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Isolation of Early Neurogenesis Genes with *Xenopus* cDNA Microarray

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Neurogenesis is the process that develops neuroectoderm from ectoderm. Bone morphogenetic protein (BMP) inhibition in ectodermal cells is necessary and sufficient for neurogenesis in *Xenopus* embryos. To isolate genes involved in early neurogenesis, *Xenous* Affymetrix gene chips representing 14,400 genes were analyzed in early stage of neuroectodermal cells that were produced by inhibition of BMP signaling with overexpression of a dominant-negative receptor. We identified 265 candidate genes including 107 ESTs which were newly expressed during the early neurogenesis by blocking BMP signaling. The candidates of 10 ESTs were selected and examined for upregulation in neuroectoderm. Five EST genes were confirmed to be upregulated in neuroectoderm and examined for time-dependent expression patterns in intact embryos. Two EST genes were cloned and identified as a homology of CYP26c (Xl.1946.1.A1_at) and Kielin containing VWC domain (Xl.15853.1.A1_at). One of them, CYP26c, was further characterized for its transcriptional regulation and role of anterior-posterior patterning during neurogenesis. Taken together, we analyzed and characterized genes expressed in early neurogenesis. The results suggest that neurogenesis by inhibition of BMP provides useful system to isolate genes involved in early events of neurogenesis during early vertebrate embryogenesis.

Key words: *Xenopus*, BMP inhibition, neurogenesis, Affymetrix gene chips

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Vertebrate neurogenesis is the generation process of neural cells. The early event of neurogenesis is converting processes from presumptive ectoderm to neuroectoderm at early gastrula. It was considered from ectoderm to neuro-ectoderm as inductive processes by active signaling secreted from presumptive underlying mesoderm on overlaid ectodermal cells of early gastrula stage embryos. The process was called neural induction and several neural inducers including noggin and chordin were found using *Xenopus* embryos (Lamb *et al.*, 1993; Sasai *et al.*, 1994). They contain direct neural induction activities of ectoderm to neuroectoderm without involvement of mesoderm.

In more recent years, inhibition of bone morphogenetic protein (BMP) has been discovered to initiate neurogenesis from ectodermal cells without neural inducers (Hawley *et al.*, 1995; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995; Hemmati-Brivanlou and Melton, 1997). Neural inducers secreted from organizer were then found to fuction as BMP inhibitors through direct binding to BMP (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Blokade of BMP signaling is sufficient to express anterior neural genes, whereas posterior neural patterning requires additional factors. Neurogenesis is now considered as a default process, whereas ectodermal specification requires active BMP signaling (Munoz-Sanjuan *et al.*, 2002a.) However, it is largely unkown how absence of BMP signaling initiates neurogenesis and makes only anterior neural genes expressed.

To identify the genes involved in early neurogenesis and anterior neural patterning caused by inhibition of BMP-4 signaling, mRNA of dominant negative BMP receptor (DNBR) was ectopically expressed in *Xenopus* embryos. The gene expression profiles were analyzed using *Xenopus* Affymetrix

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gene chips containing 14,400 gene transcripts. We identified candidate genes including 107 ESTs which were newly expressed during the early neurogenesis by blocking BMP signaling. The candidates of 10 ESTs were then examined for the expression patterns. As results, we found that 5 of them were promising candidates. Among them, 2 ESTs (XI.1946, 15853) were selected and cloned. Functional analysis of one gene, CYP26c, was performed for anterior neural patterning of neurogenesis. The results suggest that gene analysis of *Xenopus* gene chip provides useful tool to identify genes involved in earliest events of neurogenesis during vertebrate embryogenesis.

Material and Methods

Embryo injection, explant culture and retinoic acid treatment

Xenopus leavis eggs were fertilized *in vitro* as described (Sive, 2000) in which embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). The vitelline membranes were removed by immersing the embryos in the thioglycolic acid solution. Embryos at the one- or two-cell stages were injected in the animal pole with messenger RNA as described in the figure legends. Animal caps were dissected from the injected embryos at stages 8-9 and cultured to stages 11.5-12 in 67% Leibovitzs L-15 medium (GIBCO/BRL, USA) with BSA (1 mg/mL), 7 mM Tris-HCl (pH 7.5) and gentamicin (50 μg/mL). All *trans*-retinoic acid (Sigma, USA) was applied to animal explants obtained from the blastula embryos or to whole embryos until early gastrula stage for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

In vitro transcription

All synthetic mRNAs used for microinjection were produced by *in vitro* transcription. The junD cDNA was inserted in the pGEM (Rauscher *et al.*, 1988), c-fos were sub-cloned into the pSP64T vector (Curran *et al.*, 1987), and DNBR cDNA was inserted in the pSP64T vector. Each of the cDNAs was linearized and used for *in vitro* synthesis of capped mRNA using *in vitro* transcription kit (Invitrogen, USA) in accordance with the manufacture's instructions. The synthetic RNA was quantified by ethidium bromide staining in comparison with a standard RNA.

Sample preparation and microarray analysis

Embryos at the one- or two-cell stages were injected in the animal pole with 2 ng/5 nl of synthetic mRNA for DNBR. Animal caps, injected or not injected, were dissected from the embryos at stages 8-9 and cultured to stages 11.5-12 in the Leibovitzs L-15 medium. About 500 animal caps were harvested and stored in the RNA *letter*, RNA stabilization reagent, (Qiagen, USA) at 4°C until RNA extraction. RNA was treated with DNase1 and purified using RNeasy kit (Qiagen, USA). One hundred micrograms of total RNA was used for cDNA synthesis and to make labeled RNA probe which was hybridized to Affymetrix *Xenopus* gene chips. Microarray experiments were performed in Seoulin Bioscience with Affymetrix *Xenopus* genome gene chip as described (www.seoulin.co.kr and www.affymetrix.com). The experiments for DNA microarray were performed 3 times for each group and excluded non-overlapping genes for gene screening analysis. The data were presented with one representing group of 3 different experiments.

Plasmid construction, RNA synthesis, and whole-mount *in situ* hybridization

We identified cDNA fragments XL069c07 (Xl 1946) and XL017b19 (Xl 15853), in our microarray screen, and subsequently found the full-length cDNA clones, in *Xenopus* EST database (NIBB XDB, http://www.Xenopus.nibb. ac.jp). Using xCYP26c (XL069c07) clone, we subcloned the cDNA clones in the pCS2 vectors to perform full sequncing for localization studies. Whole-mount *in situ* hybridization was done in principle as described (Harland, 1991), with modifications as reported (Hollemann *et al.*, 1998). Probes were prepared using the digoxigenin or fluorescein RNA-labeling mixes (Boehringer Mannheim, Switzerland) and subsequendly purified using *in vitro* transcription kit (Invitrogen, USA). The probe used was xCYP26c, which was cut with *Eco*RI and transcribed with T7 RNA polymerase.

RNA isolation and RT-PCR

Total RNA was extracted from whole embryos or cultured animal explants with TRIzol reagent (Life Technologies, USA) following the manufacturer's instruction. RT-PCR was performed as follows: first, a denaturation step of 94°C for 5 min; second, 94°C for 1 min; third, each annealing temperature for 1 min; fourth, 72°C for 1 min; fifth, repeat second, third and fourth steps 19-30 cycles of amplification was performed as described at the *Xenopus* Molecular Marker Resource (XMMR; University of Texas). The data for each sample were normalized to the expression level of the ubiquitously expressed gene *EF-1α*.

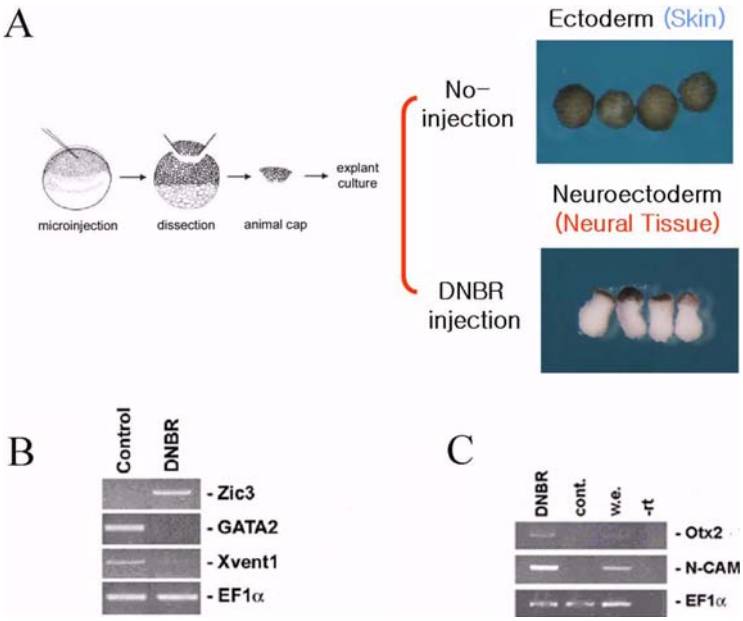


Figure 1. Analysis of neuro-specific genes involved in early stage of neurogenesis by Affiamtrix chip. Schematic diagram of experimental procedures (A). RT-PCR confirmation to examine the quality of samples with well-known genes (B and C). *EF1α* serves as RNA loading controls.

Results

Identification of candidate neural genes by microarray analysis

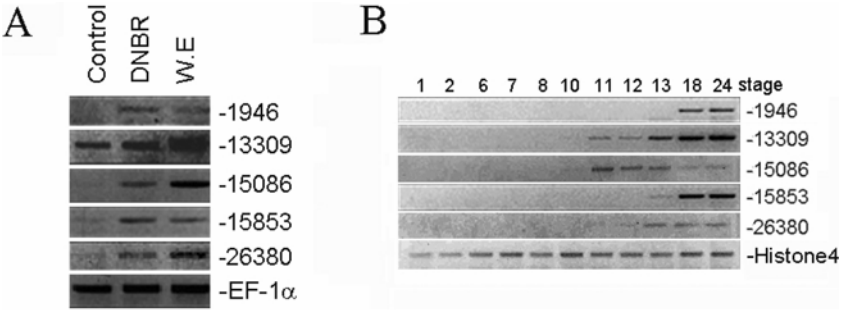
In order to identify genes involved in the early neurogenesis, homogeneous samples of animal cap explants were obtained from the embryos with/without blocking of BMP signaling. Embryos at one-cell or two-cell stage embryos were injected with 2 ng of synthetic mRNA of DNBR. Animal cap explants were isolated from blastula stage (stages 8-9) embryos and then allowed to develop until early gastrula stage (stages 11.5-12). Animal cap explants, which were derived from embryos injected with DNBR mRNA, did show elongation form (Figure 1A). The method was confirmed to be reliable by comparing the known gene expression patterns during the early neurogenesis. The expression of early neural specific marker (Zic3), anterior neural marker (Otx2) and pan-neural marker (N-CAM), known to neural gene, were analyzed by RT-PCR. The expression of all known neural marker genes were induced by DNBR (Figures 1B and C). The gene expression profiles were analyzed using *Xenopus* Affymetrix gene chips containing 14,400 gene transcripts. We identified 265 candidate genes including 151 known genes, 7 unkown genes and 107 ESTs which were newly expressed during the early neurogenesis by blocking BMP signaling. Induction level of those genes was more than 2-fold. In order to identify the neurogenesis specific genes, DNFR caused genes were excluded from genes upregulated by DNBR in Affimatrix chip because blockade of BMP signaling elicits neural gene as well as endodermal gene expression in animal cap tissues (Cha *et al.*, 2004). On the other hand, inhibition of FGF signaling causes endodermal gene expression without neural specific gene transcription. We found 20 known genes, 2 unknown genes and 17 ESTs (unpublished data). We selected the candidates of 10 ESTs and summarized in Figure 2C.

Expression pattern of EST candidates

In order to confirm the upregulation of candidate genes, we examined the expression levels of 10 ESTs. Five ESTs (Xl 1946, Xl 13309, Xl 15086, Xl 15853 and Xl 26380) were significantly upregulated in animal cap explants by blocking BMP signaling in RT-PCR analysis (Figure 2A). The temporal expression patterns of five ESTs were determined by RT-PCR analysis using RNAs extracted from embryos at various stages of development (Figure 2B). Xl 1946, Xl 13309 and Xl 15086 were expressed at gastrula stage (stage 11). However, the expression of Xl 15853 and Xl 26380 began at stage 13. All EST genes were expressed until later stage (stage 24).

Cloning and sequence analysis of EST candidates

We identified cDNA fragments of ESTs upregulated in our microarray screen. We also confirmed upregulation of ESTs in RT-PCR analysis by blocking BMP signaling in animal cap explants. We subsequently found cDNA clones of XL069c07 (Xl.1946) and XL017b19 (Xl.15853) in *Xenopus* EST database (NIBB XDB, http://www.Xenopus.nibb.ac.jp). We obtained



C

Probe set ID	Gene ID	Fold induction
XI.13309.1.S1_at	X53450.1	5.7
XI.15853.1.A1_at	BJ089315	5.3
XI.1946.1.A1_at	BJ080754	4.9
XI.12274.1.A1_at	BJ084286	4.9
XI.1712.2.A1_at	BM261508	4.8
XI.15086.1.A1_at	BJ087601	4.1
XI.26380.1.A1_at	BJ050459	4
XI.3988.1.S1_at	BF072140	3.6
XI.25136.1.A1_at	CB756627	3.5
XI.14609.1.A1_at	CD360811	3.4

Figure 2. Identification of neuro-specific transcripts and confirmation of EST gene expression by RT-PCR. Increased expression of ESTs during early neurogenesis (A). Temporal expression of EST genes. *EF1α* and *Histone4* serve as RNA loading control (B). Identification of neuro-specific EST genes by Affimatrix chip (C).

Figure 3. Amino acid sequences of subcloned two EST genes. Amino acid sequences of ORF (open reading frame) for subcloned two EST genes, 1946 (A) and 15853 (B).

and subcloned the cDNA clones in the pCS2 vectors to perform full sequncing of the clones. Sequncing analysis showed that the clones contained full length open reading frames of two genes. First, we isolated full length cDNA of Xl 1946 and identified it as *Xenopus* cytochrome P450 subfamily 26 (xCYP26) through homology analysis of amino acid sequences appeared in NCBI protein databases. xCYP26c had 2113 bp and 160 amino acids (Figure 3A). Second, Xl 15853 had 3584 bp and 225 amino acids according to sequence analysis of full length cDNA (Figure 3B). It is a novel protein containing Von Willebrand factor type C (VWC) domain. The VWC domain, also called the cysteine-rich domain, typically contains less than 100 residues and has in common the conserved C*XX*C*X*C and CC*XX*C consensuses. Many VWC-containing

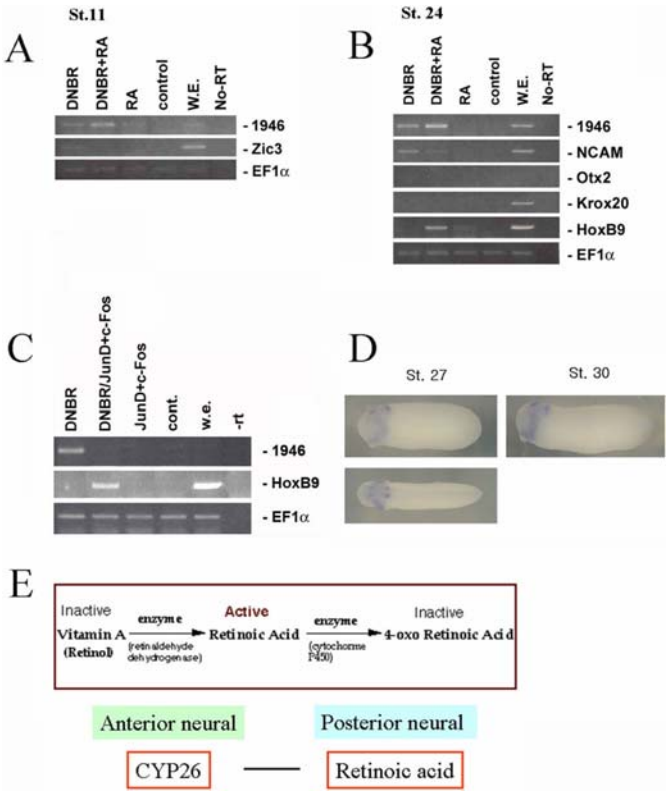


Figure 4. Characterization of a EST gene 1946 (CYP26c). The exrpession pattern of 1946 (CYP26c) induced by DNBR and RA (A), AP-1(JunD/c-Fos) (B). Spatial expression pattern of CYP26c examined by *in situ* hybridization at late stages (st.27 and st.30) (C). A model for role of CYP26c (D).

proteins act as extracellular modulators in the BMP/TGF-β signaling pathway (Abreu *et al.*, 2002; Garcia Abreu *et al.*, 2002).

The expression of CYP26c by RA and AP-1

Finally, we examined the expression pattern and regulation of CYP26c in animal cap explants. In the previous study, *Zebrsfish* CYP26 was expressed in the presumptive anterior neural ectoderm and retinoic acid (RA)-response gene (White *et al.*, 1996). Therefore, we investigated the expression of CYP26c by RA. The expression of CYP26c induced by DNBR was increased by RA in both early and late stages (Figure 4A.). However, AP-1 (JunD/c-Fos) blocked the expression of CYP26c induced by DNBR (Figure 4B). We confirmed that CYP26c was expressed in the anterior region at late stage (Figure 4C).

Discussion

In this study, we searched for newly expressed genes involved in early neurogenesis by using *Xenopus* specific Affimatrix DNA chip. The methods we used were based on the unique properties of *Xenopus* animal cap explants which have been known to be similar to stem cells in the pluripotent properties and could be induced to the specific fate of cells by treatment of specific reagents (Ariizumi *et al.*, 2009). When the animal cap explants were treated with a specific reagent or a specific gene involved in the early development of vertebrate, they have been well known to develop to the specific germ layer. In this study, we used DNBR injected animal cap explants and found 265 transcrips including 107 ESTs upregulated by DNBR in Affimatrix gene chip.

The neurogenesis mechanism is best established in *Xenopus* embryos. Blocking of BMP signaling in ectodermal tissues leads to default neurogenesis containing anterior neural genes (Zaraiskii, 2007). The animal cap explants from embryos injected with DNBR mRNA elongated and expressed neural specific markers (Figures 1A and B). Among 107 ESTs, which were upregulated by DNBR in animal cap explants, we excluded common transcripts upregulated also by DNFR. Blockade of BMP signaling elicits neural gene as well as endodermal gene expression in animal cap tissues. On the other hand, inhibition of FGF signaling causes endodermal gene expression without influence on neural specific gene transcription. We selected 10 transcripts based on induction folds after elimination of common transcripts found in DNFR

injected samples (Figure 1C). Several papers have been published to identify neural specific genes using DNA microarrays (Munoz-Sanjuan *et al.*, 2002b; Shin *et al.*, 2005; Zaraiskii, 2007; Karsten *et al.*, 2008; Cornish *et al.*, 2009). However, endodermal gene contamination was not considered in animal cap samples produced by blocking BMP signaling. The transcripts which we identified by subtracting transcripts expressed in control and DNFR injected animal cap explants were more likely to be the genes specifically involved in neurogenesis. This methods was reliable since we identified 20 known genes including retinal homeobox 1A (Rx1A), Zic3A (Zic family member 3 heterotaxy 1), brain factor2 (BF2), Sox2 and Sox3 (unpublished data) which have been known to be specific in neuroectoderm (Nakata *et al.*, 1998; Feledy *et al.*, 1999; Ohuchi *et al.*, 1999). Blocking of BMP signaling in ectodermal tissues led us to find upregulation of 265 transcripts including 107 ESTs, 151 known genes and 7 unknown genes. However, many genes were eliminated, but known neural specific genes were not with subtracting DNFR induced genes. When we examined upregulation of candidate transcripts, 5 out of 10 candidate transcripts were actually shown to be significantly upregulated in DNBR injected animal cap explants (Figure 2). DNA microarray is a powful method in identifying genes in a large scale, but it requires additional effort to eliminate false-positive reaction. Fifty percent of true positive selection with our methods indicates that *Xenopus* Affimatrix gene chip is a reliable method to screen the genes involved in early events of development, which is largely dependent on transcriptional regulation. Further improvement of fidelity in selection of neuroectoderm specific genes may be achived not only by subtraction of DNFR induced genes, also by selection of common transcripts induced both by DNBR and activin.

We cloned and sequenced two genes based on EST sequnce information. Sequncing analysis showed that the clones contained full length open reading frames of two genes, CYP26c and a novel protein containing Von Willebrand factor type C (VWC) domain. Since many VWC-containing proteins act as extracellular modulators in the BMP/TGF-β signaling pathway (Zhang *et al.*, 2007), this protein may have a modulation role of signaling in early neurogenesis. The other gene we identified was CYP26c. CYP26 plays a central role in degradation of the RA signal that is known as a posteriorizing factor in CNS development (Abu-Abed *et al.*, 2001; Sakai *et al.*, 2001). The expression pattern and regulation of CYP26c were examined. The expression of CYP26c was upregulated by DNBR and further upregulated in the presence of RA (Figure 4A). However, coinjection of DNBR and AP-1 (JunD/c-Fos) posteriorized the explants and abolished the expression of CYP26c (Figure 4B). CYP26c expression was localized in midbrain region of anterior neural tissues at stages 27 and 30 (Figure 4C).

It is interesting to note that CYP26c was induced during the early neurogenesis caused by DNBR in *Xenopus* animal cap explants. Blocking of BMP signaling induces the ectodermal cells to form neural tissue of an anterior type, while the second signal (transformer) including Wnt, FGF and RA convert the neural tissue into progressively more posterior types of neural tissue (hindbrain and spinal cord) in a concentration-dependent manner (Kudoh *et al.*, 2002). Our results suggest that CYP26c may play a specific role in anterior-posterior neural patterning during the early development of vertebrate embryos and *Xenopus* embryos. Further characterization of the obtained genes may provide new insights into the unknown mechanisms of neurogenesis caused by blocking BMP signaling during *Xenopus* embryogenesis.

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