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Short Communication

Unequal distribution of *otx1* mRNAs among cleavage stage blastomeres in the teleost, *Leucopsarion petersii* (shiro-uo)

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ABSTRACT The *otx* genes belong to the *orthodenticle* gene family and play important roles in anterior brain development in vertebrates. We isolated two cDNA sequences, one homologous to human and zebrafish otx1 and another homologous to zebrafish otx3, from the teleost *Leucopsarion petersii* (shiro-uo), which belongs to the family of gobies in the Perciformes. During embryogenesis of shiro-uo, *otx1* and *otx3* were expressed in the fore- and mid-brain throughout development in a manner similar to that observed in other vertebrates so far studied. However, *otx-1* mRNA was also present at earlier stages and we obtained unique results using *in situ* hybridization and RT-PCR analysis demonstrating that *otx-1* signals showed a distinct increase in the upper blastomeres, but not in the lower blastomeres, at the 8-cell stage. These stronger signals were maintained in the animal pole blastomeres during the 16-cell to 64-cell stages, followed by a gradual decrease during blastula stages. Such unexpected unequal distribution of *otx1* mRNA revealed that blastomeres at early cleavage stages already showed non-equivalence in the embryogenesis of shiro-uo.

KEY WORDS: *teleost embryogenesis, otx1, otx3, 8-cell stage blastomeres, mRNA distribution*

Recently, vigorous efforts have been concentrated to investigate the developmental processes of fish embryos using the zebrafish and medaka. However, it is also significant to study embryogenesis in other teleost species, especially those belonging to evolutionary distant groups. We have described several advantages of the shiro-uo, *Leucopsarion petersii*, belonging to the family of gobies in the Perciformes group. One unique feature of its early development is that the third cleavage is horizontal and thus results in formation of the upper and lower tiers of blastomeres at an early stage (Nakatsuji *et al.*, 1997; Arakawa *et al.*, 1999). Also, using an explant culture study of lower blastomeres or upper blastomeres isolated from the yolk cell mass at the 8-cell stage, we found an unequal presence of very early signaling for mesoderm induction and differentiation of the blastomeres from the very early cleavage stage (Saito *et al.*, 2001).

Here, we describe the cloning and expression patterns of shiro-uo *otx* genes, belonging to the orthodenticle gene family, which play important roles for anterior brain development in vertebrates and are conserved throughout evolution (Finkelstein and Boncineli, 1994; Leuzinger *et al.*, 1998).

otx1 *and* otx3 *Genes in Shiro-uo*

From a shiro-uo cDNA library made from embryos at various stages, we isolated and characterized two *otx* clones. The amino acid sequences deduced from the nucleotide sequences of shiro-uo *otx1* and *otx3* full-length cDNAs are shown in Fig. 1A. The putative shiro-uo otx1 ORF was encoded from nucleotides 108 to 1089 and consisted of 326 amino acids. The putative shiro-uo otx3 protein consisted of 331 amino acids. About 100 amino acids in the N-terminal region and about 30 amino acids in the C-terminal region were conserved between otx1 and otx3. The comparison of otx protein sequences from the initiation methionine residue to the homeodomain among shiro-uo, zebrafish, human, and lamprey is presented in Fig. 1B (Mori *et al.*, 1994; Simeone *et al.*, 1993; Tomsa and Langeland, 1999). The amino acids 36 through 100 encoded the homeodomain and were conserved among many species including amphioxus (data not shown). FASTA analysis indicated that 87% and 76% of the full peptide sequence of shiro-uo otx1 were identical

Abbreviations used in this paper: RT-PCR, reverse-transcription polymerase chain reaction.

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Footnote: The relevant DDBJ accession numbers are: AB066289 *(otx1)* and AB066290 *(otx3).*

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A

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otx1  MMSYLKQPPYGVNGLGLSGAAMDLLHPSVGYPATPRKQRRERTTFTRSQLDVLEALFAKTRYPDIFMREEVALKINLPES 80
otx3  MMSYLKQSPYGMGGLGL-GGAVDLLHPTVGYPTNPRKQRRERTTFTRTQLDILESLFAKTRYPDIFMREEVALKINLPES 79
                                                                    HOMEODOMAIN
otx1  RVQVWFKNRRAKCRQQQQSG--------------SSAKARPPKKKSSPARESTGSES-SGQFTPPAVSSAGSSSS-SST- 143
otx3  RVQVWFKNRRAKCRQQQQSSNSANPNSASNGNNTNTSKAKPLKKKASPIRESSGSESGGGHFTPASTSASSSTPPLPSSM 159

otx1  NHSG--P-VLSTSTSIST-VSSIWSPAISPGNAPAPAVAPLPEPGPPSNASCMQRSMTG-SATAATSYPMSYNQT-PGYS 218
otx3  ASSACPPALMNSSTSVTAPVSSIWSPA--P-ISPALAPVPMPDPVANGSASCMQRSSAGVGSTSAASYPVPYGQTVAAYG 236

otx1  -QGYP-P-SSSYFSSVDCSSYLAPMHSHHHPHQLSPMAASSMSGHPHHHISQSSGHHHHHHHQAYGGSGLAFNSSDCLDY 294
otx3  HQGYPAAGSGSYF-G-DCGSYL-PMHS-HH-H----MANPAMSG--HHHIGQ-SGHHHSHHHAP-AHHQPYYSASDCLDY 303

otx1  KEQSAASSWKLNFNATDCLDYKDQASWRFQVL 326
otx3  KEQ--A--WKLNFNASDCWDYKDQAAWRFQVL 331
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B

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g otx1 MMSYLKQPPYGVNGLGLSGAAMDLLHPSVGYPATPRKQRRERTTFTRSQLDVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQQQ 98
z otx1 ----------AM-----------------------------------------I---------------------------------------- 98
h Otx1 ----------M-----A-P-------------------------------------------------------------------------- 98
z otx2 ----------T----S-TTSG-------------------------A---------------------------------------------- 98
h Otx2 ----------A----S-TTSG-------------------------A---------------------------------------------- 98
g otx3 -------S---MG----G-- V-----T----TN------------T---I--S------------------------------------------ 97
z otx3 -------A--AM-----G--T-----------TN------------T---I--S------------------------------------------ 98
l otx  --AFI--A--PM--M---A-G-E-F-SA--F--P------------A------S--S------------------------------------- 98
                                                     HOMEODOMAIN
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C

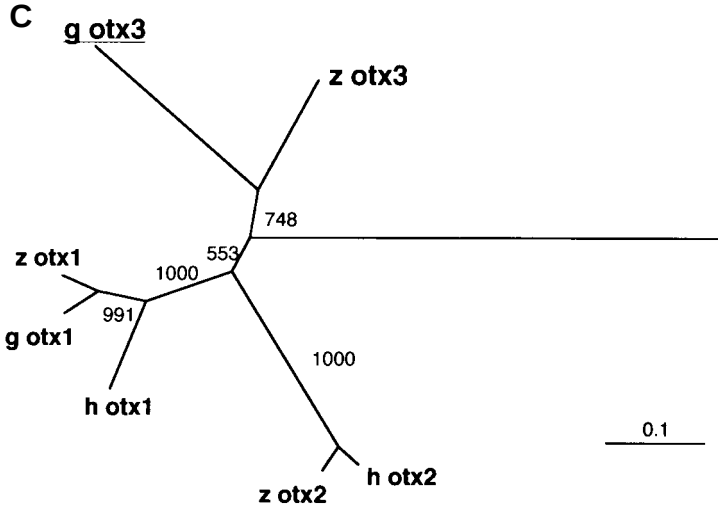

Fig. 1. Sequence and phylogeny of deduced amino acid sequences of shiro-uo otx homeoproteins. (A) *Alignment of the deduced amino acid sequences of shiro-uo otx1 and otx3 peptides. Sets of identical amino acid residues between two proteins are shadowed. The homeodomain is double underlined. The nucleotide sequence data of these proteins have been submitted to the DDBJ under the accession numbers AB066289 and AB066290, respectively.* **(B)** *Comparison of the amino acid sequences from the initiation methionine residue to the homeodomain among shiro-uo (g) otx1, otx3; zebrafish (z) otx1, otx2, otx3 (Mori et al., 1994); human (h) otx1, otx2 (Simeone* et al., *1993) and lamprey,* Petomyzon marinus, *(l) otx (Tomsa and Langeland, 1999). Bars indicate the amino acid residues identical to those of shiro-uo otx1 in the aligned position.* **(C)** *Phylogeny of the otx homeoproteins. Numbers after internal branches indicate the bootstrap value (value/1000) for each group. The dentrogram was constructed using the neighbor-joining method and based on a cluster alignment of full peptide sequence of the otx homeoproteins excluding regions with gaps (Thompson* et al., *1994). Bar, 0.1 amino acid substitutions per position in the sequences.*

with zebrafish otx1 and human otx1, respectively. Whereas, 62% of shiro-uo otx3 was identical with zebrafish otx3. A radial phylogenetic tree of otx proteins by the Neighbor-joining method indicated that the deduced shiro-uo otx1 protein falls into the cluster of human otx1 and zebrafish otx1 proteins, and that the shiro-uo otx3 protein clustered with zebrafish otx3 protein (Fig. 1C). The zebrafish otx2 homeoprotein was identified by having high homology with the mouse otx2. The otx2 is an anterior neural marker and suggested to play roles in epiblast differentiation into anterior neuroectoderm (Koshida *et al.*, 1998; Perea-Gomez *et al.*, 2001). In shiro-uo, however, no *otx2* gene was isolated from the embryonic cDNA library in our study.

Expression Patterns during Embryogenesis

Our results of whole mount *in situ* hybrdization in embryogenesis of shiro-uo showed that *otx3* and *otx1* were expressed in the fore- and mid-brain (Fig. 2 A-C, D-F) and in the eyes (data not shown) throughout their development, in a similar manner as in other vertebrates so far studied (Mori *et al.*, 1994; Li *et al.*, 1994; Simeone *et al.*, 1993). Also, a weak *otx3* signal was observed in the tail bud (Fig. 2C).

Otx3 gene expression became slightly detectable at the blastula to 100%- epiboly stage (Fig. 2A), but no signals were found during cleavage stages. At the bud stage, expression signals were found in the dorsal anterior portion of forebrain (Fig. 2B). From the 2-somite to 30-somite stages, strong signals were found in the forebrain and mid-brain, and they showed clear boundaries of expression between the mid-brain and hindbrain (Fig. 2C).

Otx1 mRNA signals were detectable from the cleavage stages as described later. During blastula stages, expression signals were distributed uniformly among all cells, followed by a gradual decrease (Fig. 2L). At the beginning of epiboly to 50%-epiboly, expression signals had almost disappeared (data not shown). At the 100%-epiboly stage, strong expression signals were observed in a V shape, which indicated the posterior limit of the expression zone (Fig. 2D, dorsal view). After the 100%-epiboly stage, shiro-uo *otx1* expression signals were found in the fore- and mid-brain in a similar manner as in other vertebrates so far studied (Fig. 2 E,F).

Expression of otx1 *during Cleavage Stages*

Otx1 hybridization signals were very weak during cleavage stages from the 1-cell to early 8-cell stage (Fig. 2 G,H). However, *otx1* signals showed a distinct increase in the upper blastomeres, but not in the lower blastomeres, at the late 8-cell stage (Fig. 2 I,J). These stronger signals were maintained in the animal pole blastomeres

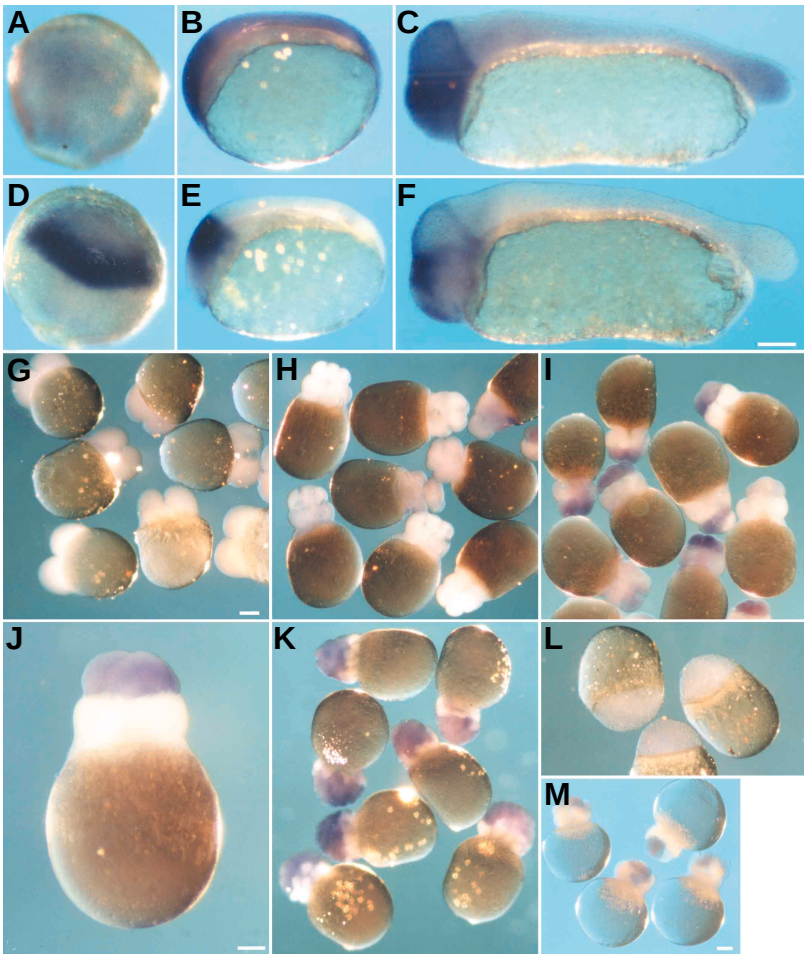

Fig. 2. Expression of *otx3* and *otx1* during shiro-uo embryogenesis examined by whole mount *in situ* hybridization. (A-C) otx3 *expression at the 100%-epiboly (A), bud (B) and 13-somite (C) stages.* **(D-F)** otx1 *expression at the 100%-epiboly (D), bud (E) and 13-somite (F) stages.* **(G-L)** otx1 *expression during cleavage stages; 2-cell (G), early 8-cell (H), late 8-cell (I,J), 64-cell (K) and blastula (L) stages. (J) Enlarged view of one of embryos in (I).* **(M)** otx1 *expression in embryos cultured for 1h after removal of upper blastomeres at the 8-cell stage. Pictures are lateral view (A-C, E,F) or dorsal view (D). A-C and D-F are at the same magnification. G-I and K,L are at the same magnification. Bar, 100 μm.*

during the 16-cell to 64-cell stages (Fig. 2K). When we removed all upper 4 blastomeres at the 8-cell stage, the remaining lower blastomeres with the yolk cell mass cleaved and reproduced two tiers as reported previously (Saito *et al.,* 2001). In such embryos, we observed that the *otx1* hybridization signals increased in these newly reproduced upper blastomeres, but not in lower blastomeres (Fig. 2M). In contrast, *ef1-α* signals did not show such clear differences between the upper and lower blastomeres at the cleavage stage (Fig. 3).

In *zebrafish*, *otx1* mRNAs are present in fertilized eggs and distributed uniformly in all cells at 8-cell stage. Thus, it has been suggested that such *otx1* mRNAs are maternal and they may play a role in very early embryogenesis (Mori *et al.*, 1994).

RT-PCR Analysis of otx-1 *mRNA*

To confirm the distribution patterns of *otx1* mRNAs at the late 8-cell stage, RT-PCR analysis was performed using mRNA extracts from whole embryos, the isolated upper blastomeres or those from the remaining lower blastomeres and yolk cell mass. We observed stronger signals from the upper blastomeres than the lower blastomeres and yolk cell mass (Fig. 4C). Then, in order to examine whether the *otx1* mRNAs in the cleavage stage embryos are maternal or zygotically expressed, we carried out RT-PCR analysis of the unfertilized eggs and embryos from cleavage to somite stages (Fig. 4 A,B). It showed that *otx1* mRNAs were already present in the unfertilized eggs. Also, the actinomycin D (50μ l/ml, Wako) treatment from the fertilization produced no effects on such increase of the *otx1* signals in the upper blastomeres at the late 8-cell stage, although it inhibited the normal development from the epiboly to later stages (data not shown).

These results indicate that the *otx1* signals at cleavage stages of shiro-uo embryos are maternal mRNAs. Therefore, they should undergo interesting uncovering and /or translocation in early embryos, because the increased signals were detectable only in the upper blastomeres from the late 8-cell to 64-cell stages. Thus, in the embryogenesis of shiro-uo, blastomeres at early cleavage stages already showed nonequivalence as revealed by unequal distribution of *otx1* mRNAs. Such localized *otx1* mRNAs at early cleavage stages in shiro-uo may play important roles in the regulation of cell adhesion, as reported in zebrafish (Bellipanni *et al.*, 2000), or in the initial events of axis formation by regulating expression of other genes, as reported in *Xenopus* (Pannese *et al.*, 1995).

Experimental Procedures

Embryos

Shiro-uo embryos were obtained by artificial insemination as described previously (Arakawa *et al.*, 1999). Fertilized eggs and embryos were cultured at 19°C in diluted (10%) sterile Hank's salt solution.

Cloning of shiro-uo otx1 *and* otx3

The mRNAs were extracted from 6-somite to 8-somite stage embryos of shiro-uo using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). First strand cDNAs were synthesized using an oligo

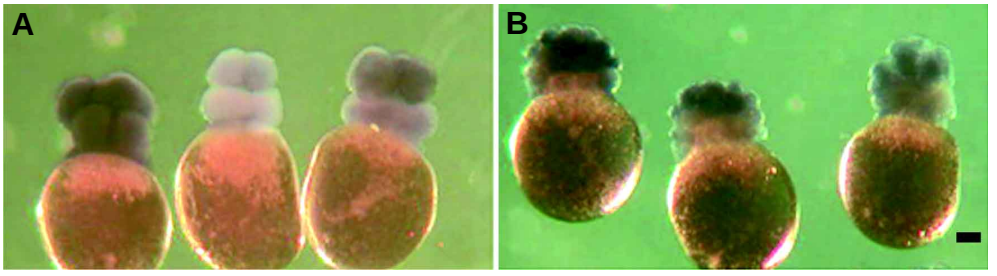

Fig. 3. Expression of *ef1-α* at the cleavage stage of shiro-uo embryo examined by the whole mount *in situ* hybridization. *Expression at the 8-cell* **(A)** *and 32-cell* **(B)** *stages is shown. Bar, 100 μm.*

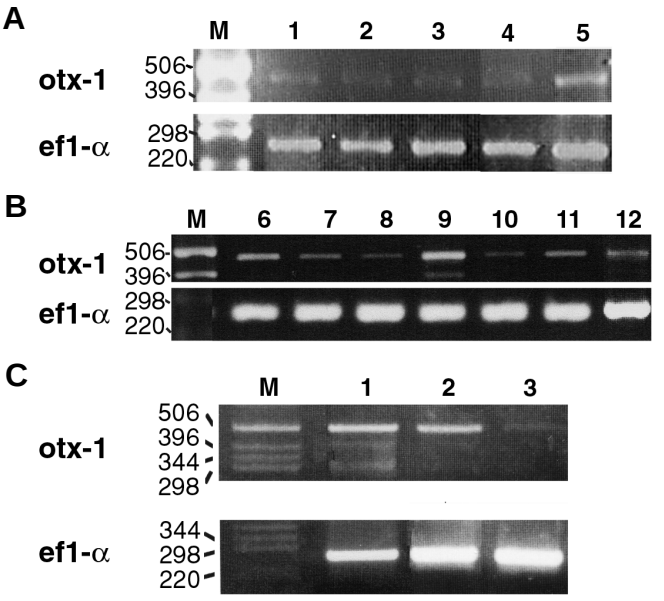

Fig. 4. RT-PCR analysis of *otx-1* mRNA expression. (A) *Unfertilized eggs (lane1), 2-cell (lane 2), 8-cell (lane 3), 64-cell (lane 4) and bud stage embryos (lane 5).* **(B)** *Morula (lane 6), late blastula (lane 7), 50%-epiboly (lane 8), bud (lane 9), 3-somite (lane 10), 12-somite (lane 11) and 24-somite stage embryos (lane 12).* **(C)** *Whole shiro-uo embryos (lane 1), isolated upper blastomeres (lane 2) and remaining lower blastomeres and yolk cell mass (lane 3) at the late 8-cell stage. (M) bp size marker.*

$(dT)_{12-18}$ primer, and 2nd strand cDNAs were generated using the cDNA QuickPrep cDNA Synthesis Kit (Amersham Pharmacia Biotech). The following PCR primers were designed to amplify 658 bp and 725 bp cDNA fragments of *otx* genes including the homeobox domain; 5'IsoGT(ATCG)CA(AG)GT(ATCG)TGGTT(TC)AA(AG)AA-3'(upper) and 5'-GC(ATGC)GT(TC)TG(TC)TC(TC)TT(AG)TA(TG)TC-3'(lower). Fragments obtained in this manner were then used to isolate full length coding sequences (*otx1*; DDBJ AB066289, *otx3*; DDBJ AB066290) from a shiro-uo embryonic cDNA library in λ…ZAPII vector (Stratagene).

Whole Mount In Situ *Hybridization*

Staged embryos were fixed overnight at 4°C with 4% paraformaldehyde dissolved in 50% PBS. *In situ* hybridization was performed as described (Jowett and Lottice, 1994) using Digoxigenin (Dig)-labeled riboprobes, which were prepared from the full length cDNA clones, shiro-uo *otx1* and *otx3*. Antisense mRNA probes were generated using T7 RNA polymerase (Roche). Similar probes were also generated for shiro-uo *ef1-α.*

RT-PCR Analysis

mRNA samples used for each RT-PCR analysis were extracted from 10 (Fig. 4A) or 5 (Fig. 4B) unfertilized eggs and embryos at 2-cell stage to 24-somite stage of shiro-uo. Also, mRNA samples were extracted from the isolated upper 4 blastomeres of 20 embryos at late 8-cell stage or the remaining lower 4 blastomeres with yolk cell mass of 20 embryos, using the QuickPrep Micro mRNA purification Kit (Amersham Pharmacia Biotech). First strand cDNAs were synthesized using a *Not*I $(dT)_{18}$ primer and the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). A pair of primers:

5'-AGAGTGCAGGTGTGGTTCAAGAAC-3' and

3'-CTGCAGTCCACGCTGCTGAAGTAG-3' were used for amplifying a 458 bp fragment of shiro-uo *otx1*. Amplification was performed with 33 cycles for the whole embryos and separated blastomeres. The parameters of PCR were 95°C for 0.5 min, 63°C for 1 min and 72°C for 2 min.

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