Int. J. Dev. Biol. 52: 893-901 (2008)
doi: 10.1387/ijdb.082683mt

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Retinoic acid metabolizing factor *xCyp26c* is specifically expressed in neuroectoderm and regulates anterior neural patterning in *Xenopus laevis*

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ABSTRACT Anterior-posterior neural patterning is determined during gastrulation when head structure is induced. Induction of anterior neural structures requires inhibition of Wnt signaling by several Wnt antagonists. We performed microarray analysis to isolate genes regulated by canonical Wnt signaling and abundantly expressed in the anterior neuroectoderm at the early neurula stage. We identified *xCyp26c*, a Cyp26 (RA-metabolizing protein)-family gene. *In situ* hybridization showed *xCyp26c* expression restricted to the anterior region of neurula, while *xCyp26a* was expressed in both anterior and posterior regions. At the tadpole stage, *xCyp26c* was also expressed in restricted sets of cranial nerves. Microarray, RT-PCR and *in situ* hybridization analyses revealed decreased *xCyp26c* expression with overexpression of β-catenin, suggesting regulation by Wnt/β-catenin signaling. We also assessed the effects of retinoic acid (RA) on *xCyp26c* expression. Embryos treated with 10^{-7} M RA showed an anterior shift in the spatial expression of *xCyp26c*, reflecting a posteriorization effect. Conversely, expression patterns in embryos treated with more than 10^{-6} M RA were less affected and remained restricted to the most anterior region. Moreover, injection of *xCyp26c* mRNA into animal poles caused head defects, and exogenous expression of *xCyp26c* rescued the posteriorizing effect of RA treatment. Taken together, these results implicated a role for *xCyp26c* in anterior patterning via RA signaling.

KEY WORDS: *Xenopus, retinoic acid, Wnt signaling, AP neural patterning, rhombencephalon*

Introduction

Anterior-posterior (AP) neural patterning and head structure formation is determined during gastrulation in *Xenopus*. Several intracellular signaling pathways are important in AP neural patterning including Wnt, fibroblast growth factor (FGF), and retinoic acid (RA) signaling (Niehrs, 1999; Sasai and DeRobertis, 1997). RA is a posteriorizing factor that induced loss of head due to posteriorization in *Xenopus* embryos treated with RA at the mid-blastula stage (Ruiz i Altaba and Jessell, 1990). RA can also increase types of organs induced by treatment with Activin, reflecting the posteriorization effect (Ariizumi and Asashima, 2001). Although not supported by direct evidence, a presumptive gradient of RA concentration along the midline is considered a determining factor for AP neural patterning. RA concentration is mainly determined by two major factors. The first is retinaldehyde dehydrogenase (RALDH). RA production largely occurs via the oxidative activity of RALDH. RalDH2 is a RALDH-family gene expressed in a range of species including human, mouse, and rat (Zhao *et al.,* 1996; Wang *et al.,* 1996; Hsu *et al.,* 2000). In *Xenopus*, RalDH2 is expressed around the yolk plug in the gastrula stage and overexpression of the gene caused an anterior shift in brain marker expression, implicating RalDH2 function in AP neural patterning (Chen *et al.,* 2001).

Abbreviations used in this paper: AP, anterior-posterior; FGF, fibroblast growth factor; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase.

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Published online: 29 August 2008

0214-6282/2008/$35.00

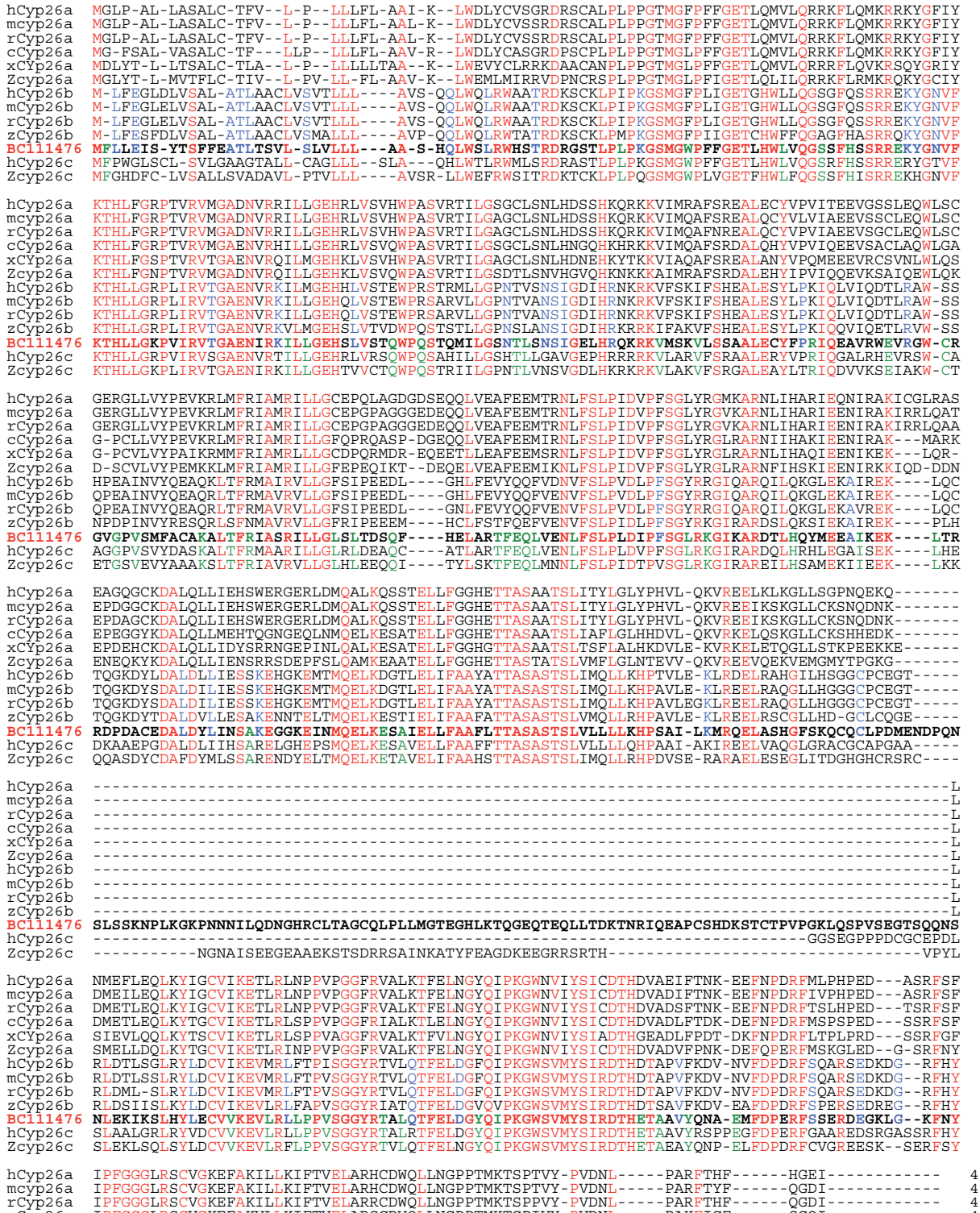


Fig. 1. Comparison of deduced amino acid sequence of BC111476 with that of several *Cyp26* family genes. *Amino acid residues in red are identical among all* Cyp26 *genes, whereas those in green are identical among only* Cyp26c *genes.*

Cyp26 is a RA-metabolizing factor first identified as a novel cytochrome P450 (P450RAI; White *et al.,* 1996). Like RalDH2, Cyp26 is expressed in many species (White *et al.,* 1997; Fujii *et al.,* 1997). Cyp26 genes comprise at least 3 subgroups: Cyp26a, Cyp26b, and Cyp26c. *Xenopus* Cyp26a is found in the circumblastoporal ring and dorsal animal hemisphere, and overexpression of xCyp26a mRNA induces anteriorization (Hollemann *et al.,* 1998; de Roos *et al.,* 1999). Similar expression characteristics were shown in zebrafish (Kudoh *et al.,* 2002). In fact, a recent triple knockdown of Cyp26a, Cyp26b, and Cyp26c in zebrafish caused complete loss of krox20 expression (Hernandez *et al.,* 2007), suggesting that these three genes function coordinately in hindbrain development.

FGF and Wnt signaling both regulate xCyp26a expression (Kudoh *et al.,* 2002). As described above, Wnt signaling is also important in AP neural patterning. Injection of a truncated form of bone morphogenetic protein receptor tBR into the ventral side of embryos induced secondary axis formation without head structure (Glinka *et al.,* 1997). Coinjection of tBR and dominant-negative xWnt8 (dn-xWnt8) induced complete axis with head structure, suggesting that inhibition of Wnt signaling is crucial for head formation (Glinka *et al.,* 1997). Moreover, several canonical Wnt signaling (late canonical Wnt signaling) pathway components such as β-catenin and glycogen synthase kinase -3β (GSK-3β) are also critical for head formation (Onai *et al.,* 2004, Yamamoto *et al.,* 2005, Michiue *et al.,* 2004; Funato *et al.,* 2006). However, the target genes of late canonical Wnt signaling remain unknown.

We therefore sought to identify genes expressed in the presumptive head region of early neurula and negatively regulated by late canonical Wnt signaling. To do this, we prepared cDNAs from different parts of the presumptive anterior neuroectoderm and compared gene expressions by microarray analysis; specifically, between the anterior region of early neurula from normal embryos and the anterior region of early neurula from β-catenin-injected embryos (described on M&M section in detail). We isolated several genes that are negatively regulated by Wnt signaling. We started to characterize the molecular features of xCyp26c from this list. Different from xCyp26a, xCyp26c expression was restricted to the anterior region where it was found in some sets of cranial nerves. XCyp26c expression was positively regulated by inhibition of canonical Wnt signaling, and high-dose RA treatment induced xCyp26c expression only in the anterior region in neurula. Injection of xCyp26c mRNA into the animal pole region of *Xenopus* embryos rescued this posteriorizing effect of RA. Together, these results suggested an important role for xCyp26c in anterior neural development via regulating RA concentration in the anterior neural region.

Results

Identification of xCyp26c *as a head-specific gene*

We earlier identified several genes that were downregulated by activation of β-catenin in the anterior neuroectoderm of early-neurula embryo. One of these (Genbank No. BG264135) was not described as an identified gene, but BLAST searching indicated homology with the gene for hypothetical protein MGC131069 (Genbank No. BC111476, Unigene No. xl.82105). The BC111476 sequence encodes a 603-amino acid protein. Homology search analysis also revealed a BC111476 gene product with an obvious Cyp (Cytochrome P450) domain (Fig. 1). Different from other Cyp26 genes, the BC111476 gene product contains a long sequence gap of about 90 amino acids in length within the Cyp26 domain (Fig. 1). Thus, we divided the BC111476 sequence into N-terminal region (aa 1-342) and C-terminal region (aa 438-602), and compared these regions among other Cyp26 gene products. The BC111476 N-terminal region was most similar with human Cyp26c (hCyp26c) (65.0% homol-

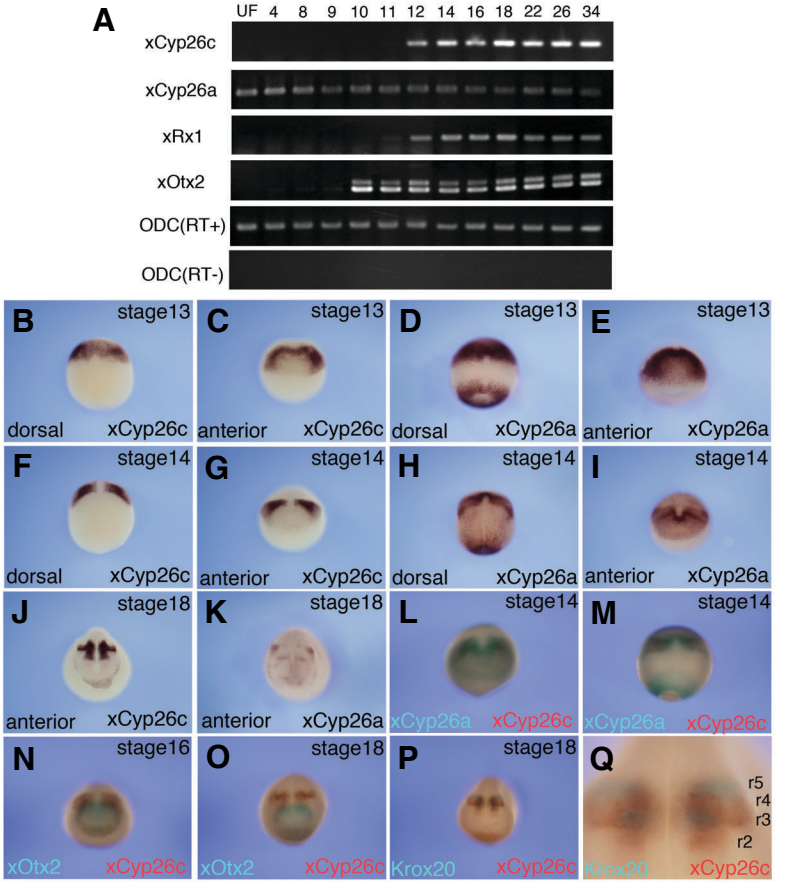


Fig. 2. Temporal and spatial pattern of *xCyp26c* expression. (A) *Temporal pattern of* xCyp26c *expression was examined by RT-PCR analysis with cDNAs prepared from embryos at various stages.* XCyp26c *expression increased from the mid-late gastrula stage. This pattern is different from* xCyp26a, *which is ubiquitously expressed.* **(B-K)** *The spatial pattern of* xCyp26c **(B,C,F,G,J)** *and* xCyp26a **(D,E,H,I,K)** *expression was analyzed by whole-mount* in situ *hybridization. Different from* xCyp26a, xCyp26c *was expressed only in the presumptive anterior neural region at the early neurula stage (B,C, compared with D,E). At the mid-neurula stage, the midline of* xCyp26c *expression decreased* **(F,G)**. *By the late neurula stage,* xCyp26c *expression was restricted to a set of rhombencepharic cells* **(J)**, *whereas* xCyp26a *expression was greatly reduced* **(K)**. **(L-Q)** *Double* in situ *hybridization. Brown staining indicates* xCyp26c *expression, while the light blue staining indicates* xCyp26a **(L,M)**, xOtx2 **(N,O)**, *and* xkrox20 **(P,Q)** *staining, respectively. Anterior view (L,N,O,P). Dorsal view (M).* **(Q)** *Magnified image of (M). r2-5 show rhombomeres 2 to 5, respectively. These data indicated that* xCyp26c*-positive cells were mainly identical with r2, r3, and r4.*

ogy; Table 1), and also showed high homology with zebrafish Cyp26c (zCyp26c) (58.9%; Table 1). Compared with Cyp26c, Cyp26a and Cyp26b showed less homology with BC111476. Similarly, the C-terminal domain of BC111476 was most similar to human Cyp26c (69.9%). Therefore, we defined BC111476 as xCyp26c and further investigated the role of this gene.

Expression of xCyp26c *gene in gastrula-neurula-stage embryo*

We next used RT-PCR to analyze the temporal expression of xCyp26c, using cDNAs prepared from various *Xenopus* embryonic stages. XCyp26c expression was low prior to gastrulation, and increased from the mid-gastrula stage (Fig. 2A). This was different from that of xCyp26a, which was expressed throughout development (Fig. 2A). It should be pointed out that xCyp26a expression gradually decreased after the tailbud stage (Fig. 2A). Microarray analysis revealed 40-fold higher signal intensity for xCyp26c expression in the anterior region compared to that in the posterior region (Table 2), suggesting that xCyp26c expression was restricted to the head region of neurula. To evaluate this result and further examine the spatial expression of xCyp26c, we carried out *in situ* hybridization, using xCyp26a expression to compare spatial patterns. At stage 13, xCyp26c was expressed in presumptive anterior neuroectoderm, in an arch-like pattern similar to xCyp26a (Fig. 2C, E). In contrast to xCyp26a, however, there was no posterior expression of xCyp26c around the yolk plug (Fig. 2B, D). At stage 14, xCyp26c expression was strong in the presumptive hindbrain region and decreased around the midline (Fig. 2F, G). The expression patterns of xCyp26a and xCyp26c were similar in the anterior neuroectoderm (Fig. 2G, I); xCyp26a expression was also seen at the rhombencephalic region, but the expression was most intense around the midline and the anterior end of hindbrain, whereas the expression area of xCyp26c was more posteriorly located (Fig. 2G, I). XCyp26a was moderately expressed at the anterior neural plate and adjacent regions including the presumptive cement gland (Fig. 2I). Moreover, xCyp26c was not expressed in the posterior ectoderm even at the mid-neurula stage (Fig. 2F). At stage 18, the region of xCyp26c expression was further narrowed, and one of the rhombencephalic expression areas extended laterally (Fig. 2J). Expression of xCyp26c was also seen outside of the neural plate including presumptive cement gland region at this stage (Fig. 2J). XCyp26a expression was markedly decreased at the anterior neural plate by stage 18 (Fig. 2K), whereas posterior expression remained (data not shown).

We next used double *in situ* hybridization with digoxygenin (DIG)-labeled and fluorescein (Flu)-labeled probes to determine the exact expression region of xCyp26c. First, we used DIG-labeled xCyp26a and Flu-labeled xCyp26c mRNAs. At the early neurula stage, expression patterns around the yolk plug were different between the two transcripts (Fig. 2M), while the intense expression patterns in the anterior neural plate seemed nearly identical, with slightly weaker expression of xCyp26c than xCyp26a at the midline (Fig. 2L). The circular patterns of expression in anterior neuroectoderm were also similar, although not identical in this region (Fig. 2M). Next, we compared the anterior and posterior regions of xCyp26c expression using a mid-brain marker gene (Xotx2). The posterior ends of the Xotx2 expression area only partially overlapped with xCyp26c expression, suggesting that xCyp26c was mainly expressed in rhombencephalon (Fig. 2N). At stage 18, the most anterior part of the xCyp26c expression area overlapped closely with that of Xotx2, indicating that the anterior end of xCyp26c expression extended into the cement gland region (Fig. 2O). To identify which rhombomeric region expressed xCyp26c, we performed double *in situ* hybridization

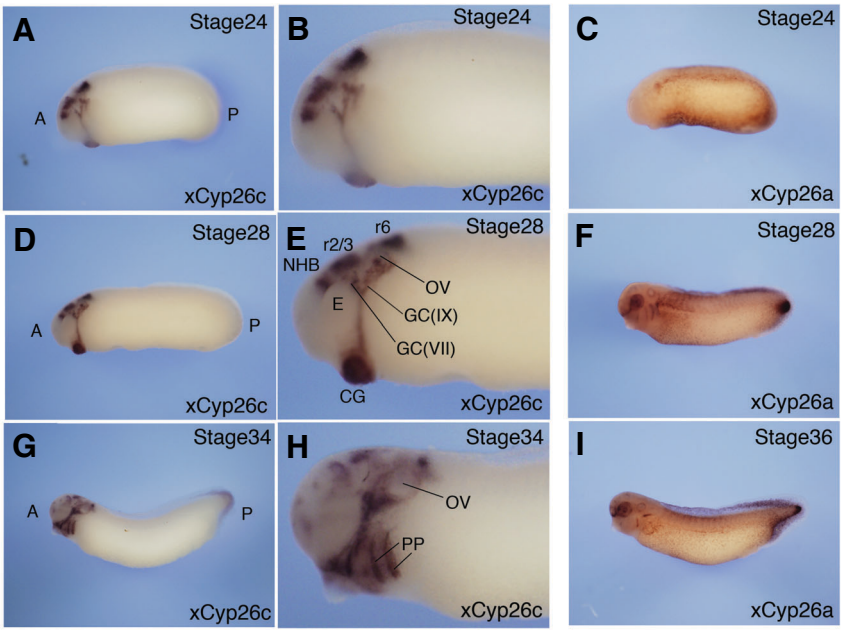


Fig. 3. Spatial expression of *xCyp26c* after tailbud stage. (A-B, D-E, G-H) XCyp26c *expression.* **(C,F,I)** XCyp26a *expresion. Stage 24* **(A-C)**, *stage 28* **(D-F)**, *and stage 36* **(G-I)**. XCyp26c *was expressed in restricted sets of cranial nerves (GC(VII) and GC(IX)). XCyp26c was also expressed in the pharyngeal pouch (PP) and rhombomeric cells (r2/3 and r6). These patterns were obviously different from those of* xCyp26a *(C,F,I).*

TABLE 1

% HOMOLOGY BETWEEN *BC111476* AND OTHER CYP26 PROTEINS

	% homology	
	BC111476-N (a.a. 1-342	**BC111476-C (a.a. 438-602)**
hCyp26a	45.4	52.0
mCyp26a	44.8	51.3
rCyp26a	45.1	52.7
cCyp26a	45.0	52.4
xCyp26a	45.0	50.3
zCyp26a	42.4	52.3
hCyp26b	54.6	58.3
mCyp26b	54.0	59.5
rCyp26b	53.7	58.9
zCyp26b	52.3	60.1
hCyp26c	65.0	69.9
zCyp26c	58.9	65.8

These data indicate that BC111476 is likely to be a *Xenopus* homolog of Cyp26c

TABLE 2

MICROARRAY SIGNAL INTENSITIES OF *XCYP26A* AND *XCYP26C* EXPRESSION

		slide#1			slide#2		
No.	**genename**	**Ant.**	**Pos.**	**A/P**	**Ant.**	**Post.**	**A/P**
Xl.1946	xCyp26c	9850	232	42.5	9130	182	50.2
Xl.456	xCyp26a	12600	15500	0.8	9900	16200	0.6
		slide#3			**slide#4**		
No.	**genename**	**bcat.**	**No**	**No/bcat.**	**No**	**bcat.**	**No/bcat.**
Xl.1946	xCyp26c	3770	8380	2.22	2910	7100	2.44
Xl.456	xCyp26a	7770	10000	1.28	7450	11100	1.49

Ant. and Pos. shows target RNAs derived from the anterior and posterior region, respectively. Expression of xCyp26c was obviously decreased by b-catenin (b-cat) injection.

with xCyp26c and Xkrox20 probes. As expected, stage-18 embryos showed partially overlapping rhombencephalic expressions of xCyp26c and xkrox20 on low-magnification imaging (Fig. 2P). Higher magnifications localized the xCyp26c expression to r2, r3, and r4 (Fig. 2Q).

XCyp26c *expression in cranial nerves at the tailbud-tadpole stage*

At stage 24, strong xCyp26c expression was seen in cement grand primordia (Fig. 3A) and in some rhombencephalic regions. From here, the expression of xCyp26c extended to the cement glands, although the two regions of expression did not connect (Fig. 3A, B). At stage 28, xCyp26c expression in cranial nerves from the rhombencephalon further elongated, obviously connecting with that in cement glands (Fig. 3D, E). Spatial comparison of the xCyp26c expression pattern and otic vesicle position indicated the xCyp26c-positive nerves to be typeVII and type IX (Fig. 3E). At stage 34, xCyp26c expression was still restricted in the head region (Fig. 3G). Expression in the cranial nerves was maintained and became complicated especially around the cement gland (Fig. 3H). XCyp26c expression was also apparent in the rhombencephalon was also seen, although here it was less intense and restricted (Fig. 3H). Moreover, xCyp26c-positive cells were also seen at the ventral side of the otic vesicle and pharyngeal pouch (Fig. 3H). Finally, the xCyp26c expression pattern differed from that of xCyp26a (Fig. 3 C, F, I).

XCyp26c *expression is regulated by canonical Wnt signaling*

The microarray analysis suggested that canonical Wnt signaling regulates xCyp26c transcription, although not whether xCyp26c is a direct target gene. Thus, we next examined how modulating the Wnt pathway affected xCyp26c expression. We injected various doses of β-catenin mRNA into the animal poles of 8-cell embryos and assayed expression by RT-PCR analysis and *in situ* hybridization. Injection of 1 ng of β-catenin mRNA decreased xCyp26c expression (Fig. 4A, column 1), but had little effect on the expression of xCyp26a (Fig. 4A, column 2). *In situ* hybridization of the β-catenin-injected embryos revealed clearly reduced xCyp26c expression in the anterior neural plate (Fig. 4D, E). These results suggested that xCyp26c expression is negatively regulated by canonical Wnt signaling.

XCyp26c *expression was upregulated by retinoic acid treatment*

Cyp26a expression changes in response to low doses of RA (1.8×10^{-7} M; Hollemann *et al.,* 1998). Conversely, in chick embryo, Cyp26c was downregulated by RA treatment (Reijntjes *et al.,* 2005). We used *in situ* hybridization to assess the effect of RA treatment on xCyp26c expression. As described above, normal embryos showed intense expression of xCyp26c mRNA in the rhombencephalic region of mid-neurula stages (Fig. 5A, Fig 2G). Increasing the concentration of RA gradually shifted the xCyp26c expression area to the anterior end of the neuroectoderm (Fig. 5B, C, D, and E). High magnification images showed that most anterior edges of xCyp26c expression were not shifted, whereas the rhombencephalic expression was slightly extended and shifted both anteriorly and medially, resulting in overall reduction of the expression area (Fig. 5E, compared with 5A). The xCyp26a expression pattern was also

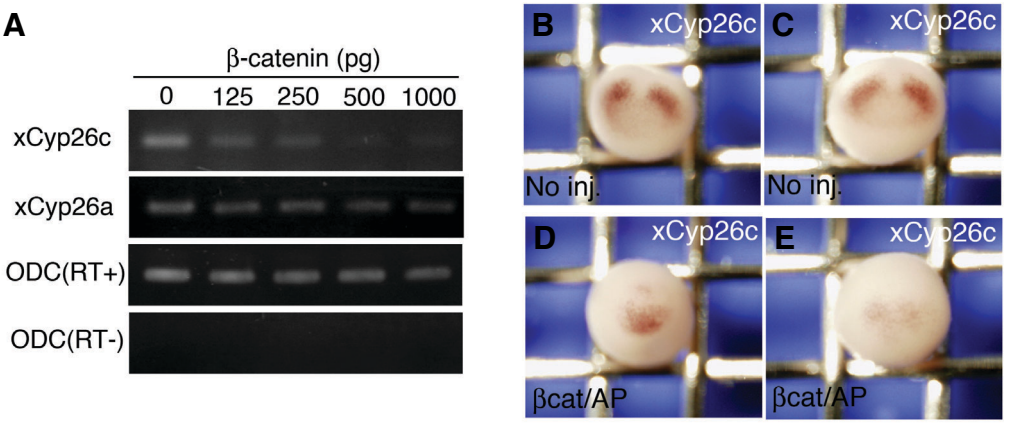
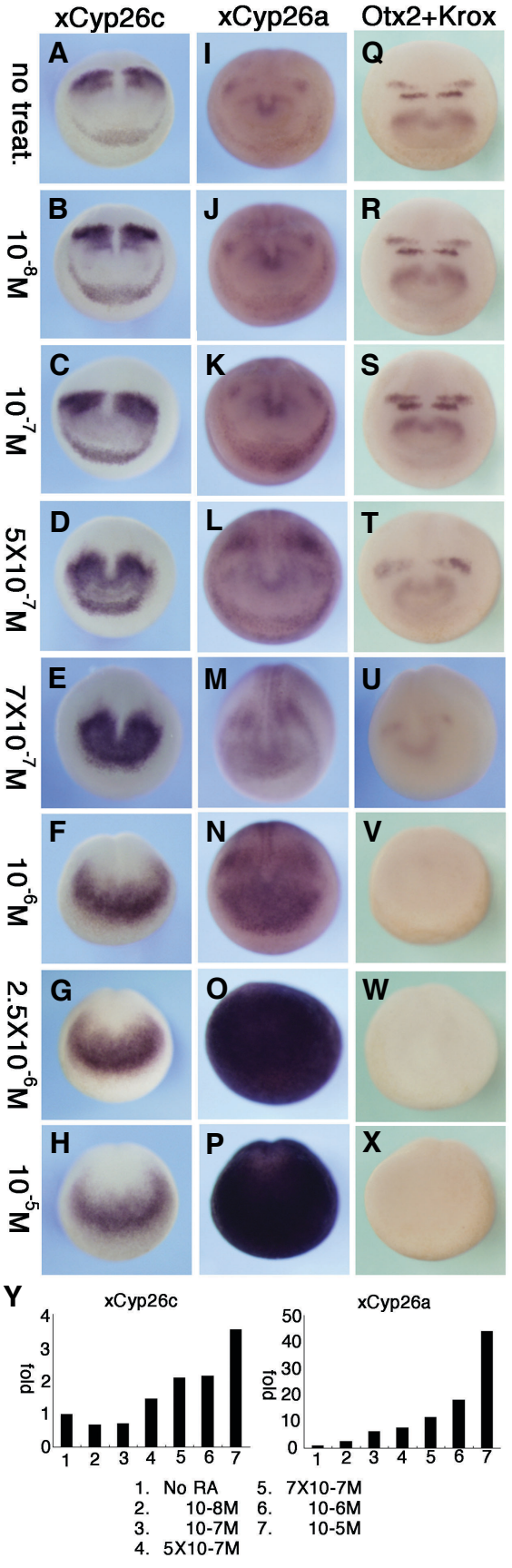


Fig. 4. Regulation of *xCyp26c* expression by canonical Wnt signaling. (A) *RT-PCR analysis with cDNAs synthesized using* β-catenin*-injected embryos. Injection of* β-catenin *mRNA into the animal pole region of embryos decreased* xCyp26c *expression in a dose-dependent manner.* XCyp26a *expression was not clearly decreased by* β-catenin *injection.* **(B-E)** *Expression of* xCyp26c *in mid-neurula embryos injected into the animal pole with nothing* **(B,C)** *or* β-catenin **(D,E)**. β-catenin *clearly inhibited* xCyp26c *expression (D,E).*



changed by RA treatment, although not in the same manner as observed with xCyp26c (Fig. 5I, J, K, and L). Doses of RA higher than 10^{-6} M no longer affected the xCyp26c expression pattern, which remained condensed in an arched shape (Fig. 5 F, G, and H). In contrast, xCyp26a became expressed throughout the whole embryo as the RA dose increased above 10^{-6} M (Fig. 5N, O and P). We also observed xOtx2 and xkrox20 gene expressions in these embryos. Up to 7 x 10^{-7} M RA, the marker gene expression patterns shifted similarly to that of xCyp26c, before disappearing with RA concentrations above 10^{-6} M (Fig. 5Q-V). Furthermore, we ensured change of the expression level of xCyp26c and xCyp26a by real-time PCR analysis. XCyp26c was, like as xCyp26a, increased by elevation of RA concentration (Fig. 5Y). Though expression pattern did not show obvious difference, xCyp26c expression was further increased more than 10^{-6}M RA (Fig. 5Y lane 6 and 7, compared with Fig. 5F and H).

Overexpression of xCyp26c *could attenuate the anterior neural RA effect*

To examine whether overexpression of xCyp26c induces morphogenesis of the anterior neural region, we injected xCyp26c mRNA into *Xenopus* embryos and observed the morphological effects. Injection of 1 ng of xCyp26c mRNA into the dorsal blastomere occasionally induced a small head, although the phenotype was weak (Fig. 6D). However, histological examination of the injected embryos revealed no morphological change in brain structure (Fig. 6E, F, compared with B and C). Ventral injection with xCyp26c caused no defect (data not shown).

Next we speculated that if xCyp26c regulates patterning in the anterior neural region at the neurula stage, overexpression of xCyp26c could weaken the observed RA effect. Accordingly, we treated the xCyp26c mRNA-injected embryos with RA and observed the external morphology, while mock-injected embryos treated with 10^{-6} M RA showed loss of head structure due to posteriorization (Fig. 6G). On the other hand, 1 ng of xCyp26c mRNA injected into animal poles still caused head defects, but the severity was less, and tadpoles still showed eye structures (Fig. 6H). Subsequent RT-PCR experiments on lacZ mRNA-injected embryo treated with 10^{-6} M RA showed decreased expression of both xBF-1 (telencephalic marker) and xRx1 (diencephalic marker) (Fig. 6J, lanes 2 and 3). Conversely, the decrease was significantly attenuated in xCyp26c-injected embryos (Fig. 6J, lanes 5 and 6). These results suggested that xCyp26c expression rescues the loss of forebrain marker ex-

Fig. 5. Effect of retinoic acid (RA) on *xCyp26c* expression. (A-X) *Embryos were treated with various doses of RA at stage 8, cultured until stage 13, and then prepared for WISH analysis. No treatment* **(A-C)**. *RA treatment* **(D-X)**. *Concentration of RA (M) is shown on left side. Expression pattern of* xCyp26c **(A-E)**, xCyp26a **(I-P)** *and* xOtx2 *and* xkrox20 **(Q-X)**. *Moderate doses of RA increased* xCyp26c *expression, but narrowed the area of expression* **(A-M)**. *Over* 10^{-6} *M of RA,* xCyp26c *expression became restricted in the anterior neuroectoderm and underwent no further change* **(F-H)**. XCyp26a *was expressed throughout the embryo* **(O,P)**, *whereas* xOtx2 *expression completely disappeared* **(V-X)**. **(Y)** *Real-time PCR analysis with whole embryos treated with RA. These graphs are expressed as ratio of expression level of RA-treated embryo (lane 2-7) to that of no-RA treated embryo (lane 1).*

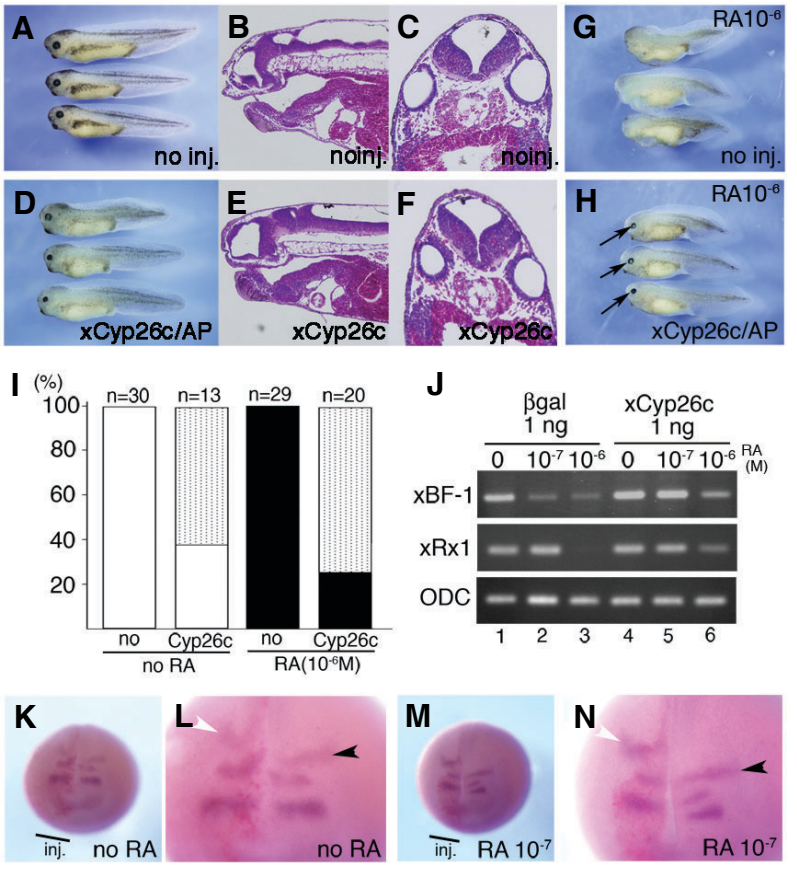


Fig. 6. Overexpression of *xCyp26c* could attenuate retinoic acid (RA)-induced posteriorization. (A-F) *Phenotype of tadpole injected with* xCyp26c *mRNA.* **(A-C)** *Normal tadpoles.* **(D-F)** *Tadpoles injected with 1 ng of* xCyp26c *mRNA into the animal pole region.* **(B,C,E,F)** *Histological section of* xCyp26c*-injected tadpoles. Sections were stained by hematoxylin-eosin.* **(B,E)** *Sagittal sections, whereas (C,F) show transverse sections of (A,D), respectively.* **(G-I)** *Head defect by RA treatment was rescued by* xCyp26c *injection. Embryos not injected (G) or* xCyp26c *mRNA-injected embryos (H) were treated with* 10^{-6} *M of RA. In* xCyp26c*-injected embryos, loss of eye structure was rescued (arrow). These results are summarized in graph* **(I)**. *White box, dotted box, and filled box show normal eye, small eye, and no eye structure, respectively. Point out that the size of eye vesicle reflects the severity of posteriorizaion by RA treatment.* **(J)** *RT-PCR analysis with cDNAs synthesized from whole embryo injected with* β-gal *(1 ng; lanes 1-3) or* xCyp26c *(1 ng; lanes 4-6). Embryos were treated with* 10^{-7} *M (lane 2, 5) or* 10^{-6} *M (lane 3, 6) of RA, or untreated (lane 1, 4). Overexpression of* xCyp26c *attenuated the inhibition of* xBF-1 *(telencephalic marker; column 1) and* xRX1 *(diencephalic marker; column 2) expression by RA treatment (lane 6).* **(K-N)** *Change of expression patterns of* En2 *and* xkrox20 *by* xCyp26c *injection. (L,N) are magnified views of (K,M), respectively. Injected side (inj. In (K) and (M)) was indicated by lineage tracer (Red-gal).* **(K,L)** *show no RA-treated embryos and* **(M,N)** *show* 10^{-7} *M RA-treated embryos. Normal embryos showed a slight posterior shift of* xkrox20 *expression in the* xCyp26c*-injected area (white arrowhead, compared with noninjected region (black arrowhead)). RA-treated embryos showed an obvious difference in* xkrox20 *expression patterns between injected and noninjected regions.*

pression induced by RA treatment. To observe this rescue spatially, we injected xCyp26c mRNA into half the blastomere of animal poles and treated with RA. Analysis of anterior marker-gene expressions by *in situ* hybridization showed the expected anterior shift in En2 and xkrox20 expression in xCyp26c mRNA-injected embryos (Fig. 6 K, L), and treatment with 10^{-7} M RA increased this shift (Fig. 6 M, N).

Discussion

XCyp26c *expression in anterior ectoderm*

In situ hybridization experiments showed xCyp26c expression restricted to the anterior region of early neurula, while xCyp26a was expressed both in anterior neuroectoderm and around the yolk plug (Hollemann *et al.,* 1998). In support of this, our microarray analysis indicated 40-fold higher expression of xCyp26c in the anterior embryonic regions than in posterior parts, whereas abundant xCyp26a expression was seen posteriorly (Table 2). RA has a posteriorizing effect and this phenotype is severe only in the head region. We therefore propose that xCyp26c functions mainly in AP axis formation.

After the late neurula stage, xCyp26c was strongly expressed in a rhombencephalic region that corresponded to r2, r3, and r4 by double *in situ* hybridization. These data correlate with a recent zebrafish study (Hernandez *et al.,* 2007), showing evolutionary conservation. At the tadpole stage, xCyp26c was expressed only in a subset of cranial nerves. Neuroanatomically, the root of cranial nerve VII is r4, whereas nerve IX is r6, supporting the rhombencephalic expression of xCyp26c. Furthermore, our results showed these nerves extending to cement gland primordial, and nerves VII and IX are related to saliva secretion, supporting this result.

The effect of RA on xCyp26c *expression*

In this study, xOtx2 expression shifted anteriorly, indicating shrinkage of the forebrain region. XCyp26c expression showed a similar shift until the RA concentrations exceeded 10^{-6} M, when expression did not change further temporally or spatially. At these RA levels, xkrox20 and En2 expression completely disappeared. These results may show existence of two independent systems for regulating xCyp26c transcription. XCyp26c expression was not quantitatively enhanced at low RA levels, with the xCyp26c expression area dependent simply on AP patterning. On the other hand, exposure to RA in high concentrations expanded xCyp26c expression only in the limited region of embryos. In addition and unlike for other family member xCyp26a, the area of ectopic xCyp26c expression was limited. This limitation might reflect that the competent region of xCyp26c expression is restricted in the anterior neuroectoderm. It is unclear, however, how this competent area is defined and further studies need to be done.

XCyp26c function in rhombencephalon

Our results revealed strong xCyp26c expression in the rhombencephalic region. RA signaling is involved with rhombencephalic differentiation (Papaopulu *et al.,* 1991; Kolm *et al.,* 1997), so we predicted that overexpression of xCyp26c would cause defects in hindbrain development. However, xCyp26c injection had no obvious effect on rhombencephalon, suggesting

that RA signaling in hindbrain is sufficiently maintained at low RA levels and that any effect of xCyp26c is hard to observe. We have not yet done downregulation assays with morpholino antisense oligos, and recent studies in mouse and zebrafish showed that knockdown of only xCyp26c had almost no effect, and that simultaneous disruption of both xCyp26a and xCyp26c was required for the hindbrain defect (Uehara *et al.,* 2007; Hernandez *et al.,* 2007). Together with the expression patterns of these genes, these findings implicate a cooperative action of xCyp26a and xCyp26b, in addition to xCyp26c alone, in rhombencephalon development.

Transcriptional regulation of RA signaling-related genes by Wnt signaling

Finally, transcription of xCyp26c was inhibited by upregulation of canonical Wnt signaling. Interestingly, our microarray analysis revealed that RalDH2, a RA synthesizing-factor gene, was positively regulated by canonical Wnt signaling (data not shown). It is thought that a presumptive gradient of canonical Wnt signaling is formed along with the AP axis. We therefore propose that formation of a RA gradient that is high in the posterior region and low in the anterior region might be actualized by complementary expression of xCyp26c and RalDH2 via Wnt signaling. However, xCyp26a is expressed both anteriorly and posteriorly, suggesting that RA signal regulation for AP pattern determination is complicated. More analyses are clearly needed to understand the crosstalk between RA and Wnt signaling in AP neural patterning.

Materials and Methods

DNA construction

A DNA fragment containing the xCyp26c gene was obtained by RT-PCR (described below). Primers for cloning were 5'-AGG ACT GTC ACT AGT GAG ACC ACT GG-3' and 5'-TCA GGC AAG TGA CCC ATT TCT TGC TGC-3'. For microinjection, the xCyp26c fragment was inserted into the EcoRI and XhoI sites of pCS2+.

RT-PCR

RT-PCR analysis was performed as described above (Suzawa *et al.,* 2007). Primers for PCR were as follows: xCyp26c; 5'-ACG AGG GGA AAC TGG GCA AAT TCA AC-3' and 5'-TCA GGC AAG TGA CCC ATT TCT TGC TGC-3', xCyp26a; 5'-CTT GCG GAG GTG GAG TGA GGT G-3' and 5'-GCT TAA ATA GAG CTG GAG AAG GG-3', ODC; 5'-GCC ATT GTG AAG ACT CTC TCC ATT C-3' and 5'-TTC GGG TGA TTC CTT GCC AC-3', xRX1; 5'-GGC TAT GGA GAT CCA TAT TCA GG-3' and 5'-CTC TTC TCT GCT GTA TAC GTC GG-3', xOtx2; 5'-GGA TGG ATT TGT TAC ATC CGT C-3' and 5'-CAC TCT CCG AGC TCA CTT CCC-3'.

Real time PCR analysis was performed with Chromo4 real-timePCR analysis system (BioRad). For detection, we used iQ CYBR Green Supermix (BioRad).

Microinjection

Messenger RNAs for microinjection were synthesized with a mMessage mMachine SP6 kit (Ambion) and were cleaned up with a RNeasy RNA purification kit (QIAGEN). Embryos were obtained by artificial fertilization, dejellied with 4.6% L-cysteine solution, and then microinjected using a PLI100 microinjector (Harvard Medical Instruments). For phenotype observation, injected embryos were cultivated in 0.1 x Steinberg's solution for 3 days.

In situ hybridization (WISH)

WISH was performed as described in Michiue *et al.* (2007). In brief, embryos were fixed in MEMFA solution (formaldehyde-MOPS solution), bleached in hydrogen peroxide-methanol, and then dehydrated using ethanol solutions. After rehydration, embryos were hybridized with DIG-labeled probe for 24 hours at 60°C. Embryos were then incubated with 2000 x anti-DIG antibody (Roche) for 12 h, washed 5 times, and detected by reaction in NBT/BCIP solution. Embryos injected with β-gal mRNA to mark the injection area were stained with Red-Gal before MEMFA fixation. For double-labeled WISH, RNA probes were labeled with DIG or Flu RNA mixture (Roche) and detected with BCIP or Vectorblack AP substrates (VECTOR) respectively.

Microarray analysis

Overview of the microarray analysis we performed was described previously (Michiue *et al.,* 2007). For synthesizing target RNAs, stage 13 embryos were cut into three equal parts (anterior, middle, posterior) and total RNA was prepared from the anterior part and the posterior part of dissected embryo.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (M. A. and T. M) and a Grant from the National Institute of Advanced Industrial Science and Technology.

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