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Evolution of Developmental Control Mechanisms

Evolution of *Otx* paralogue usages in early patterning of the vertebrate head

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ABSTRACT

To assess evolutional changes in the expression pattern of *Otx* paralogues, expression analyses were undertaken in fugu, bichir, skate and lamprey. Together with those in model vertebrates, the comparison suggested that a gnathostome ancestor would have utilized all of *Otx1*, *Otx2* and *Otx5* paralogues in organizer and anterior mesendoderm for head development. In this animal, *Otx1* and *Otx2* would have also functioned in specification of the anterior neuroectoderm at presomite stage and subsequent development of forebrain/midbrain at somite stage, while *Otx5* expression would have already been specialized in epiphysis and eyes. *Otx1* and *Otx2* functions in anterior neuroectoderm and brain of the gnathostome ancestor would have been differentially maintained by *Otx1* in a basal actinopterygian and by *Otx2* in a basal sarcopterygian. *Otx5* expression in head organizer and anterior mesendoderm seems to have been lost in the teleost lineage after divergence of bichir, and also from the amniotes after divergence of amphibians as independent events. *Otx1* expression was lost from the organizer in the tetrapod lineage. In contrast, in a teleost ancestor prior to whole genome duplication, *Otx1* and *Otx2* would have both been expressed in the dorsal margin of blastoderm, embryonic shield, anterior mesendoderm, anterior neuroectoderm and forebrain/midbrain, at respective stages of head development. Subsequent whole genome duplication and the following genome changes would have caused different *Otx* paralogue usages in each teleost lineage. Lampreys also have three *Otx* paralogues; their sequences are highly diverged from gnathostome cognates, but their expression pattern is well related to those of skate *Otx* cognates.

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Introduction

Otx/otd genes encode transcriptional factors that play central roles in the patterning and formation of the anterior parts of the body, collectively called the "heads" throughout the metazoans. The vertebrate head is a unique structure that has no comparable structure in invertebrates (Gans and Northcutt, 1983); it is also a structure that has most dramatically changed with vertebrate evolution as seen in the six-layered mammalian cortex with a series of areas. *Otx* orthologues also play essential roles in its development. Mouse has three *Otx* paralogues, *Otx1*, *Otx2* and *Crx*. *Otx2* is earliest expressed in the epiblast (Simeone et al., 1992, 1993), though its significance is not clear yet; *Otx2* might function in cooperation with a transcriptional factor downstream of *Nodal*/*Cripto* signaling to induce the embryonic part of the visceral endoderm and the distal visceral endoderm cells that uniquely express a series of head organizer genes (Brennan et al., 2001; Kimura et al., 2001).

Otx2 is also expressed in the distal visceral endoderm cells and is essential for their anterior movement to generate the anterior visceral endoderm (Kimura et al., 2000). In the anterior visceral endoderm thus formed, *Otx2* protects the adjacent epiblast against caudalizing signals to specify it as the anterior neuroectoderm. During gastrulation, *Otx2* is expressed in the anterior definitive mesendoderm that replaces the anterior visceral endoderm; *Otx2* expression is essential in this mesendoderm to maintain the anterior neuroectoderm induced by the anterior visceral endoderm (Ang et al., 1996; our unpublished data). *Otx2* directs the expression of *mDkk1* and *mShisa* encoding Wnt- and Fgf antagonists (Kimura-Yoshida et al., 2005; Furushima et al., 2007), in distal/anterior visceral endoderm and probably in anterior mesendoderm.

Otx2 is also one of the earliest genes expressed in the anterior neuroectoderm induced by anterior visceral endoderm and anterior mesendoderm and essential to maintain the neuroectoderm (Simeone et al., 1993; Kurokawa et al., 2004a). *Otx1* expression starts when anterior neuroectoderm further develops into forebrain and midbrain around 3–6 somite stage; the *Otx1*-positive domain is less extensive within the *Otx2*-positive domain (Simeone et al., 1992, 1993). At this stage the rostral forebrain, which corresponds to future telencephalon

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and hypothalamus, becomes insensitive to caudalizing signals, and *Otx2* and *Otx1* cooperate to protect mesencephalon and diencephalon against caudalization into metencephalon (Kurokawa et al., 2004b). *Otx2* and *Otx1* also cooperate with *Emx2* and *Pax6* in regulating the development of the diencephalon (Suda et al., 2001; Kimura et al., 2005), and *Otx2* plays essential roles in development of cephalic neural crest-derived structures (Matsuo et al., 1995; Kimura et al., 1997). Thus, among the *Otx* paralogues, it appears to be *Otx2* that plays central roles in early head development in the mouse.

A teleost ancestor has undergone whole genome duplication (WGD) prior to its radiation (Amores et al., 1998; Postlethwait et al., 1998); most of the duplicated genes have subsequently undergone either degeneration of one of the duplicates or segregation of their expression (Ohno, 1970; Force et al., 1999). Accordingly, zebrafish has two *Otx1* orthologues, *DrOtx1* and *DrOtx1-like* (*Otx3*), one *Otx2*, *DrOtx2*, with the degeneration of its duplicated counterpart, and two *Otx5* orthologues, *DrOtx5* and *DrCrx* (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995; Gamse et al., 2002; Shen and Raymond, 2004). No genetic studies have been reported on functional cooperation and segregation of each paralogue, and morpholino experiments have been unsuccessful in discriminating the endogenous functions of each paralogue (Foucher et al., 2006). However, the expression data suggests no *Otx2* prevalent usage among *Otx* paralogues for early head development in zebrafish. It is *Otx1* and *Otx1-like* that are expressed in the dorsal margin of blastoderm, embryonic shield and anterior mesendoderm in zebrafish (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995). In the anterior neuroectoderm, their expression precedes that of *DrOtx2* (Li et al., 1994). In the forebrain and midbrain, the *DrOtx2*-positive domain settles within *DrOtx1* and *DrOtx1-like* positive domains. Accordingly, it has been broadly assumed that *Otx2* prevailed in mammal and *Otx1* in zebrafish in the *Otx* paralogue usages for early head development. However, no comparative data exists on the expression pattern of *Otx* paralogues in other teleosts (Loosli et al., 1998; Kamimoto et al., 2003; Heimbucher et al., 2007).

Another question about *Otx* genes concerns *Otx5* orthologues. *Crx* is their highly diverged mammalian orthologue (Plouhinec et al., 2003). It is specifically transcribed in the retinal photoreceptors and the pinealocytes of the epiphysis and essential to the differentiation of retinal photoreceptors and circadian entrainment (Furukawa et al., 1997, 1999). In addition, the expressions of zebrafish orthologues, *DrOtx5* and *DrCrx*, are also unique to eyes and epiphysis (Gamse et al., 2002; Shen and Raymond, 2004). *Xenopus* has two *Otx5* paralogues by tetraploidization unique to this animal; *XlOtx5* and *XlOtx5b* (Kuroda et al., 2000; Vignali et al., 2000). In this animal *XlOtx5* and *XlOtx5b* are expressed not only in eyes and epiphysis, but also in organizer and anterior mesendoderm together with *XlOtx2*. No functional analyses exist to demonstrate their endogenous organizer functions, though over-expression studies indicated their anteriorizing potency (Kuroda et al., 2000; Vignali et al., 2000). The question is whether *XlOtx5* and *XlOtx5b* expression in organizer and anterior mesendoderm was acquired uniquely in this lineage or whether *Otx5* was ancestrally expressed at these sites and the expression was lost in zebrafish and mammalian *Otx5* orthologues independently. Recently, a dogfish orthologue, *ScOtx5*, was reported to be expressed in the fish organizer (Coolen et al., 2007).

The third question relates to *Otx* orthologues in agnatha. Previously we reported two *Otx* orthologues in *Lethenteron japonicum*: *LjOtxA* and *LjOtxB* (Ueki et al., 1998). On the other hand, Tomsa and Langeland (1999) reported that *Petromyzon marinus* has only one *Otx* orthologue, *PmOtx*, proposing that the divergence of *Otx1* and *Otx2* took place after the gnathostome/lamprey divergence. *PmOtx* does not cluster either to *LjOtxA* or *LjOtxB*, and it has also been proposed that an independent duplication took place in *L. japonicum* lineage. However, a hagfish harbors three *Otx* paralogues: *MgOtxA*, *MgOtxC* and *MgOtxD* (Germot et al., 2001). These *Otx* orthologues in agnatha show no clear relationship to the three orthology classes identified in gnathostomes; moreover, neither *LjOtxB* nor *PmOtx* exhibits orthology to any hagfish *Otx* paralogues (Plouhinec et al., 2003; Fig. 1).

To address these questions we have identified *Otx* orthologues and examined their expression in fugu (*Fugu niphobles*), bichir (*Polypterus senegarus*), skate (*Raja eglanteria*/*Leucoraja erinacea*) and lamprey (*L. japonicum*) with particular focus on *Otx* functions in early embryogenesis. The study, together with studies reported on model vertebrates, suggests that a gnathostome ancestor would have utilized all of *Otx1*, *Otx2* and *Otx5* paralogues in organizer and early anterior mesendoderm for head development. In the ancestor, *Otx1* and *Otx2*, but not *Otx5*, would have also functioned in specification of the anterior neuroectoderm and subsequent development of forebrain/midbrain. With vertebrate evolution the paralogues used in each step and site of head development seem to have been selected differentially by lineages. In a teleost ancestor prior to WGD *Otx1* and *Otx2* would have been expressed both in each site and step of head development. Subsequent whole genome duplication and the following genome changes would have caused different *Otx* paralogue usage in each teleost lineage. Lampreys also have three *Otx* paralogues; their expression pattern is well related to those of skate *Otx* cognates.

Materials and methods

Animal embryos

Freshly laid *F. niphobles* eggs were collected on Arai beach, Kanagawa prefecture, Japan and cultured in filtered seawater at 20 °C with vigorous aeration. Embryos were staged according to Uno (1955). *Polypterus senegalus* and *Polypterus endlicheri* embryos were obtained by natural crossbreeding in our aquarium (Bartsch et al., 1997), and cultured at 28.5 or 23 °C in embryonic media (EM: 15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2.2H_2O$,1 mM $MgSO_47H_2O$, 0.15 mM KH_2PO_4, 0.05 mM $Na_2HPO_4.2H_2O$, and 0.7 mM $NaHCO_3$, pH 7.0–7.5). *L. erinacea* embryos were supplied by the Marine Biological Laboratory, Woods Hole, USA and staged according to the dogfish staging table by Ballard et al. (1993). *L. japonicum* embryos were obtained as described (Kuratani et al., 1997) and staged according to Tahara (1988).

Isolation of Otx genes

F. niphobles Otx genes were isolated by RT-PCR with adult brain total RNAs using primers at 5′ and 3′ UTRs of each mRNA. The 5′ and 3′ UTR sequences are regarded to be the same as those of *Fugu rubripes Otx* genes in v4.0 whole genome shotgun assembly (Aparicio et al., 2002). Primers used and lengths of amplified products are: *FnOtx1a*, 1131 bp with sp (5′- AACCACTGGCCTGAGCTGGGTG -3′) and ap (5′- AATGAATATGCTGATCTGGAAAAGC -3′); *FnOtx1b*, 1102 bp with sp (5′- TACAGCGGGACATCTTGCGCCTTTC-3′) and ap (5′-AAAGACTTGCGGGAAGTCTGTGAGG-3′); *FnOtx2a*, 1072 bp with sp (5′- ACCAAGTACATCGACGGAGATC-3′) and ap (5′- AGAAAACGGATCAGTCCGATGT-3′); *FnOtx2b*, 1129 bp with sp (5′- TGCGCGGATCAGCAAGGATTAC-3′) and ap (5′- TGCGCGGATCAGCAAGGATTAC-3′); *FnOtx5a*, 1020 bp with sp (5′- GAGTGAGTGTTTTAAAGCCGACAGG-3′) and ap (5′- GGTTCATTTCATTGTCTTAAAATCC-3′); *FnOtx5b*, 981 bp with sp (5′- CATACAGCGTCAGGACTTAAAAGGC-3′) and ap (5′-CTTCACATGACCTCACATATCTGTG-3′). *P. senegalus*, *R. eglanteria*, *L. japonicum* and *P. marinus Otx* gene fragments were obtained by semi-nested degenerate PCR for the 3rd exon with the degenerate primers starting from each genomic DNA (Germot et al., 2001). The primers used were sp (5′-GTNTGGTTYAARAAYMG-3′ and 5′-GCNAARTGYMGNCARCA-3′) and ap (5′-ARNACYTGRAAYTTCCA-3′). Sizes of the amplified products were: *PsOtx1* 759 bp, *PsOtx2* 638 bp, *PsOtx5* 651 bp, *ReOtx1* 669 bp, *ReOtx2* 600 bp, *ReOtx5* 601 bp,

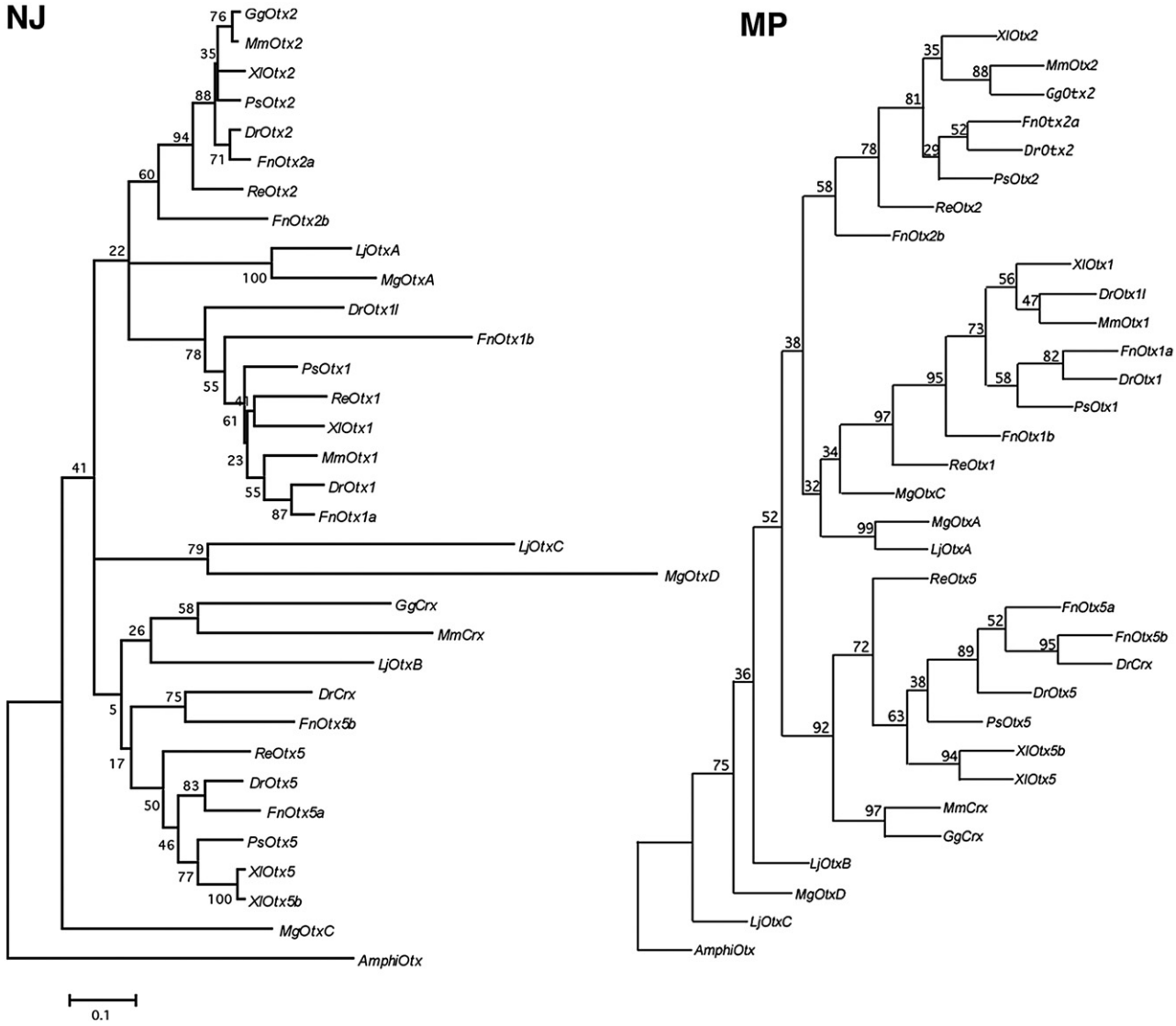

Fig. 1. Phylogenetic relationships of *Otx* genes in vertebrates. Phylogenetic trees calculated by NJ and MP algorithms are presented. Numbers indicate the bootstrap values supporting the corresponding node. Abbreviations: Amphi, amphioxus; Dr, zebrafish; Fn, fugu; Gg, chick; Lj, lamprey; Mg, hagfish; Mm, mouse; Ps, bichir; Re, skate; Xl, *Xenopus*; *Otx1l*, *Otx1-like* (*Otx3*/*Otx1b*).

PmOtx (*PmOtxC*) 860 bp, *PmOtxA* 617 bp, *PmOtxB* 677 bp and *LjOtxC* 876 bp. Amplified fragments were subcloned and sequenced; sequences were deduced from the consensus of at least three independent clones. Full-length *L. japonicum OtxA* and *OtxB* cDNAs were previously isolated (Ueki et al., 1998). Full-length *LjOtxC* cDNA was obtained by 5′- and 3′-rapid amplification cDNA end (RACE) with total RNAs of gastrula embryos.

Database searches for Otx genes

Mouse, chick, *Xenopus*, zebrafish, hagfish and amphioxus *Otx* protein sequences were retrieved from the GenBank database as described in Germot et al. (2001) and Plouhinec et al. (2003).

Phylogenetic analysis

All amino acid sequences were aligned using the CLUSTAL W multiple alignment program of the MEGA version 4 (Tamura et al., 2007). Phylogenetic trees were constructed using neighbor-joining (NJ) and maximum-parsimony (MP) algorithms using the MEGA version 4 and PHYLIP version 3.67 (Felsenstein, 1989), respectively.

RNA in situ hybridization

Whole mount and section in situ hybridization were performed using digoxigenin-11-UTP labeled antisense RNA probes as described (Harland, 1991; Suda et al., 2001) with minor modifications. The expression analyses in skate embryos were performed with *L. erinacea* (*Le*) embryos using the 3rd exon fragments of *Otx* genes isolated with *R. eglanteria* (*Re*) DNAs as in situ probes. In the dogfish *ScOtx5* is expressed in future posterior margin as early as stage 5/6 when the blastocoele is not yet visible; its intensity is comparable to that of the *ScOtx5* expression at later stages (Coolen et al., 2007). However, none of the *Otx* paralogues is expressed at all in skate embryos at these early stages (see Results). The use of *Re*-derived probes in the expression analysis with *Le* embryos is unlikely to explain the difference. *Re* and *Le* species are so closely related that the use of *Re* probes should not affect the hybridization efficiency (Amemiya et al., unpublished). Indeed, the *Otx* expressions at later stages in the skate embryos were observed to be similar to those in dogfish embryos. The probes for fugu *Otx* genes are the full-length cDNAs isolated above; those for bichir *Otx* genes are their 3rd exon fragments isolated above; those for lamprey *Otx* genes are their full-length cDNAs described

above. The chick *GgOtx1* probe was the kind gift of Dr. K. Shimamura (Shimamura et al., 1995), the *GgOtx2* probe the gift of Dr. H. Nakamura (Funahashi et al., 1999) and the *GgOtx5* probe the gift of Dr. T. Furukawa (Furukawa et al., 1997).

RT-PCR

Total RNAs were extracted from *P. senegalus* (*Ps*) embryos at each stage (Fig. 3a) or from each dissection of *P. endlicheri* (*Pe*) embryos at early gastrula (Fig. 3b) using ISOGEN (Nippon Gene) according to the manufacturer's instructions. The RNAs were reverse-transcribed using SuperScriptIII (Invitrogen) with random hexameric primers in vitro, and the cDNA products were amplified by PCR. Primers used and lengths of amplified products are: *PsOtx1*, 393 bp with sp (5′-TGGTC-AGTTTACTCCACCGG-3′) and ap (5′-ATAGACGACGGTGCCATTGG-3′); *PeOtx1*, 251 bp with sp (5′-TGCGTCTTGCATGCAGCGTTCAG-3′) and ap (5′-GATGATGGTGACCAGACGACTGG-3′); *PsOtx2*, 296 bp with sp (5′-TCAGAGAGTGGAGCGAGTGG-3′) and ap (5′-GGACTTAAGGTAGA-CCCTGG-3′); *PeOtx2*, 225 bp with sp (5′-AGAGAGGCAAGCT-CAGAGAGTGG-3′) and ap (5′-CGTAGATCCGGCATAGCCTTGGC-3′); *PsOtx5*, 391 bp with sp (5′-AAGCACAAATGGGCAATACAGTCC-3′) and ap (5′-GAAGCCAAGGCCAGCAGTGC-3′); *PeOtx5*, 258 bp with sp (5′-CCAATGCCACGGTGTCCATCTGG-3′) and ap (5′-CCATGGTCGGAGTA-GTGATTGGG-3′); *PeSox2*, 193 bp with sp (5′-GACGAAGCCAAGCGTC-TTCGTGCGC-3′) and ap (5′-TTTGATTGACCCCGGCACCCAAGCC-3′); *PsHistoneH4*, 218 bp with sp (5′-CGTCATCGTAAAGTGCTCCG-3′) and ap (5′-GCATACACTACATCCATGGC-3′); *PeHistoneH4*, 153 bp with sp (5′-CCTGCTATCCGCCGTCTAGC-3′) and ap (5′-GGTCACGGTCTTCCTCTTGG-3′).

Results

Otx paralogue expression in F. niphobles

In teleosts fugu (tetraodontiformes) is distantly related to zebrafish (cypriniformes) (Miya et al., 2003), and we have chosen fugu to represent the expression patterns of *Otx* orthologues in teleosts. The *F. niphobles* we analyzed in this study is so closely related to *Fugu rubripes*, on which genome analysis has been conducted, that they can interbreed to generate fertile F1 heterozygotes (Kai et al., 2005). In contrast to zebrafish that has five *Otx* paralogues, we found six orthologues in fugu genome. Genome and EST data indicate that among fishes close to fugu in *Percomorpha*, stickleback and flounder

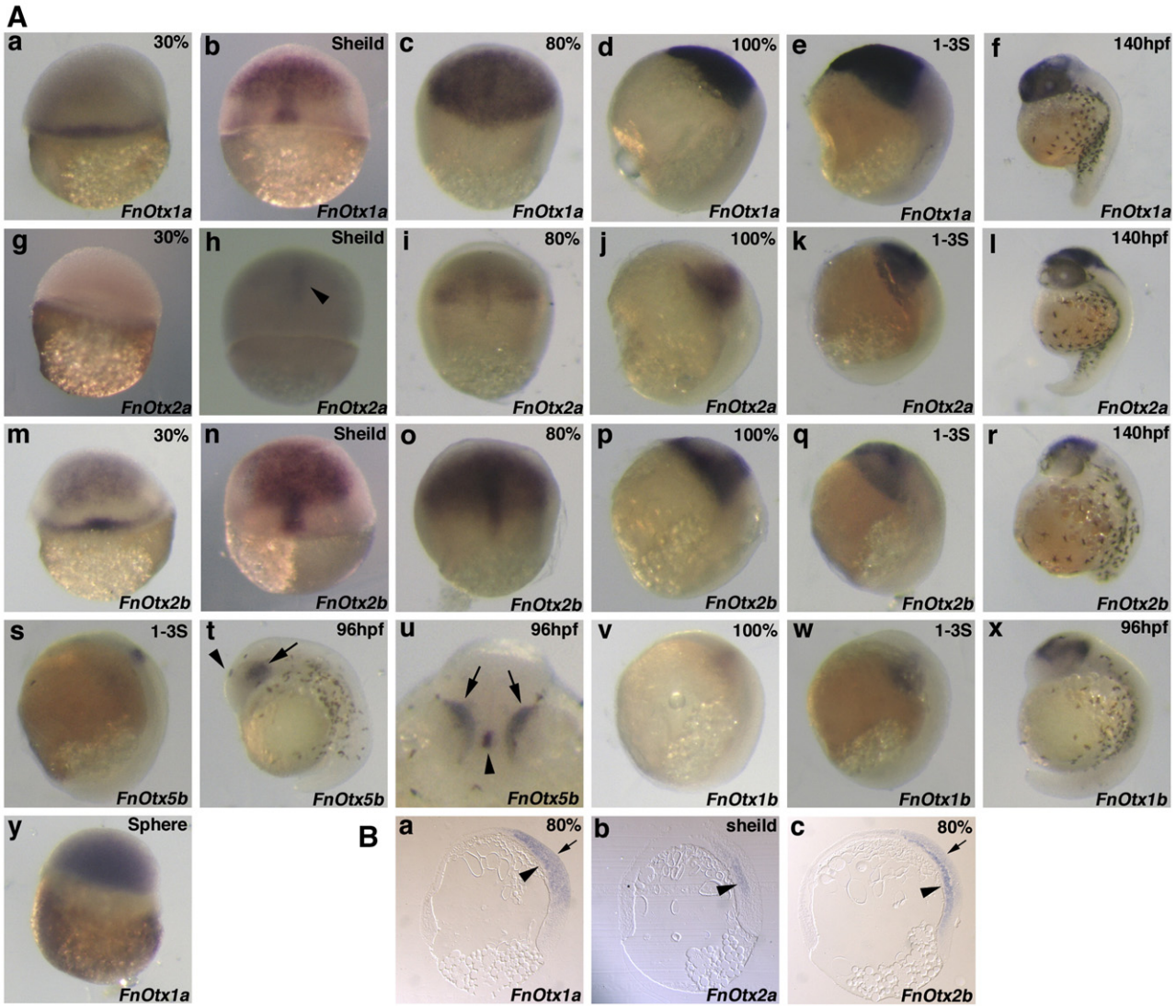

Fig. 2. *Otx* expression in *Fugu niphobles*. (A) *Otx* paralogue expression in whole mounts of *F. niphobles* embryos at stages indicated. (a–c, g–i, m–o) dorsal views, (d–f, j–l, p–t, v–y) lateral views (anterior is at the top) and (u) an anterior view. (B) *Otx* paralogue expression in sagittal sections of *F. niphobles* embryos at stages indicated; anterior is at the top. Arrowheads indicate the expressions in anterior mesendoderm (Ah, Ba–c) and epiphysis (At, u); arrows those in eyes (At, u) and anterior neuroectoderm (Ba, c).

also have six *Otx* orthologues, while medaka has five (Wittbrodt et al., 1998; Kasahara et al., 2007).

Phylogenetic analyses using the NJ and MP methods both assign these six *Otx* orthologues to two *Otx1* (*FnOtx1a* and *FnOtx1b*), two *Otx2* (*FnOtx2a* and *FnOtx2b*) and two *Otx5* (*FnOtx5a* and *FnOtx5b*) paralogues; zebrafish and medaka lack an *Otx2b* orthologue. In addition, *FnOtx2b* as well as *FnOtx2a* retain several cis-domains conserved among tetrapod *Otx2* orthologous gene loci (Kurokawa et al., 2006), while neither *FnOtx1a* nor *FnOtx1b* does. Therefore, we conclude that *FnOtx2b* corresponds to an *Otx2* duplicate that was lost in zebrafish and medaka.

FnOtx5b expression is characteristic to eyes and epiphysis (Figs. 2As–u), and not found during early embryogenesis; *FnOtx5a* expression is not apparent at all by tailbud stage (data not shown). *FnOtx1b* also exhibits no apparent expression until 80% epiboly and a faint expression at 100% epiboly in midbrain area; at subsequent stages moderate expression continues in midbrain (Figs. 2Av–x). The following description is limited to *FnOtx1a*, *FnOtx2a* and *FnOtx2b*.

It is only *FnOtx1a* that is expressed maternally in fugu (Fig. 2Ay). Zygotically *FnOtx1a* and *FnOtx2b*, but not *FnOtx2a*, are expressed in the dorsal margin and embryonic shield (Figs. 2Aa, b, g, h, m, n). *FnOtx1a* and *FnOtx2b* are also expressed in the anterior mesendoderm (Figs. 2Ab, c, n, o; Ba, c); the *FnOtx2a* expression in this tissue was found faintly at 60% epiboly and weakly at 80% epiboly (Figs. 2Ah, i, Bb). *FnOtx1a* and *FnOtx2b* are expressed in the entire anterior neuroectoderm at 60% and 80% epiboly stages (Figs. 2Ab, c, n, o, Ba, c). *FnOtx2a* expression is insignificant in the neuroectoderm at 60% epiboly (Fig. 2Ah) and faint at 80% epiboly (Figs. 2Ai, Bb). *FnOtx2b* expression diminishes in the rostral brain at 100% epiboly (Fig. 2Ap) when the *FnOtx2a* expression is complementarily enhanced in this region (Fig. 2Aj). *FnOtx1a*, *FnOtx1b*, *FnOtx2a* and *FnOtx2b* expressions are overlapping in mesencephalon and diencephalon at late somite stages with several unique features in each paralogue (Figs. 2Af, l, r, x). *FnOtx1a*, *FnOtx2a* and *FnOtx2b* are also expressed in eyes and epiphysis (data not shown).

Otx paralogue expression in P. senegarus

Bichir is an animal that diverged from the teleost lineage prior to its whole genome duplication at the stem of actinopterygian lineage. *P. senegarus* possesses only one *PsOtx1*, *PsOtx2* and *PsOtx5* paralogue similar to what has been reported in the reedfish *Erpetoichthys calabaricus* (Germot et al., 2001). RT-PCR analysis indicated it is only *PsOtx1* that is maternally expressed (Fig. 3a). In situ hybridization analysis of *Otx* expression has been unsuccessful in our hand at early gastrula stage. Embryos at the initiation of gastrulation were thus dissected into several pieces as shown in Fig. 3b, and the RT-PCR analysis was conducted on each paralogue in these pieces. The analysis suggested that all three *Otx* paralogues are expressed in the dorsal marginal zone, probably *PsOtx1* being the most extensive and *PsOtx2* the least (Fig. 3b). The animal pole pieces that express *Sox2*, a neural marker, do not express *PsOtx1*, *PsOtx2* or *PsOtx5*, suggesting that the expression of these *Otx* paralogues does not start in the fish anterior neuroectoderm at this stage.

At late gastrula stage in situ hybridization demonstrated the expression of all three paralogues in anterior mesendoderm, and of *PsOtx1* and *PsOtx2*, but not of *PsOtx5*, in anterior neuroectoderm (Figs. 4a, b, i, j, p, q). At the early neurula stage *PsOtx1* continues to be intense and extensive in anterior neuroectoderm, but *PsOtx2* becomes less (Figs. 4c, k). *PsOtx5* starts to be expressed at late neurula stage and is evident at tailbud stage in epiphysis and the attachment gland but not in eyes (Figs. 4r–t). At tailbud stage *PsOtx1* expression covers forebrain and midbrain except for the most rostral forebrain (Figs. 4d, e). Its expression is extended caudally beyond midbrain into the hindbrain area (Fig. 4d). In contrast, *PsOtx2* expression is limited to the midbrain region (Fig. 4l). *PsOtx1* was also found in eyes and attachment gland, but not in epiphysis; *PsOtx2* was not found in these sites. At early tadpole stage, *PsOtx1* positive region is further regressed caudally in forebrain but it still covers caudal forebrain and midbrain (Figs. 4f, g). *PsOtx2* continues to be expressed in midbrain (Figs. 4n, o), and its caudal limit coincides with the midbrain/hindbrain boundary (Fig. 4n), but that of *PsOtx1* is extended beyond the boundary (Fig. 4f) as it is at tailbud stage. It is expressed in neuroectoderm but not in surface ectoderm (Fig. 4h). The *PsOtx5* expression continues in epiphysis and the attachment gland (Figs. 4u, v). At this stage, the expression of *PsOtx5*, but not of *PsOtx1 or PsOtx2,* is apparent in otic vesicles (Fig. 4u).

Otx paralogue expression in L. erinacea

Expression of *ScOtx1*, *ScOtx2* and *ScOtx5* orthologues during gastrulation has been reported in a dogfish (*Scyliorhinus canicula*) (Coolen et al., 2007), and we have chosen skate (*L. erinacea*) to represent the expression pattern of *Otx* orthologues in *Elasmobranchii*. The skate also has only one *Otx1*, *Otx2* and *Otx5* orthologue (*LeOtx1*, *LeOtx2* and *LeOtx5*). None of these are expressed in skate embryos at

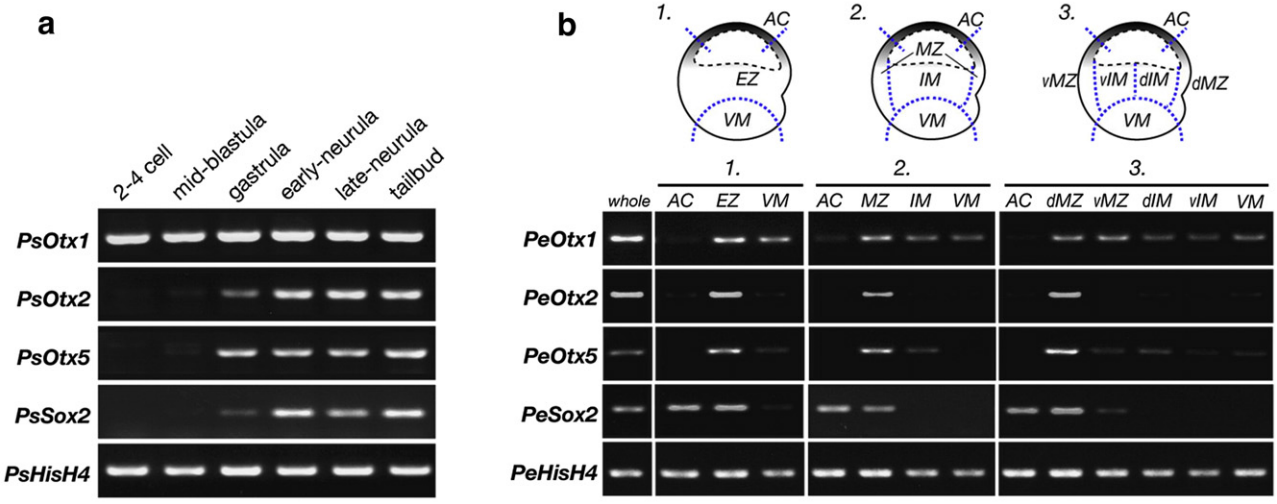

Fig. 3. *Otx* paralogue expression in bichir by RT-PCR. The expression in *Polypterus senegalus* (*Ps*) embryos at stages indicated (a) and in pieces dissected as indicated at the start of gastrulation (b). The experiments in (b) were performed with *Polypterus endlicheri* (*Pe*) embryos which are larger than *P. senegalus* embryos. Abbreviations: *AC*, animal cap; *EZ*, equatorial zone; *IM*, inner mass; *dIM*, dorsal *IM*; *vIM*, ventral *IM*; *MZ*, marginal zone; *dMZ*, dorsal *MZ*; *vMZ*, ventral *MZ*; *VM*, vegetal mass.

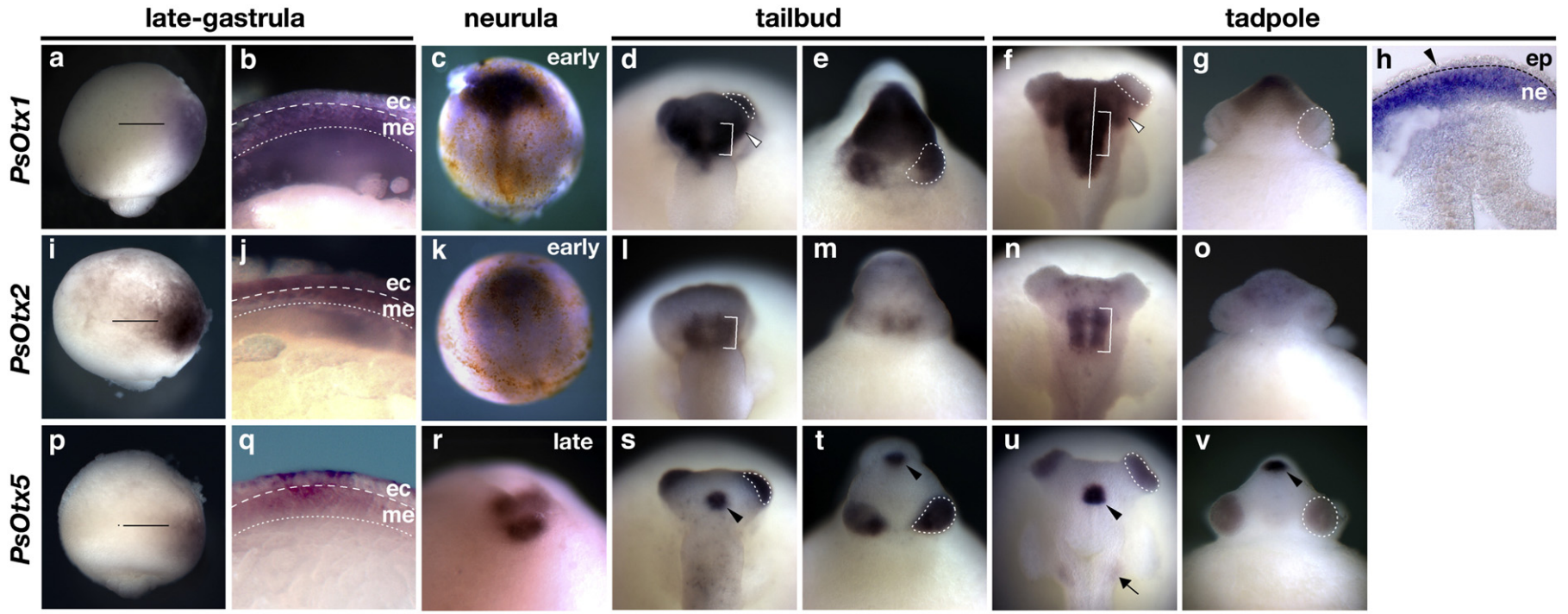

Fig. 4. *Otx* paralogue expression in *Polypterus senegarus* by in situ hybridization. (a–g, i–v) give whole mount and (h) section in situ hybridization; (a, i, p) lateral views (anterior is at the top, and dorsal at the right), (c, d, f, k, l, n, s, u) dorsal views (anterior is at the top), (e, g, m, o, r, t, v) anterior views (dorsal is at the top) and (h) a sagittal view at the plane indicated by a white line in (f) (anterior is at the left). (c, k) show early neurula embryos, and (r) a late one. (b, j, q) are views dissected at planes indicated by black lines in (a, i, p), respectively; dotted lines demarcate neuroectoderm and mesendoderm layers. *PsOtx1* and *PsOtx2* are expressed in both layers, but *PsOtx5* is only in mesendoderm layer. Dotted lines in (d–g, s–v) indicate attachment glands, the dotted line in (h) the boundary between surface ectoderm and neuroectoderm, and brackets in (d, f, l, n) midbrain. Arrowheads in (d, f) indicate eyes, and in (s–v) epiphysis. In (h) an arrowhead indicates the position of mid/hindbrain boundary. Abbreviations: ec, ectoderm; me, mesendoderm; nec, neuroectoderm; sec, surface ectoderm.

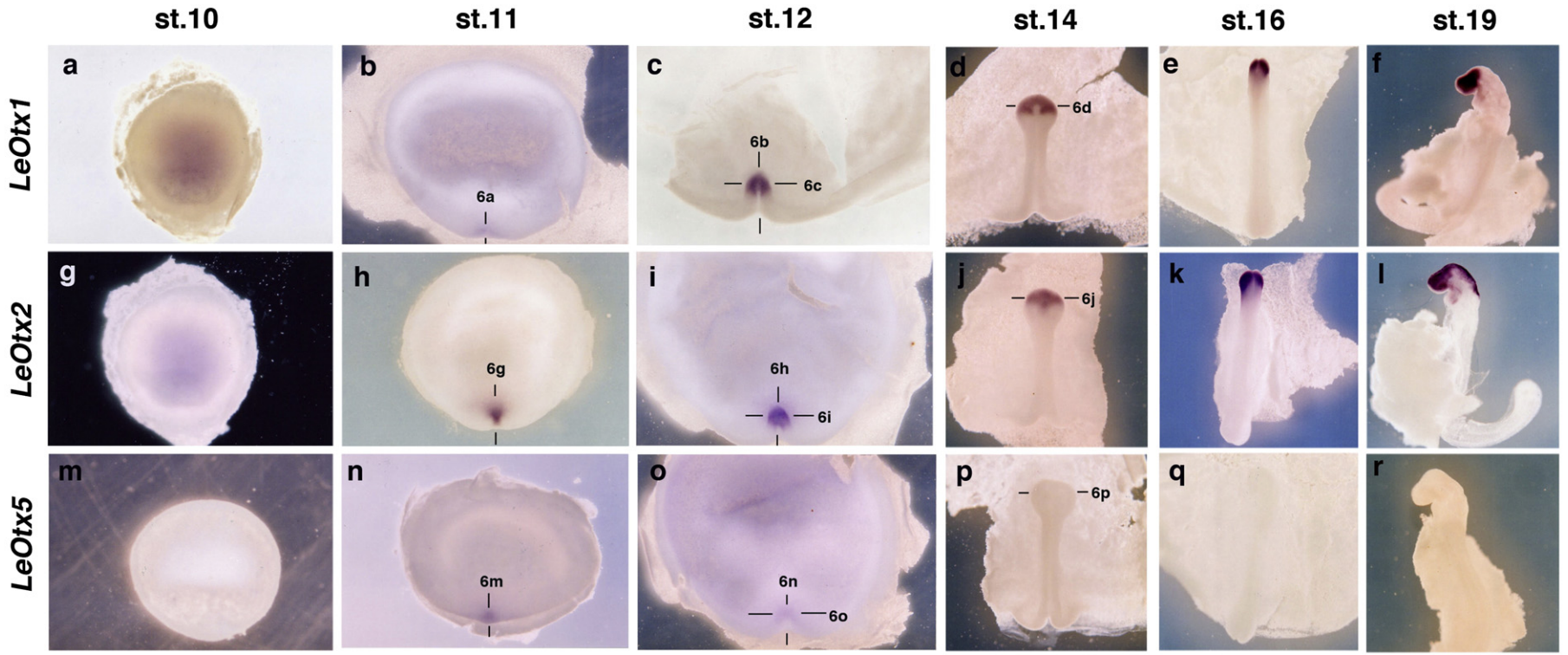

Fig. 5. *Otx* paralogue expression in *Leucoraja erinacea* by whole mount in situ hybridization. (a–e, g–k, m–q) dorsal views (anterior is at the top) and (f, l, r) lateral views (anterior is at the left) at stages and with probes indicated. The st. 10 is late blastula, st. 11 early gastrula, st. 12 late gastrula, st. 14 early neurula, st. 16 late neurula and st. 19 early pharyngeal stage, respectively. Lines in (b–d, h–j, n–p) indicate the planes of the sections in Figs. 6a–d, g–j, m–p.

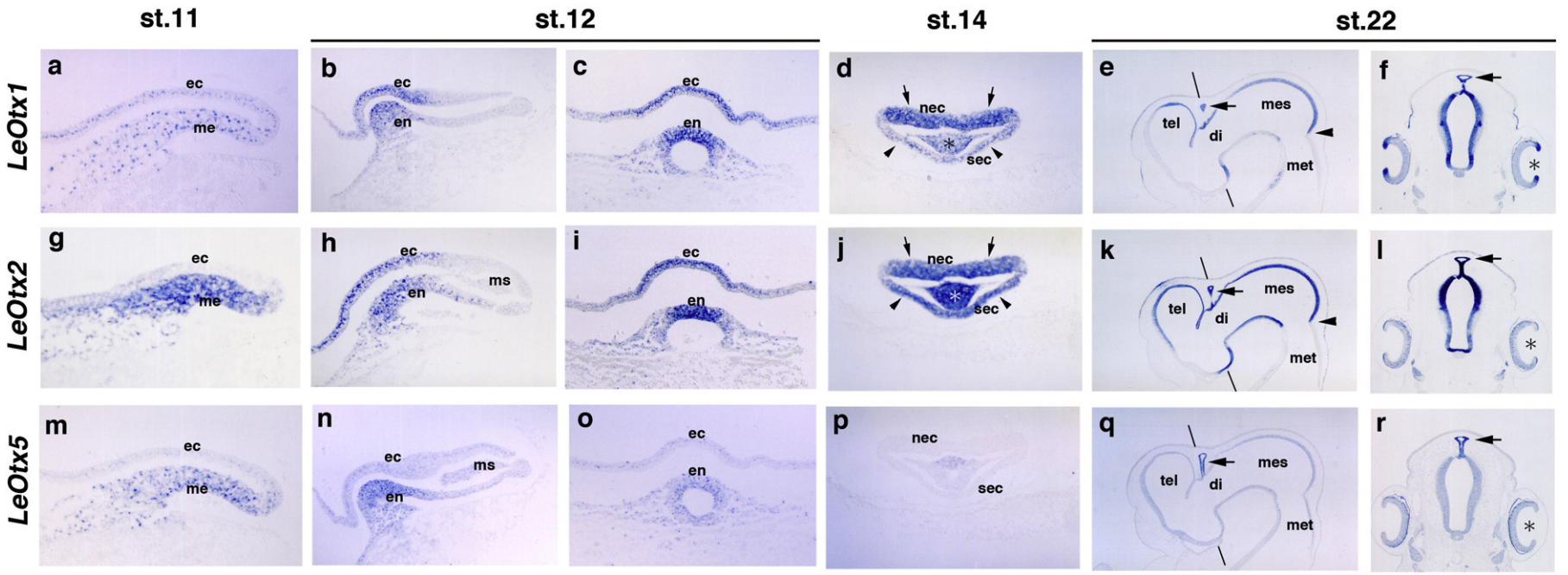

Fig. 6. *Otx* paralogue expression in *Leucoraja erinacea* by in situ hybridization on section. (a, b, g, h, m, n) give sagittal sections at midlines (anterior is at the left) indicated in Figs. 5b, c, h, i, n, o; (c, d, i, j, o, p) cross sections at planes indicated in Figs. 5c, d, i, j, o, p; (e, k, q) para-sagittal sections (anterior is at the left) and (f, l, r) cross sections at planes indicated in (e, k, q). Probes and stages are as indicated. Arrows, arrowheads and asterisks in (d, j) indicate neural plate, surface ectoderm and anterior mesendoderm, respectively. Arrowheads in (e, k) indicate mid/hindbrain boundary; arrows in (e, f, k, l, q, r) epiphysis; asterisks in (f, l, r) eyes. Abbreviations: di, diencephalon; en, endoderm; ec, ectoderm; me, mesendoderm; mes; mesencephalon; met, metencephalon; ms, mesoderm; nec, neuroectoderm; sec, surface ectoderm; tel, telencephalon.

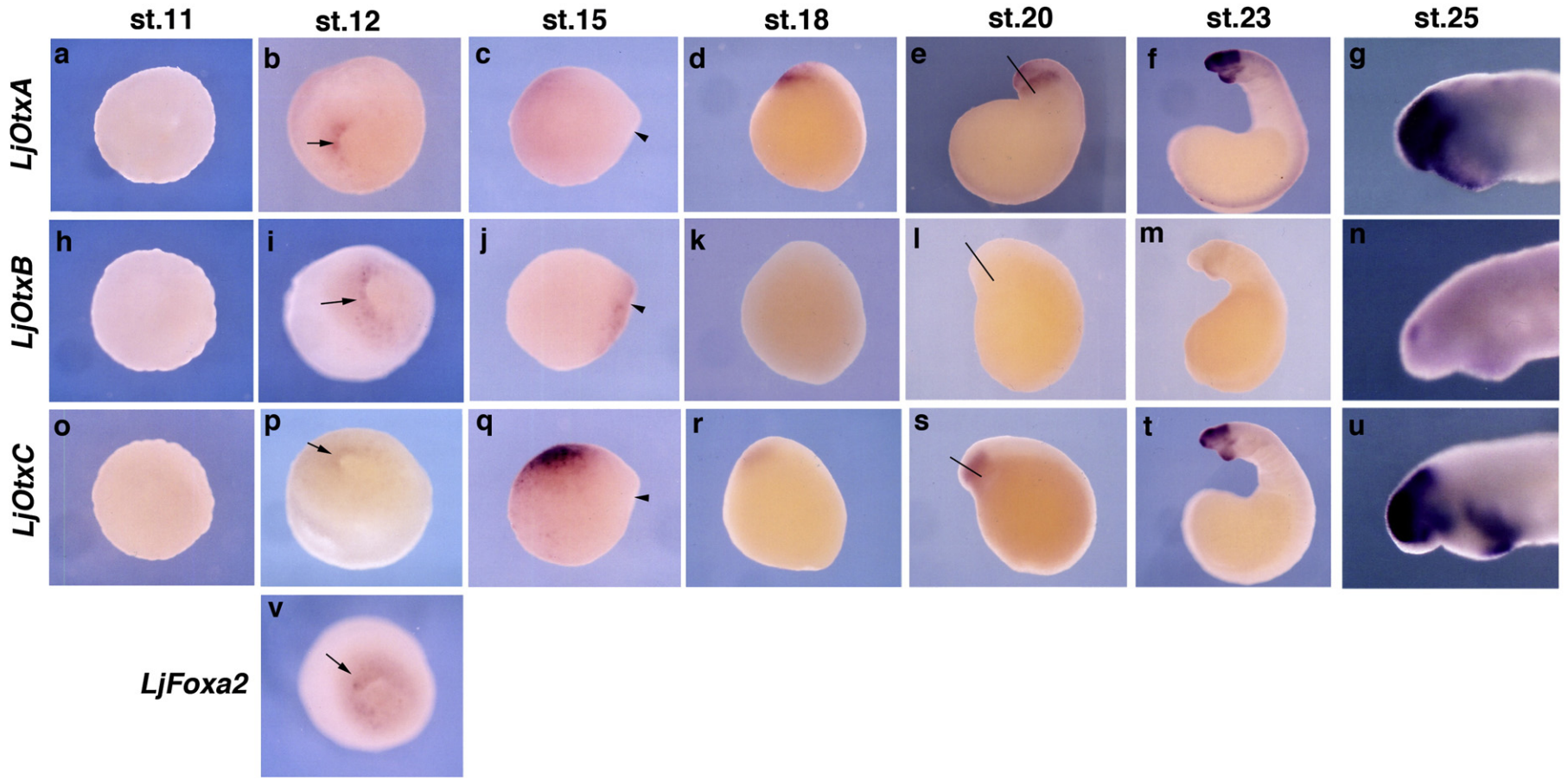

Fig. 7. *Otx* paralogue expression in *Lethenteron japonicum* by whole mount in situ hybridization. (a, c–h, j–o, q–u) give lateral views, and (b, i, p, v) views at the prospective blastopore, at stages and with probes indicated. Arrows in (b, i, p, v) indicate dorsal, and arrowheads in (c, j, q) blastopore; in (d–g, k–n, r–u) anterior is at the left. The st. 11 is late blastula, st. 12 the start of gastrula, st. 15 mid-gastrula, st. 18 early neurula, st. 20 late neurula and st. 25 early tailbud, respectively. Lines in (e, l, s) indicate the planes of the sections in (Figs. 8b, h, n), respectively.

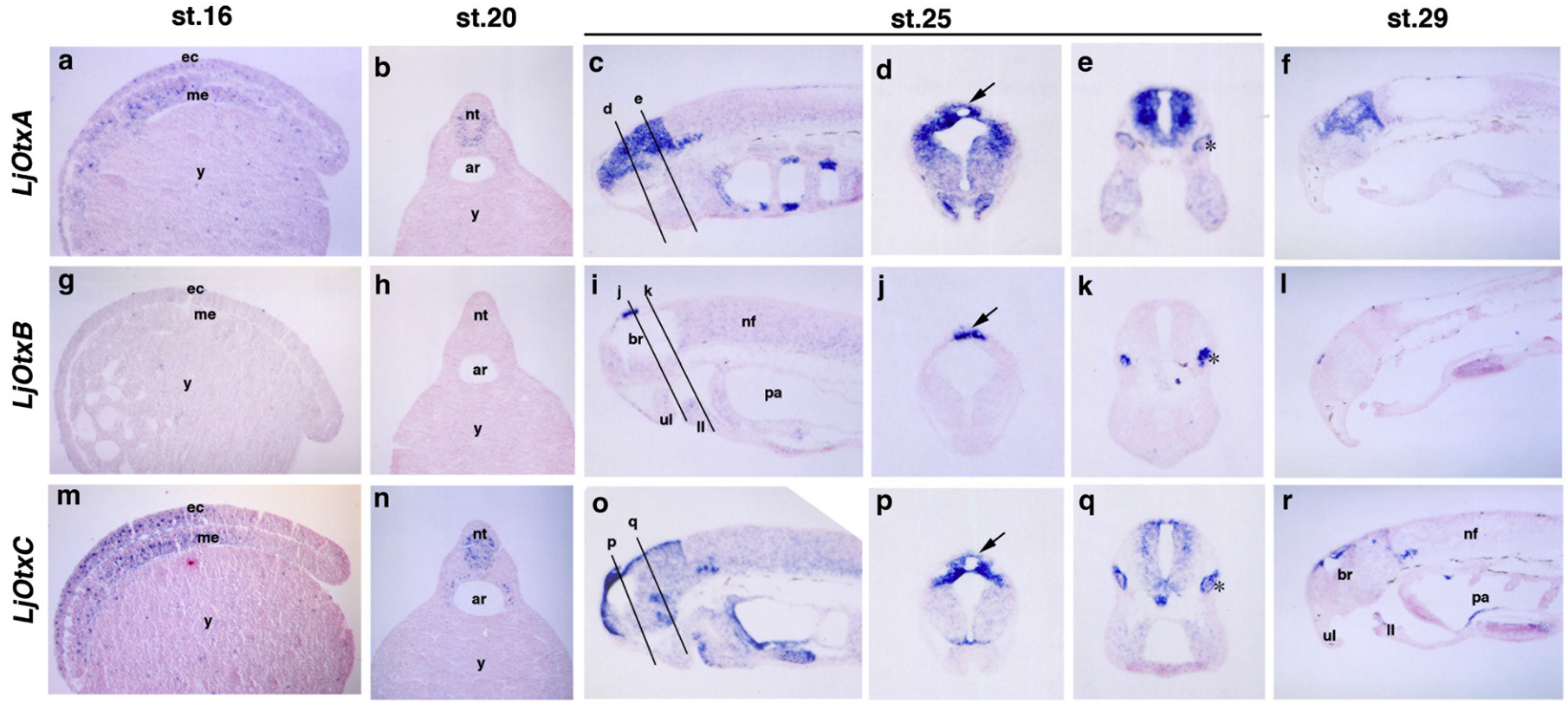

Fig. 8. *Otx* paralogue expression in *Lethenteron japonicum* by in situ hybridization on section. (a, c, f, g, i, l, m, o, r) give sagittal sections (anterior is at the left), and (b, d, e, h, j, k, n, p, q) cross sections. Planes of the sections in (b, h, n) are indicated in Figs. 7(e, l, s), and those in (d, e, j, k, p, q) in (c, i, o), respectively. Probes and stages are as indicated. Arrows in (d, j, p) indicate epiphysis; asterisks in (e, k, q) eyes. Abbreviations: ar, archenteron; br, brain; ec, ectoderm; ll, lower lip; me, mesendoderm; nt, neural tube; pa, pharyngeal arches; ul, upper lip; y, yolk.

blastula stage (data not shown). At stage 10 prior to gastrulation there is also no expression of these *Otx* orthologues in the skate embryos (Figs. 5a, g, m). In skate the expressions of all *Otx* paralogues start at stage 11 with the onset of gastrulation (Figs. 5b, h, n); this is in the invaginating mesendoderm or in the lower layer (Figs. 6a, g, m). That is the site where head organizer genes, *Foxa2*, *Lim1* and *Gsc*, are also expressed in the dogfish (Coolen et al., 2007). There are some differences in the expression intensity and the expression domain among the three paralogues. However, there would be active convergence of cells along the margin toward the posterior midline and then toward the anterior in the midline at this stage. Subtle changes in the embryonic stages analyzed would explain the differences. At this stage neither *LeOtx1*, *LeOtx2* nor *LeOtx5* is expressed in either ectoderm at the posterior or the central part of the blastoderm (Figs. 5b, h, n; 6a, g, m).

Subsequently at stage 12, *LeOtx1* and *LeOtx2*, but not *LeOtx5*, become expressed in anterior ectoderm overlaying *Otx*-positive anterior mesendoderm (Figs. 5c, i, o; 6b, c, h, i, n, o). At stage 14 and 16, *LeOtx1* is more intense in anterior neuroectoderm than in surface ectoderm, but *LeOtx2* is also intense in anterior surface ectoderm (Figs. 5d, e, j, k; 6d, j). *LeOtx5* expression is not apparent at these stages (Figs. 5p, q; 6p). At stage 19–22 in the early pharyngeal stage, *LeOtx2* expression was found in entire forebrain except for the optic chiasma region, but *LeOtx1 expression* was somewhat less extensive in rostral forebrain (Figs. 5f, l; 6e, f, k, l); both are also found in eyes and epiphysis. The caudal limit of both *LeOtx1* and *LeOtx2* expression in the neuroectoderm is sharply delineated at the future midbrain/hindbrain boundary (Figs. 6e, k), while the *LeOtx2* expression in surface ectoderm extends into hindbrain region (Fig. 5l). *LeOtx5* expression is expressed only in epiphysis and eyes at these stages (Figs. 5r; 6q, r).

Otx paralogue expression in L. japonicum

A hagfish is reported to have three *Otx* paralogues: *MgOtxA*, *MgOtxC* and *MgOtxD* (Germot et al., 2001). Here we have also identified three paralogues in both *L. japonicum* and *P. marinus*; they are called *OtxA*, *OtxB* and *OtxC*. *PmOtx* by Tomsa and Langeland (1999) is *PmOtxC* of which the orthologue, *LjOtxC*, is also present in *L. japonicum*. Phylogenetic analyses with the NJ and MP methods suggest that *LjOtxA* and *PmOtxA* are orthologous with *MgOtxA* (Fig. 1). However, *LjOtxC*/*PmOtxC* or *LjOtxB*/*PmOtxB* display no clear

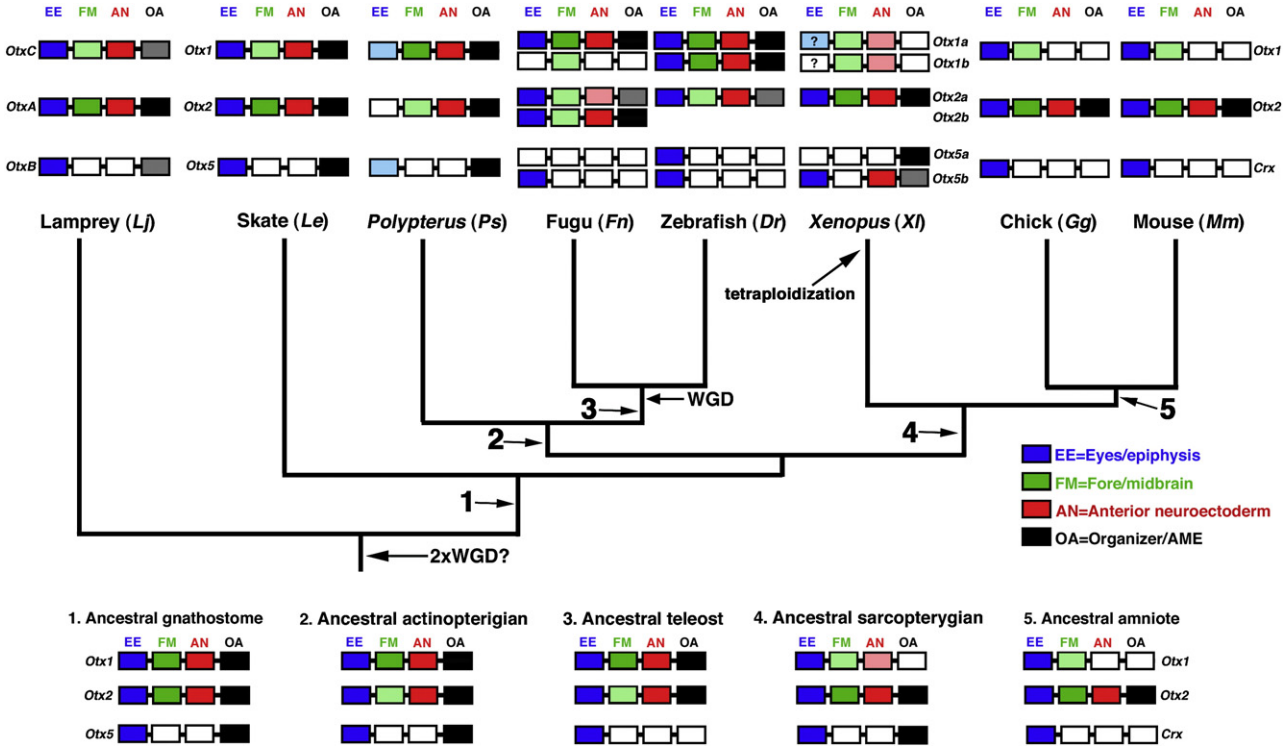

Fig. 9. Lineage-specific usage of *Otx* paralogues in vertebrate head development. On the basis of our enhancer analyses on *Otx2* gene (Kurokawa et al., 2004a,b, 2006; Kimura-Yoshida et al., 2007), the divergence in the expression of *Otx* genes in organizer and anterior mesendoderm (OA), anterior neuroectoderm at presomite stage (AN), forebrain/midbrain at somite stage (FM) and eyes/epiphysis (EE) is schematized. The figure does not suggest that the conservation of the expression among different vertebrate lineages is brought about by orthologous enhancers. For example, *Otx2* expression in anterior neuroectoderm and forebrain/midbrain is directed by the same enhancer in teleosts, but by two different enhancers in other gnathostomes (Kurokawa et al., 2006). Color depth represents the expression intensity and/or extent. The mouse data are by Simeone et al. (1992, 1993) and Furukawa et al. (1997). The chick *Otx2* data are by Bally-Cuif et al. (1995), Bovolenta et al. (1997) and Plouhinec et al. (2005). *GgOtx1* and *GgOtx5* expressions are by our unpublished data; the *GgOtx1* expression in anterior mesendoderm remains to be examined in detail. The *Xenopus* data are by Blitz and Cho (1995), Pannese et al. (1995, 2000), Kablar et al. (1996), Kuroda et al. (2000) and Vignali et al. (2000); *XlOtx1* in these references is *XlOtx1a*, and *XlOtx4* is *XlOtx1b*. In forebrain/midbrain *Otx1* orthologue expressions are less extensive than *Otx2* in mouse, chick and *Xenopus*. In *Xenopus Otx1a* and *Otx1b* are expressed in the anterior neuroectoderm at stage 12 (late gastrula) though less extensive than *Otx2* (Kablar et al., 1996); their expression might be present in anterior mesendoderm or prechordal plate at very low levels. *XlOtx1a* is expressed in eyes, but its expression in epiphysis is not reported; *XlOtx1b* expression in eyes or epiphysis is also not reported. *XlOtx5b* is expressed in organizer and anterior neuroectoderm as *XlOtx2* is, but not in anterior mesendoderm at stage 12 (Vignali et al., 2000). The zebrafish data are by Li et al. (1994), Mori et al. (1994), Mercier et al. (1995), Gamse et al. (2002), and Shen and Raymond (2004). *DrOtx2* is expressed in anterior mesendoderm of gastrula embryos, but not in the dorsal margin of blastoderm or embryonic shield (Li et al., 1994). The data on fugu are from this study. *FnOtx1b* is moderately expressed in midbrain region at 96 hpf. *FnOtx2a* expression is not expressed in the dorsal margin of blastoderm, embryonic shield or early anterior mesendoderm, but was found weakly in anterior mesendoderm at 80% epiboly. The data in bichir are from this study. *PsOtx1* is expressed in eyes but not in epiphysis, while *PsOtx5* is expressed in epiphysis but not in eyes. In these actinopterygian forebrains and midbrains the *Otx1* expression is more intense and extensive than the *Otx2* expression. The skate data are from this study; we consider they are largely the same as those in dogfish by Sauka-Spengler et al. (2001), Plouhinec et al. (2005) and Coolen et al. (2007). The data in lamprey are by Ueki et al. (1998), Tomsa and Langeland (1999) and this study. *LjOtxC* is expressed in anterior mesendoderm, but is faint in dorsal blastopore, while *LjOtxB* is expressed in dorsal blastopore but not in anterior mesendoderm. *LeOtx1* and *LjOtxC* expressions are less intense and extensive than *LeOtx2* and *LjOtxA*, respectively, in forebrain and midbrain.

relationships to either of the hagfish *Otx* genes (Fig. 1). The high degree of divergence of agnatha *Otx* sequences does not allow us to relate them to any of the three paralogous classes (*Otx1*, *Otx2* and *Otx5*) identified in gnathostomes.

None of these *Otx* paralogues is expressed in *L. japonicum* embryos at the blastula stage (Figs. 7a, h, o). The expressions of all three *Otx* paralogues start prior to gastrulation at the future dorsal blastopore lip that was demonstrated to be the lamprey organizer (Yamada, 1938) and where *Foxa2* is also expressed; *LjOtxC* staining is faint (Figs. 7b, i, p, v). At stage 15 with the onset of gastrulation, *LjOtxA* and *LjOtxC* expressions are found in the anterior part of invaginating mesendoderm, while *LjOtxB* expression remains at the blastopore region, being expanded into the vegetal side that is fated to become anterior ventral archenteron (Figs. 7c, j, q; 8a, g, m; Weissenberg, 1934). Subsequently *LjOtxB* expression is lost (Figs. 7k, l; 8g, h). In contrast, *LjOtxA* and *LjOtxC* continue to be expressed in the anterior part of invaginating mesendoderm and of ectoderm overlaying it (Figs. 7d, e, r, s; 8a, b, m, n) as *LeOtx1* and *LeOtx2* are in skate embryos. At stage 23 they are apparent in rostral brain with the caudal boundary at the midbrain/hindbrain junction (Figs. 7f, t); they are also expressed in lower lip. At stage 25 *LjOtxA* is still expressed in the entire forebrain/midbrain, but *LjOtxC* expression is reduced throughout the brain; *LjOtxC* is rather intense in surface ectoderm at the forebrain/midbrain region (Figs. 7g, u; 8c–e, o–q). *LjOtxA*, *LjOtxB* and *LjOtxC* expressions were also found in epiphysis and eyes at this stage; *LjOtxA* and *LjOtxC* are also expressed in the pharyngeal region (Figs. 8c–e, i–k, o–q). At stage 29, *LjOtxA* expression is lost in rostral forebrain, but yet intense in caudal forebrain and midbrain (Fig. 8f). In contrast, *LjOtxC* expression is scarcely expressed in forebrain and midbrain (Fig. 8r). The *LjOtxC* expression pattern is quite similar to that of *PmOtxC* reported by Tomsa and Langeland (1999), and the *LjOtxB* expression pattern is reminiscent of gnathostome *Otx5* expression. Moreover, the expression pattern of *LjOtxA* is more or less similar to *LeOtx2*, and that of *LjOtxC* to *LeOtx1*. The expression patterns among the three *Otx* paralogues thus appear to be conserved from agnatha to gnathostome, implying their early evolutionary establishment by the vertebrate common ancestor (Fig. 9).

Discussion

Otx paralogues play essential roles in each step and site of mouse head development. Their expression is regulated by a series of unique enhancers (Kimura et al., 1997, 2000; Kurokawa et al., 2004a,b, 2006; Kimura-Yoshida et al., 2007). Several upstream factors for each *Otx* expression and downstream factors regulated by *Otx* gene products have been characterized (Boncinelli and Morgan, 2001; Spieler et al., 2004; Kimura-Yoshida et al., 2005, 2007; Kurokawa et al., 2004a,b; Furushima et al., 2007; Ogino et al., 2007; Takasaki et al., 2007). Comparative analyses have been undertaken on several *Otx2* enhancers among gnathostomes (Kurokawa et al., 2006; unpublished results). The present result (Fig. 9) is the basis to develop these analyses further to elucidate how each *Otx cis*-regulatory sequence and its upstream and downstream cascade during head development could have evolved in each vertebrate lineage.

Neither amniote nor teleost *Otx5*/*Crx* orthologue is expressed in organizer tissues or anterior mesendoderm (Furukawa et al., 1997; Gamse et al., 2002; this study; Fig. 9). However, *XlOtx5* and *XlOtx5b* are expressed in *Xenopus* organizer and anterior mesendoderm (Kuroda et al., 2000; Vignali et al., 2000), and *PsOtx5* in bichir organizer and anterior mesendoderm (this study). Moreover, both *ScOtx5* and *LeOtx5* are also expressed in these tissues of dogfish (Coolen et al., 2007) and skate embryos (this study). The most parsimonious interpretation would be that the ancestral gnathostome would have utilized all of *Otx1*, *Otx2* and *Otx5* paralogues in organizer and anterior mesendoderm (Fig. 9). In this animal *Otx1* and *Otx2* would have also been, but *Otx5* would have been not, utilized for anterior neuroectoderm and forebrain/midbrain development; in the neuroectoderm *Otx5* expression would have been already specialized in epiphysis and eye development. This condition may be more or less conserved in the extant elasmobranches. Regarding *Otx1*/*Otx2* functions for anterior neuroectoderm/brain development, subsequently, *Otx1* would have prevailed in a basal actinopterygian and *Otx2* in a basal sarcopterygian. The domain positive to *Otx2* orthologues in the brain is contained within or less extensive than that positive to *Otx1* orthologues in zebrafish, fugu and bichir, and the reverse is true in tetrapods. *Otx5* expression in head organizer and anterior mesendoderm must have been lost in teleost lineage after divergence of bichir and in amniotes after divergence of amphibian, independently. In addition, teleost lineage would have retained both *Otx1* and *Otx2* expression in organizer and anterior mesendoderm, while *Otx1* expression in organizer must have been lost in tetrapod lineage (Fig. 9).

The expression data in fugu together with those reported in zebrafish suggest that in a teleost ancestor prior to WGD *Otx1* and *Otx2* would have both been expressed in the dorsal margin of blastoderm, embryonic shield, anterior mesendoderm, entire anterior neuroectoderm and forebrain/midbrain at each step of head development. Subsequent whole genome duplication and the following genome changes would have caused different *Otx* paralogue usage in each teleost lineage. Zebrafish lost the *DrOtx2b* gene, and fugu would have silenced *FnOtx1b* for proper *Otx* dosage compensation in early head development. *Leucopsarion petersii* (Shiro-uo), which is relatively close to fugu in the *Periciformes* group, did not silence *Otx3* (*Otx1b*) (Kamimoto et al., 2003). In organizer and early anterior mesendoderm zebrafish coopted *DrOtx1* and *DrOtx1-like* (*Otx3*/*Otx1b*) (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995) and fugu *FnOtx1a* and *FnOtx2b* (this study).

We have reported that an enhancer homologous to the enhancer for the *Otx2* expression in mouse distal/anterior visceral endoderm and anterior mesendoderm exists at the 15 kb 3′ downstream of the translational start site in fugu *Otx2a* gene locus (Kimura-Yoshida et al., 2007). However, the present study demonstrates that *FnOtx2a* is not expressed in the fish organizer or early anterior mesendoderm. This may suggest that the putative 3′ enhancer in *FnOtx2a* gene does not function in vivo; the activity may be artificially generated by the dissection of the genome or by the assay with transgenic mouse embryos. However, *FnOtx2a* is expressed in late anterior mesendoderm at 80% epiboly, and the enhancer might be responsible for this *FnOtx2a* expression.

This study together with the study of hagfish (Germot et al., 2001) indicates that ancestral agnatha vertebrates must have already established three *Otx* paralogues. Their sequences underwent a high degree of divergence, and they show no clear relationship with either the gnathostome *Otx1*, *Otx2* or *Otx5* orthologues, but the expression data strongly suggests that *LjOtxB* would correspond to gnathostome *Otx5* orthologues. *OtxB*/*Otx5*/*Crx* usage in eyes and epiphysis would have been established in ancestral agnatha and conserved throughout vertebrate evolution. Of note is that *LjOtxB* is also expressed in the dorsal lip of blastopore or the lamprey organizer (Yamada, 1938), though it is not expressed in anterior mesendoderm probably due to a divergence in lamprey lineage. The expression pattern suggests that *OtxA* and *OtxC* would be related to either *Otx1* or *Otx2*, respectively. They are expressed in the dorsal lip, anterior mesendoderm, anterior neuroectoderm, caudal forebrain /midbrain and eyes/epiphysis. Of note is that the expression pattern of *LjOtxA* is more or less similar to that of *LeOtx2* and *LjOtxC* to *LeOtx1*. *LjOtxA* and *LeOtx2* expression dominates *LjOtxC* and *LeOtx1* expression in their continuity and/or extensiveness in forebrain and midbrain. Apparently the expression pattern is more conserved than the coding sequence among gnathostome and agnatha *Otx* orthologues, and the analysis of *OtxA* and *OtxC* noncoding cis-sequences or enhancers would demonstrate this.

There are several features that would be lineage-specific or which cannot be consistently explained by the data available. An *Otx5* orthologue is expressed in early anterior neuroectoderm of *Xenopus* (Vignali et al., 2000). However, *Otx5* orthologues are never found in anterior neuroectoderm of other vertebrates; the *Otx5* expression in the anterior neuroectoderm would have been acquired uniquely in *Xenopus*. None of the *Otx* homologues has been known to be expressed beyond midbrain/hindbrain boundary in any vertebrate. The counteraction between *Otx* and *Gbx2* has been shown to determine the boundary in amniotes (Joyner et al., 2000; Simeone, 2000). However, in bichir *Otx1* is expressed beyond the boundary in hindbrain. Another feature is maternal expression. In teleosts and bichir only one *Otx1* orthologue (Mori et al., 1994; this study) and in *Xenopus* all *Otx* orthologues are maternally expressed (Pannese et al., 1995, 2000; Kablar et al., 1996; Kuroda et al., 2000). In contrast, none is maternally expressed in lamprey, skate or mouse. Zygotic *Otx2* expression occurs in dogfish blastoderm (Coolen et al., 2007), and in chick and mouse epiblast prior to gastrulation (Simeone et al., 1993; Bally-Cuif et al., 1995). Zygotic expression is not apparent in lamprey or skate blastoderm or in *Xenopus* animal pole cells. The functional significance of the maternal *Otx* expression and the zygotic expression in blastoderm or epiblast has never been demonstrated in any vertebrates. Uniquely in bichir *Otx5* expression is lost in eyes, and *Otx1* and *Otx2* in epiphysis and/or eyes. The significance of these lineage-specific expression patterns remains to be determined in future studies.

In the dogfish *ScOtx5* is expressed in the future posterior margin as early as stage 5/6 when blastocoele is not yet visible (Coolen et al., 2007), but none of the *Otx* paralogues is expressed at all in skate embryos at these early stages. Although at stage 10 prior to gastrulation we did not observe the expression of any of the *Otx* orthologues in the skate embryos, all *Otx* paralogues were reportedly expressed at the posterior margin in the dogfish embryos; *ScOtx2* is also expressed in the dogfish blastoderm at this stage (Coolen et al., 2007). We have been unable to get a sufficient number of embryos at these critical stages; it is probable that the difference is a matter of staging of embryos, and that all *Otx* paralogues are expressed in the posterior margin just prior to the onset of gastrulation. Furthermore, Coolen et al. (2007) propose two distinct domains at stage 11 in the mid-posterior region expressing each *Otx* paralogue, *Foxa2, Lim1* and *Gsc* in a different combination, but we could not confirm this in skate embryos. Nevertheless, we consider that *LeOtx1*, *LeOtx2* and *LeOtx5* expressions in skate embryos are essentially the same as those of *ScOtx1*, *ScOtx2* and *ScOtx5*, respectively, in dogfish embryos (Sauka-Spengler et al., 2001; Plouhinec et al., 2005; Coolen et al., 2007; Fig. 9).

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