signaling.

Results

Microinjection with xCOUP-TF-B mRNA caused head defects and misexpression of neural marker genes

As described in previous studies, dorsal injection with *xCOUP-TF-B* mRNA disrupted anterior neural development (Schuh and Kimelman, 1995). To confirm this effect, we injected *xCOUP-TF-B* mRNA into the animal region of 4-8-cell embryos. Injection of 100 pg of *xCOUP-TF-B* mRNA into the dorso-animal (DA) region caused obvious head defects characterized by small eyes and a slightly bent axis (Fig. 1 A,B), while ventro-animal injections induced abnormalities in the abdominal region of tadpoles (Fig. 1C). We also observed transverse sections of the DA-injected embryos. Sections dissected at the eye vesicle showed that xCOUP-TF-B caused shrinkage of the diencephalon and loss of eye vesicles (Fig. 1 D,F), and sections cut across the otic vesicles revealed hindbrain enlargement (Fig. 1 E,G).

In a previous study (Schuh and Kimelman, 1995), overexpression of *xCOUP-TF-B* mRNA altered the spatial expression of several genes such as *En2* (a mid-hind brain marker), *Krox20* (a rhomben-

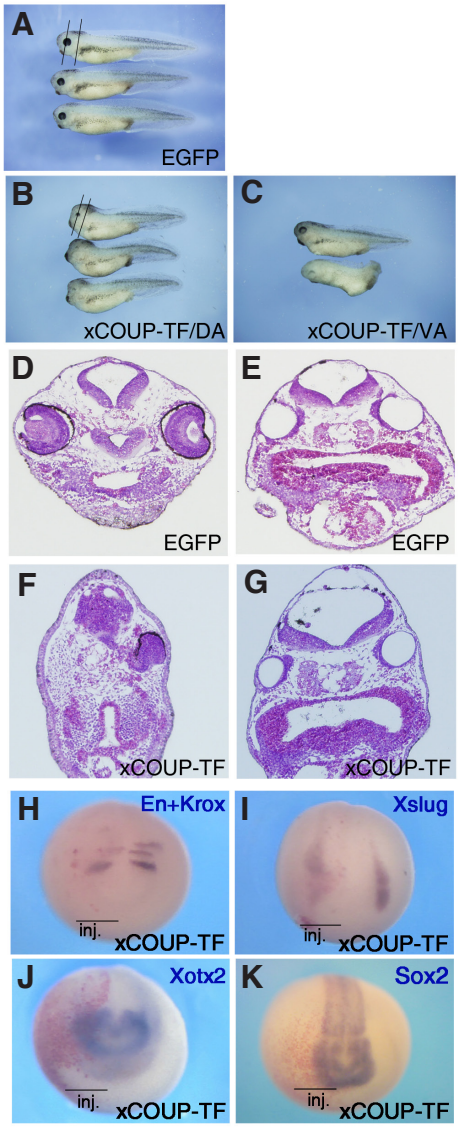

Fig.1 Phenotype caused by *xCOUP-TF-B* mRNA injection. XCOUP-TF-B *mRNA was injected into the dorso-animal* **(B,F,G)** *or ventro-animal* **(C)** *regions of 4-cell embryos, and the superficial phenotype* **(A,B,C)** *of 3-day tadpoles was observed. Thin lines in A or B indicate the position of thin sections.* **(D-G)** *Transverse section of embryo injected with* EGFP **(D,E)** *or* xCOUP-TF-B **(F,G)**. **(H-K)** *Whole-mount* in situ *hybridization of embryos injected with 100 pg of xCOUP-TF-B mRNA.* En2 *and* Krox20 *(H),* Xslug *(I),* Xotx2 *(J), and* xSox2 *(K) expression was observed at the mid-late neurula stage.*

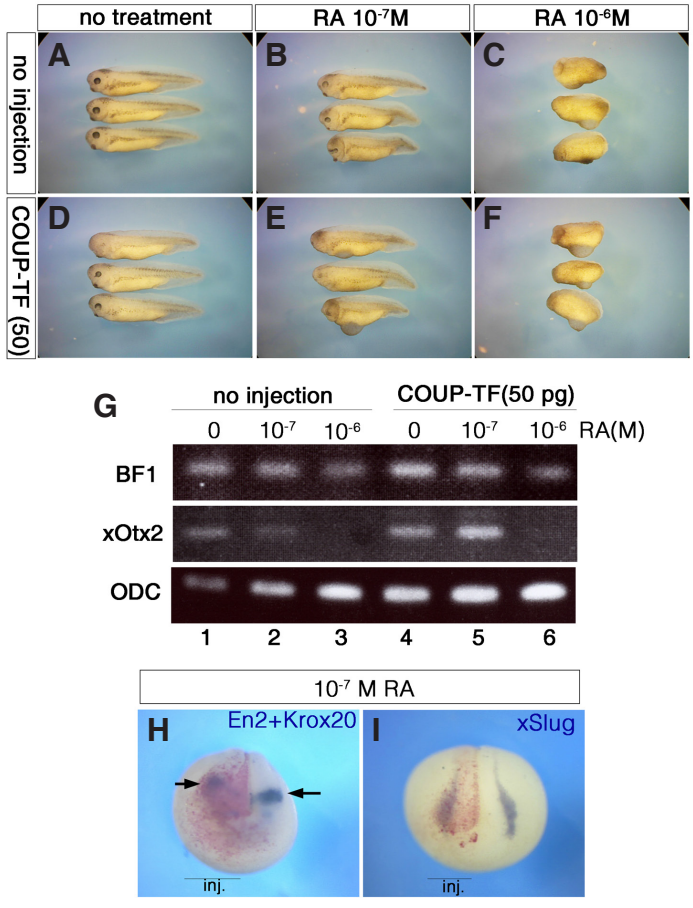

Fig. 2. COUP-TF-B could attenuate the retinoic acid (RA) effects. (A-F) *Superficial phenotype of* xCOUP-TF-B*-injected embryos treated with* 10^{-7} *M* **(B,E)** *or* 10^{-6} *M* **(C,F)** *of RA.* **(G)** *RT-PCR analysis showing gene expressions of* xBF1 *(column 1) and* Xotx2 *(column 2).* **(H,I)** *Whole-mount* in situ *hybridization with xCOUP-TF-B-injected embryos treated with* 10^{-7}*M RA. Injected side was marked with Red-gal staining by co-injection with* lacZ *mRNA. Transcription of* En2 *and* Krox20 *(H) or* xSlug *(I) were shown.*

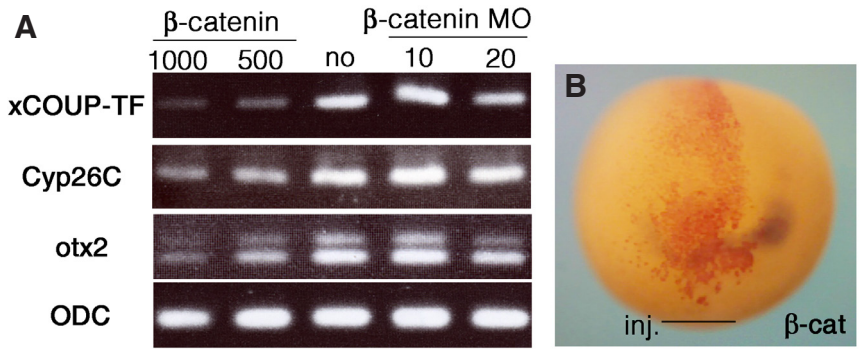

Fig. 3. Brain marker expression in *xCOUP-TF-B*-injected embryo. (A) *RT-PCR analysis with cDNA derived from the anterior half of stage-13 embryo injected with 1000 pg (lane1) or 500 pg (lane 2) of* βcatenin *mRNA, and 10 ng (lane 4) or 20 ng (lane 5) of* βcat *MO.* **(B)** *Whole-mount* in situ *hybridization with β-catenin -injected embryo. For tracing the injected area,* lacZ *mRNA was co-injected and injected embryos were stained with Red-Gal.*

cephalon marker), and *Xtwist* (a neural crest marker). To confirm these results, we performed whole-mount *in situ* hybridization. As described previously, *xCOUP-TF-B mRNA* injection disrupted *En2* and *Krox20* expression (Fig. 1H). Indeed, a complete absence of *Krox20* expression (Fig. 1H), while the pattern of En2 expression was slightly broadened and weakened (Fig. 1H). We also examined other neural markers. In *xCOUP-TF-B* injected embryo, *xslug* expression shifted posteriorly, suggesting anteriorization (Fig. 1I), although *Xotx2* and *Sox2* expressions were unchanged by *xCOUP-TF-B* (Fig. 1 J,K).

XCOUP-TF-B attenuated posteriorization by RA

Previous reports implicated an inhibitory role for xCOUP-TF in RA signaling, and RA treatment is known to induce posteriorization in *Xenopus* embryo. We therefore examined whether xCOUP-TF could inhibit such an effect. First, we injected *xCOUP-TF-B mRNA* into the DA region of 4-cell embryos, followed by treatment with RA solution at Stage 8. Without injection, treatment with 10^{-7} M RA induced a slight head defect with small eye, while 10^{-6} M RA treatment caused more severe defects (Fig. 2 B,C). In addition, the head shrinkage caused by treatment with RA was not rescued by the injection of *xCOUP-TF-B mRNA* (Fig. 2 E,F).

Next, we carried out RT-PCR analysis on these embryos. *BF1* (telencephalon marker) and *Xotx2* (forebrain marker) expressions were decreased with elevated RA concentration (Fig. 2G, lane 1-3). Conversely, *xCOUP-TF-B*-injected embryos showed suppression of the 10^{-7} M RA-induced decrease in *BF1* and *Xotx2* expression (Fig. 2G, lane 2 and 5). On the other hand, xCOUP-TF-B could not recover *BF1* and *Xotx2* expression in embryo with 10^{-6} M RA treatment (Fig. 2G, lane 3 and 6).

We then assessed *Krox20* and *En2* expression further by *in situ* hybridization. Embryos treated with 10^{-7} M RA showed a posterior shift in Krox20 expression in rhombomere 5 on the injected side (Fig. 2H), and this change was more apparent than in normal embryos without RA treatment (compare with Fig. 1H). Similar to *En2/Krox20*, the *xslug* expression area (neural crest marker) was also posteriorly shifted (Fig. 2I). These results suggest that *xCOUP-TF-B* attenuates posteriorization caused by RA.

Canonical Wnt signaling downregulated expression of xCOUP-TF-B

DNA microarray results suggested that *xCOUP-TF-B* expression is affected by canonical Wnt signaling. To confirm this, we carried out RT-PCR on β-catenin-injected embryos. β-*catenin* mRNA injection decreased the level of *xCOUP-TF-B* transcription, whereas 10 ng of β-catenin MO injection slightly promoted *xCOUP-TF-B* transcription, and 20 ng injection weakly suppressed *xCOUP-TF-B* expression (Fig. 3A). Similar elevations were observed in *xCyp26C and Xotx2* expression (Fig. 3A). The β-catenin-injected embryos also showed inhibition of spatial expression of *xCOUP-TF-B* (Fig. 3B). These data together suggests that *xCOUP-TF-B* is negatively regulated by canonical Wnt signaling.

Coinjection of xCOUP-TF-B with xCyp26C enhanced the anterior defect

We also previously reported that xCyp26C is important for precise anterior neural patterning (Tanibe *et al.*, 2008). Indeed, our microarray analysis identified both *xCyp26C* and *xCOUP-TF-B* as genes whose transcripts were more abundant in the anterior half of neurula than in the posterior half. Furthermore, it is known that both *xCyp26C* and *xCOUP-TF-B* negatively regulate RA signaling. We therefore hypothesize that *xCOUP-TF-B* is involved with xCyp26C function.

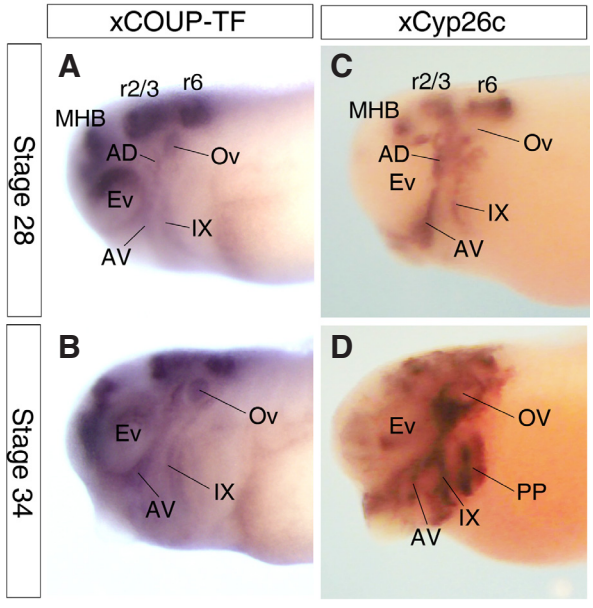

Fig. 4. Comparison of *xCOUP-TF-B* and *xCyp26C* expression in brain region of tadpole. (A-D) *Whole mount* in situ *hybridization was carried out with* COUP-TF-B **(A,B)** *and* xCyp26C **(C,D)** *probe. We observed Stage 28* **(A,C)** *or Stage 34* **(B,D)** *embryo. MHB: midbrain-hindbrain boundary. AD: anterodorsal lateral line pracode. AV: anteroventral lateral line pracode. Ev: eye vesicle. Ov: otic vesicle. IX: Epibranchial IX placode. PP: pharyngeal pouch. R2/3: rhombomere 2/3. R6: rhombomere 6.*

To assess this possibility, we first compared the spatial expression of *xCOUP-TF-B* with that of *xCyp26C*. As previously described, *xCyp26C* was expressed in restricted subsets of rhombomere and cranial placode (Fig. 4 C,D: Tanibe *et al.*, 2008). xCOUP-TF showed a similar pattern of expression to that of *xCyp26C* in rhombencephalon (Fig. 4 A,C), while both *xCOUP-TF-B* and *xCyp26C* expression was also seen in some sets of cranial placodal nerves, which were anterodorsal lateral line, anteroventral lateral line, and epibranchial IX (Fig. 4 A-D: Schlosser and Northcutt (2000); Baker and Bronner-Fraser (2001)). Although similar, there were some differences between the expression patterns of *xCyp26C* and *xCOUP-TF-B*. For example, these genes were differently expressed in each each placodal nerve (Fig. 4A-D), while only *xCOUP-TF-B* expression was observed in eye, otic vesicles, and telencephalic regions (Fig. 4 A,C and Fig. 4 B and D).

To further explore the expression of these genes, we carried out co-injection analyses using *xCOUP-TF-B* and *xCyp26C*. In this experiment, we also used xCyp26A, another xCyp26-family member. A low dose of *xCOUP-TF-B* injected into the DA region of embryos caused minimal head defect (Fig. 5B). Similarly, injection of 250 pg of *xCyp26A* showed normal phenotype (Fig. 5C). On the other hand, the co-injection of *xCOUP-TF-B* and *xCyp26A* induced a more marked head defect compared to that caused by *xCOUP-TF-B* alone (Fig. 5D). Co-injection with *xCyp26C* also showed similar effects on head defect (Fig. 5 E,F). More detailed examination confirmed that eye size was clearly reduced in the co-injected embryos (Fig. 5G).

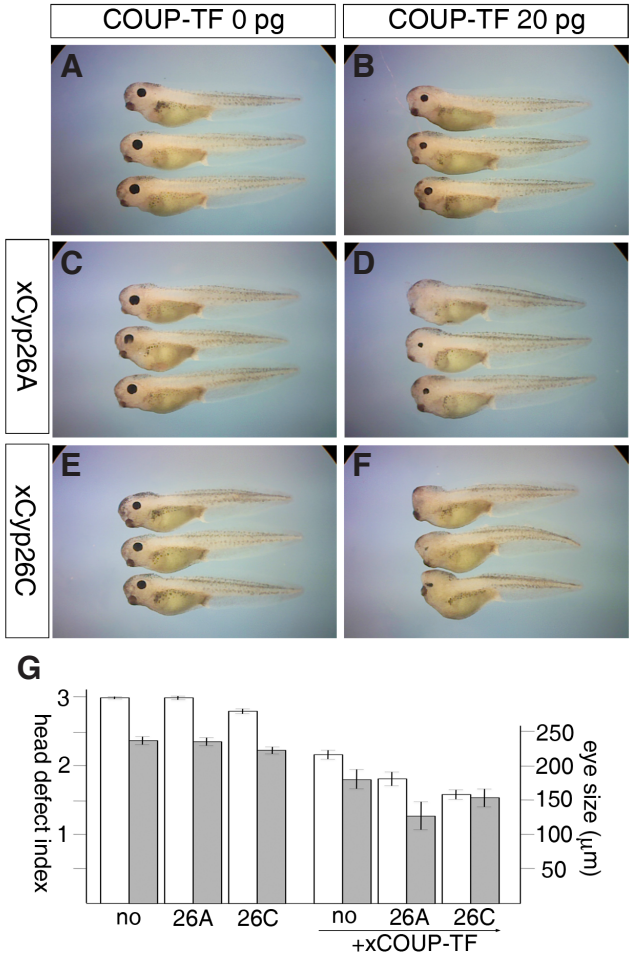

Fig. 5. Co-injection of *xCOUP-TF-B* enhanced the head defect phenotype induced by ectopic expression of *xCyp26A* or *xCyp26C*. (A-F) *500 pg of* xCyp26A **(C, D)** *or 500 pg of* xCyp26C **(E, F)** *was co-injected with* xCOUP-TF-B **(D, F)**, *and injected embryos were observed at stage 40.* **(G)** *The open graph bar indicates head defect index (left column; Michiue* et al., *2004) and gray bar indicates average eye size of injected embryo (right column). 26A and 26C indicates xCyp26A and xCyp26C, respectively. Error bar indicates S.E.*

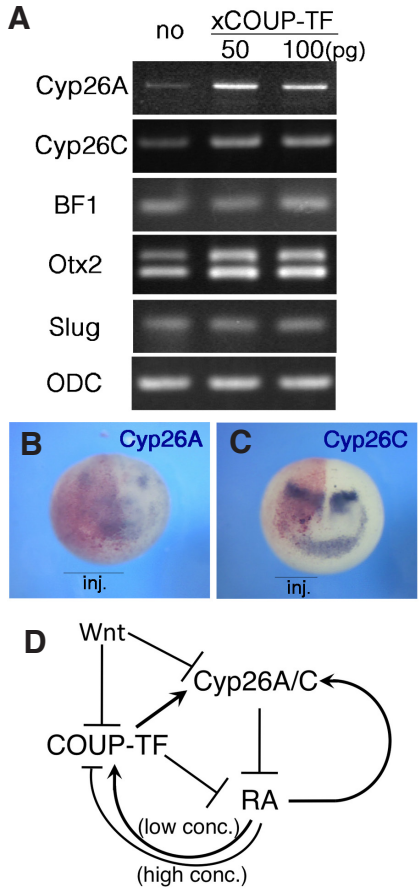

Fig. 6. *xCOUP-TF-B* enhanced transcription of both *xCyp26A* and *xCyp26C*. (A) *RT-PCR was performed with stage-13 embryos injected with 50 pg (lane 2) or 100 pg (lane 3) of* xCOUP-TF-B. *We observed gene expression of* Cyp26A *(column 1),* Cyp26C *(column 2),* XBF-1 *telencephalon marker: column 3),* Xotx2 *(forebrain marker: column 4),* xSlug *(neural crest marker: column 5), and* ODC *(quantitative control: column 6).* **(B,C)** *Whole mount* in situ *hybridization of embryos injected with 75 pg of* xCOUP-TF-B *with* xCyp26A *(B,B′) or* xCyp26C *(C,C′). The injection side was marked with Red-Gal.* **(D)** *Schematic model of xCOUP-TF function.*

xCOUP-TF-B increased xCyp26A and xCyp26C transcription

The co-injection analysis suggests that *xCOUP-TF-B* could positively affect xCyp26A/C function. Previous results indicated a direct association between xCOUP-TF and RXR that negatively regulates RA signaling by competing with RAR (Kliewer *et al.*, 1992). We therefore hypothesized that *xCOUP-TF-B* regulates *xCyp26A/C* transcription. To test this, we examined whether *xCOUP-TF-B* modulates the level of *xCyp26A/C* expression. RT-

PCR analysis revealed that *xCOUP-TF-B* overexpression increased the expressions of both *xCyp26C* and *xCyp26A* (Fig. 6A, 1st and 2nd column). Subsequent *in situ* hybridization of *xCOUP-TF-B*-injected embryos indicated little change in the expressions of *xCyp26A* (Fig. 6B); however, *xCyp26C* expression increased in *xCOUP-TF-B*-injected areas (Fig. 6C). These results suggest that *xCOUP-TF-B* upregulates *xCyp26C* transcription.

Discussion

This study showed that xCOUP-TF acts cooperatively with xCyp26A/C in A-P neural patterning of early embryogenesis. *XCOUP-TF-B* injection increased the expression of several anterior neural genes, and suppressed RA-induced effects. Indeed, these results seemed to implicate an anteriorizing function for xCOUP-TF. Ectopic expression of *xCOUP-TF-B* caused eye defect and enlarged hindbrain formation. This result could reflect an anteriorization effects, but the inhibitory function of xCOUP-TF for RA signaling is dominant in hindbrain and the eye defect may reflect a separate function independent of RA signaling. In the previous report, 10^{-6} M RA treatment suppressed COUP-TF-B expression in brain region (van der Wees *et al*., 1996). However, this result may reflect loss of COUP-TF expression area by treatment with higher RA concentration.

In this study, xCOUP-TF-B synergistically enhanced the superficial phenotype caused by xCyp26A/C, although because xCyp26A/C is not a transcription factor, xCOUP-TF-B and xCyp26A/C are unlikely to function in parallel as cofactor(s). However, our findings suggest that xCOUP-TF-B and xCyp26A/C may work in the same regulation network as described in Fig. 6D. Indeed, we showed that xCOUP-TF-B enhanced xCyp26A/C transcription, suggesting that xCOUP-TF-B physically interacts with the upstream region of xCyp26A/C gene. RA response element (RARE) sequences can be found upstream of the xCyp26A transcribed region, but not in that of xCyp26C (Tanibe *et al.,* unpublished). To clarify our working hypothesis, further investigation is needed to identify the exact regulatory regions.

We also demonstrated the dependency on Wnt signaling in *xCOUP-TF-B* transcription. Supporting the microarray analysis, *xCOUP-TF-B* transcription was decreased by β-*catenin*-mRNA injection. Interestingly, the gene expressions of both *xCyp26A/C* and *xCOUP-TF-B* are increased by the modulation of canonical Wnt signaling, suggesting crosstalk between Wnt and RA signaling. In particular, the fact that RA signaling factors are regulated by Wnt signaling may reflect epistasis of Wnt for RA signaling.

Based on the results of this study, we have proposed a model as follows (Fig. 6D). (1) Wnt signaling negatively regulates expression of both *Cyp26A/C* and *xCOUP-TF-B*. (2) RA signaling positively regulates both xCyp26A/C and xCOUP-TF-B. (3) xCOUP-TF-B positively regulates xCyp26A/C expression. (4) Both xCOUP-TF-B and xCyp26C negatively regulate RA signaling. In context of head specification, canonical Wnt signaling is negative regulated, followed by promotion of both xCOUP-TF-B and xCyp26A/C. We also propose that transcriptional enhancement of xCyp26A/C by xCOUP-TF-B will help to maintain low RA levels, and that ectopic expression of both *xCOUP-TF-B* and *xCyp26A/C* by exposure to RA could also be important for recovering normal RA level and precise anterior specification. Further extensive biochemical analyses are needed to clarify the molecular mechanisms underlying our model.

Materials and Methods

DNA constructs

DNA fragments of the *xCOUP-TF-B* gene were amplified by RT-PCR, and then ligated into pBluescriptSK(+) to make the pXCOUP-TF-B/SK construct. For the injection construct (pXCOUP-TF-B/CS), we inserted an *Eco*RI-*Xho*I fragment of pXCOUP-TF/SK into pCS2(+).

Microinjection

Messenger RNAs for microinjection were synthesized using the mMessage mMachine SP6 kit (Ambion) and were cleaned up with an RNeasy RNA purification kit (QIAGEN). Transcribed mRNA was injected using a Picoinjector (Harvard Medical Instrument). *Xenopus* embryos were obtained by artificial fertilization and dejellied with 4.6% L-cysteine hydroxychloride. Injection was performed in 5% Ficoll-Steinberg's solution, and injected embryos were cultured in the same solution for 5 hours, and then in 10% Steinberg's solution until the appropriate developmental stage.

In situ *hybridization*

In situ hybridization was carried out as previously described (Tanibe *et al*., 2008). In brief, embryos were bleached in hydrogen peroxide-methanol before fixation in MEMFA solution (formaldehyde-MOPS solution) and dehydration with ethanol. Rehydrated embryos were hybridized with DIG-labeled probe for 24 h at 60°C. Embryos were then incubated with 2000 x anti-DIG antibody (Roche) for 12 h, washed 5 times, and then visualized by reaction in NBT/BCIP solution.

RT-PCR

We used the SuperscriptII reverse transcriptase (Invitrogen) for cDNA synthesis. Primers for PCR were as follows:

xCOUP-TF-B	5'-CGAATCTTCCAAGAGCAG GTC -3' and 5'-TTA TTG TAT GGA CAT ATA GGG CC-3';
xCyp26c	5'-ACGAGG GGAAACTGGGCAAATTCAAC-3' and 5'-TCAGGCAAGTGACCCATTTCTTGC TGC-3';
xCyp26a	5'-CTTGCGGAGGTGGAGTGA GGT G-3' and 5'-GCTTAAATA GAGCTGGAGAAGGG-3';
Xotx2	5'-GGATGGATTTGTTACATCCGTC-3' and 5'-CACTCTCCGAGCTCACTTCCC-3';
XAG-1	5'-CATATGTGGTCCCAG TTATTTCCC-3' and 5'-CTCTTAGCGGTTTGAAACCGA-3';
BF1	5'-CATCGTTCAGTATCAACAGCC-3' and 5'-GACTCTCTGTCTTCACTTCCAGG-3';
En2	5'-ATGAGCAGAATAACAGGGAAGTGGA-3' and 5'-CCTCGGGGACATTGACTCGGTGGTG-3';
xSlug	5'-GGATACCCTTCATCTTTAGGTCG-3' and 5'-CATAAGCTGCACTGGAACTTCTC-3';
ODC	5'-GCCATTGTGAAGACT CTCTCCATTC-3' and 5'-TTCGGGTGATTCCTTGCCAC-3'.

For DNA amplification, we used PTC-200 thermal cycler (MJ Research / BioRad). In any cases, we performed 28 cycles of (95°C, 15sec. – 56°C, 15 sec.-72°C, 30sec.) chain reaction. Amplified DNA was detected by EtBr staining after agarose gel electrophoresis.

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