SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

August 2010 Vol.53 No.8: 1031–1040
doi: 10.1007/s11427-010-4040-2

Spatiotemporal expression of *Pax* genes in amphioxus: Insights into *Pax*-related organogenesis and evolution

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Received January 5, 2010; accepted February 5, 2010

The expression of four *AmphiPax* genes in 16 developmental stages and different organs in amphioxus (*Branchiostoma belcheri*) was investigated, finding those genes expressed throughout amphioxus life with temporal-specific (especially during embryogenesis and metamorphosis) and spatial-specific patterns. This study suggests that duplicated *Pax* genes in vertebrates might maintain most of their ancestral functions and also expand their expression patterns after the divergence of protochordates and vertebrates.

***Pax* genes, evolution, amphioxus, spatiotemporal gene expression, development**

Citation: Chen L, Zhang Q J, Wang W, *et al*. Spatiotemporal expression of *Pax* genes in amphioxus: Insights into *Pax*-related organogenesis and evolution. Sci China Life Sci, 2010, 53: 1031−1040, doi: 10.1007/s11427-010-4040-2

Chordata is characterized by a suite of apomorphies including a dorsal hollow nerve cord, notochord, pharyngeal gill, endostyle and a post-anal tail. The construction of those fundamental structures is usually controlled by a series of critically significant transcriptional regulators. A number of the transcriptional regulators have been isolated and their expression patterns have been examined during the embryonic and early larval development of amphioxus. The comparisons of their orthologous gene expressions facilitated understanding of the evolution of the chordate body plan and molecular mechanisms [1]. However, due to the difficulty of continuous culture in the laboratory, gene expressions of amphioxus in later developmental stages and in adults remain to be further illustrated. Due to the progress in laboratorial culture of amphioxus [2], we observed their gene expression patterns for various transcriptional regulators during their successive developmental states.

In addition to early embryonic development, amphioxus also undergoes several critical developmental stages throughout its life. The larva, which is strongly asymmetric after embryogenesis, develops approximately 11 gills before undergoing a radical metamorphosis which establishes the more nearly symmetrical body plan of the benthic juvenile. After this process, tissues continually undergo patterning to form the adult animal, and gonads rapidly develop before the breeding season. Thus, amphioxus passes through three key developmental phases in its life: the embryogenesis, metamorphosis and gonad development.

It was assumed that two rounds of entire genome duplications (2R hypothesis) occurred after the divergence of the protochordate and vertebrate lineages, driving the evolution of developmental and morphological complexity in vertebrates [3]. Gene duplications create new duplicated genes which may gain new functions (neofunctionalization) or partition ancestral functions between duplicates (subfunctionalization) [4]. *Pax* genes, a group of transcriptional

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regulators, are vital factors in animal embryonic patterning and development from anthozoa to vertebrates [5–14]. The proteins encoded by *Pax* genes are characterized with a highly conserved 128 amino acid paired domain and usually classified into four subfamilies: Pax1/9 (Pax1 and Pax9), Pax2/5/8 (Pax2, Pax5 and Pax8), Pax3/7 (Pax3 and Pax7), and Pax4/6 (Pax4 and Pax6). Vertebrates bear all of those nine *Pax* genes, but invertebrate chordates, tunicates and amphioxus, possess four of them [6,10–14]. Generally, embryonic expression patterns of *Pax* genes have been reported in invertebrates, while vertebrate *Pax* gene expressions are related to organogenesis and their extensive function is maintained in adulthood. Those organs include the lens tissue of the eye (*Pax6*), intestines (*Pax6*), thymus (*Pax1* and *9*), thyroid (*Pax8*), pancreas (*Pax2, 4* and *6*), oviduct (*Pax2*), vas deferens (*Pax2*), epididymis (*Pax2*), testis (*Pax5*), myogenic precursors of muscle tissue (*Pax3*) and adult satellite cells (*Pax7*) [6–14].

Previous investigations of *AmphiPax* gene expressions during embryonic and early larval development have facilitated understanding of the evolution of *Pax*-related functions [10–14]. However, the gene expressions at later developmental stages and in adult tissues are largely unknown. To address the issue, we investigated the temporal expression of *AmphiPax* genes at 16 different developmental stages throughout amphioxus life, and examined gene tissue-level expression in adults utilizing real-time quantitative PCR (RTqPCR). We found that four *AmphiPax* genes were expressed in all developmental stages with significant stage/tissue-specific variations. Considering the much higher expression levels of *AmphiPax1/9* in the pharynx area during adulthood, we further detected the expression of the gene in the adult pharynx area by *in situ* hybridization (ISH) and immunohistochemistry.

1 Materials and methods

1.1 Phylogenetic analysis

The phylogenetic trees of the *Pax* family were constructed using the neighbor-joining (complete deletion) method implemented in the program MEGA [15] version 3.1 with a *Pseudomonas* transposase sequence as an out-group [16]. Bootstrap values were estimated using 1000 replications for a confidence measure. To construct the phylogenetic tree, we adopted the sequences of four *AmphiPax* genes from a public database (GenBank accession Nos. DQ991501, DQ991502, DQ991503 and DQ991504) and the genes from following organisms (followed by gene cords and GenBank/Ensembl accession numbers in parentheses): *Caenorhabditis elegans, CePax1/9* (NM_072719), *CePax2/5/8* (NM_068112), *CePax6* (NM_001029401); *Drosophila melanogaster, DmPoxmeso* (NM_001043222), *DmPrd* (NM_078832), *Dmeyeless* (NM_079889), *DmSparkling* (NM_079894); *Danio rerio*, *Pax1* (NM_131640), *Pax7* (NM_131324), *Pax9* (NM_131298); *Xenopus tropicalis*, *Pax1* (ENSXETG00000023497), *Pax2* (ENSXETG00000014206), *Pax3* (ENSXETG00000007640), *Pax5* (ENSXETG00000006547), *Pax6* (ENSXETG00000008175), *Pax7* (ENSXETG00000018527), *Pax8* (ENSXETG00000001272), *Pax9* (ENSXETG00000002907); *Gallus gallus*, *Pax2* (NM_204793), *Pax9* (NM_204912); *Mus musculus*, *Pax1* (NM_008780), *Pax2* (NM_011037), *Pax3* (NM_008781), *Pax4* (NM_011038), *Pax5* (NM_008782), *Pax6* (NM_013627), *Pax7* (NM_011039), *Pax8* (NM_011040), *Pax9* (NM_011041); *Homo sapiens*, *Pax1* (NM_006192), *Pax2* (NM_003990), *Pax3* (NM_181460), *Pax4* (NM_006193), *Pax5* (NM_016734), *Pax6* (NM_001604), *Pax7* (NM_013945), *Pax8* (NM_013992), *Pax9* (NM_006194).

1.2 Biological materials

Ripe adult amphioxus *Branchiostoma belcheri*, identified according to the previous descriptions [17,18], were cultured in the laboratory, and the animals spontaneously spawned during breeding season. The fertilized eggs were raised in filtered seawater at room temperature. The embryos at different developmental stages were collected and immediately frozen in liquid nitrogen, and maintained at –80°C for RNA extraction. A portion of the samples were fixed in 4% paraformaldehyde in a MOPS buffer for ISH or immunohistochemical study.

1.3 RNA isolation

Total RNAs were extracted from samples of 16 developmental stages (with time after fertilization): 1 h embryo (2–4 cells), 2 h morular (32 cells), 3 h morular (64–128 cells), blastula (4 h), gastrula (5 h), neurula (10 h), 1-d larva (1 d), 2-d larva, 4-d larva, 15-d larva, late larva (at the beginning of metamorphosis, about 23-d with 11 gills), early juvenile (during metamorphosis, about 30-d with 12 gills), mid-juvenile (at the end of metamorphosis, about 40-d with full-grown hepatic diverticulum), late juvenile (without gonads, about 2-months), and female/male adult (about 1-year). In addition, we dissected late juveniles into four areas (Figure 1): anterior area, pharyngeal area, diges-

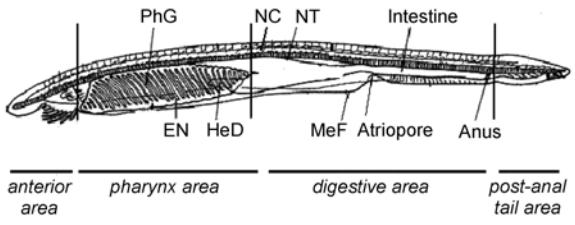

Figure 1 A diagram of juvenile amphioxus indicating the four segmentation areas (modified from R. Fox, 2001, http://webs.lander.edu/rsfox/invertebrates/branchiostoma.html).

tive area and post-anal tail area, and adult animals into eight organs: pharyngeal gill, metapleural fold, segmental muscle, hepatic diverticulum, intestine, notochord, ovary and testis. All of those samples were separately homogenized in TRNzol-A$^+$ reagent (Tiangen Co. China) for RNA extraction according to the manufacturer's instructions.

1.4 Real-time quantitative PCR

Total RNAs were reverse-transcribed to cDNAs using AMV Reverse Transcriptase XL (TaKaRa Co. Japan) in a 20 μL reaction volume, and RTqPCR was performed using SYBR Green for the detection of fluorescence during amplification. Each RTqPCR reaction contained 10 μL of SYBR Green PCR master mix (TaKaRa Co. Japan), 10 μmol L^{-1} forward and reverse primers each, and 1 μL of first strand cDNA mixture in a 20 μL volume. The reactions were carried out in a Rotor-Gene 3000 thermal cycler (Corbett Robotics, Australia), and conditions were 94°C for 1 min predenaturalization, followed by 40 cycles of 94°C for 15 s, 57°C for 15 s, and 72°C for 15 s, and detected at 84°C for 8 s. The melting curve analysis was performed to check the presence of non-specific PCR products and primer dimers. For each assay, the reaction was repeated at least 3 times. Cycle number (C_T) values were scaled at the same standard and related to copy numbers using the $2^{-\Delta\Delta C_T}$ method [19]. Normalization was performed using *β-actin* as an internal control. The primers sequences and related information are listed in Table 1.

1.5 *In situ* hybridization

For *in situ* hybridization, we made transverse frozen sections (14 μm) of adult amphioxus. After drying for 10 min at 50°C, the sections were fixed in 4% PFA (confecting with PBS) for 5 min, washed with 1% Triton-100 (confecting with PBS) and incubated in a hybridization buffer (50% formamide, 5×SSC, 1% SDS, 50 μg mL^{-1} yeast tRNA, 50 mg mL^{-1} heparin sulfate, 5 mmol L^{-1} EDTA) for 10 min. Hybridization was performed in a freshly prepared hybridization buffer containing a 2.0 μg mL^{-1} probe at 55°C overnight. After hybridization, the sections were washed in solution 1 (50% formamide, 5×SSC) for 30 min at 55°C. The solution was gradually substituted by solution 2 (0.5 mol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, pH 7.5, 0.1% Tween 20) adding RNase A with a final concentration at 50 μg mL^{-1}, and incubated for 30 min at room temperature. The sections were washed twice in a solution containing 50% formamide and 2×SSC for 30 min each at 55°C, twice in a solution containing 50% formamide and 0.2×SSC for 30 min each at 55°C, and once in 0.2×SSC for 5 min at room temperature. For the detection of digoxigenin epitopes, the sections were further blocked with a blocking solution for 60 min and incubated with 1∶4000-diluted anti-Digoxigenin-AP Fab fragments (Roche, Switzerland) at 4°C. After overnight incubation, the sections were washed five times in a washing buffer (100 mmol L^{-1} Tris-HCl buffer, pH 7.5, 150 mmol L^{-1} NaCl) for 1–2 h each at room temperature. Alkaline phosphatase activity was detected with NBT/BCIP in a detection buffer (100 mmol L^{-1} Tris-HCl buffer, pH 9.5, 100 mmol L^{-1} NaCl, 50 mmol L^{-1} $MgCl_2$). The stained sections were postfixed in PFA/PBS and photographed. *AmphiPax1/9* digoxigenin-labeled antisense riboprobe corresponding to the 3′ UTR was prepared according to the manufacturer's instructions (Roche, Switzerland), negative control probes were prepared in the opposite direction. The primer pair was *Pax1/9* (F5): 5′-ATGTTTGTATGTCCAAATCCG-3′ and *Pax1/9* (R5): 5′-CTTGTCATTCCTTCTTCCTAC-3′. In addition, whole-mount *in situ* hybridization (WMISH) was carried out according to the methods described by Holland *et al*. [20].

1.6 Immunohistochemistry

The paired domain of *AmphiPax1/9* (*AmphiPax1/9*-PD) cDNA sequence was cloned and expressed in *E. coli* BL21-Codon Plus (DE3)-RIL. Mouse *AmphiPax1/9*-PD polyclonal antiserum was generated using the recombinant protein as an antigen. To prepare an antigen column for affinity purification of *AmphiPax1/9*-PD specific antibody, recombinant His-tagged *AmphiPax1/9*-PD was purified by Ni^{2+}-charged chelating chromatography (Qiagen Co. China).

AmphiPax1/9 immunostaining was carried out on trans-

Table 1 The list of primers for real-time quantitative PCR

Gene name	Primer sequence (5′–3′)	Size of PCR products	Annealing temperature
β-actin	F: ACAACCACCGCTGAGAGGGAAAT R: CGAGGAAGGAAGGCTGGAAGAG	202 bp	58°C
AmphiPax1/9	F1: AGCCAGCTCCCCATGCACA R1: TGCGTCTGATACCTTGCACT	155 bp	
AmphiPax2/5/8	F2: ACCACGAGTGTAGCGATGAC R2: CCCGTCTGTCCTGATAGGTT	179 bp	
AmphiPax3/7	F3: GCCGCGCATGCTCAGTCCTA R3: AGACTCGTCCCGGTACTGCC	140 bp	
AmphiPax6	F4: CAGTCCGCAGACCCGCAGAT R4: TGATAGCCGTGCAGGGTCAA	191 bp	

verse frozen sections that were first bleached in 0.3% H_2O_2 confected with methanol for 30 min, washed in PBS, blocked by 10% normal goat serum confected with PBS for 60 min and incubated in 1∶25 antiserum diluted with the blocking solution. After incubating slides with secondary antibody (peroxidase-conjugated goat-anti mouse IgG) for 60 min, AEC (DAKO Co. China) was used as chromogen to incubate sections. This reaction was stopped when a uniform red color first became visible.

2 Results

2.1 Phylogenetic relationship of *Pax* genes

Sequence comparison revealed that four amphioxus *Pax* genes were about 90% similar to their vertebrate orthologs in the paired domain consistent with previous reports [10–13]. Based on the sequence data of paired domains downloaded from a public database, we reconstructed a phylogenetic tree with *Pseudomonas* transposase (*PsTrans*) as an out-group (Figure 2). The tree displayed that all *Pax* genes of vertebrates are divided into four subfamilies and four amphioxus genes are separately clustered together with each subfamily. Usually, the branches of two protostome (fruitfly and nematode) genes located at the base of each subfamily, and *AmphiPax* genes (*AmphiPax1/9, AmphiPax2/5/8, AmphiPax3/7*, except *AmphiPax6*) are between those of protostomes and vertebrates. This suggested that *AmphiPax* genes might respectively represent ancestral states of four vertebrate *Pax* subfamilies.

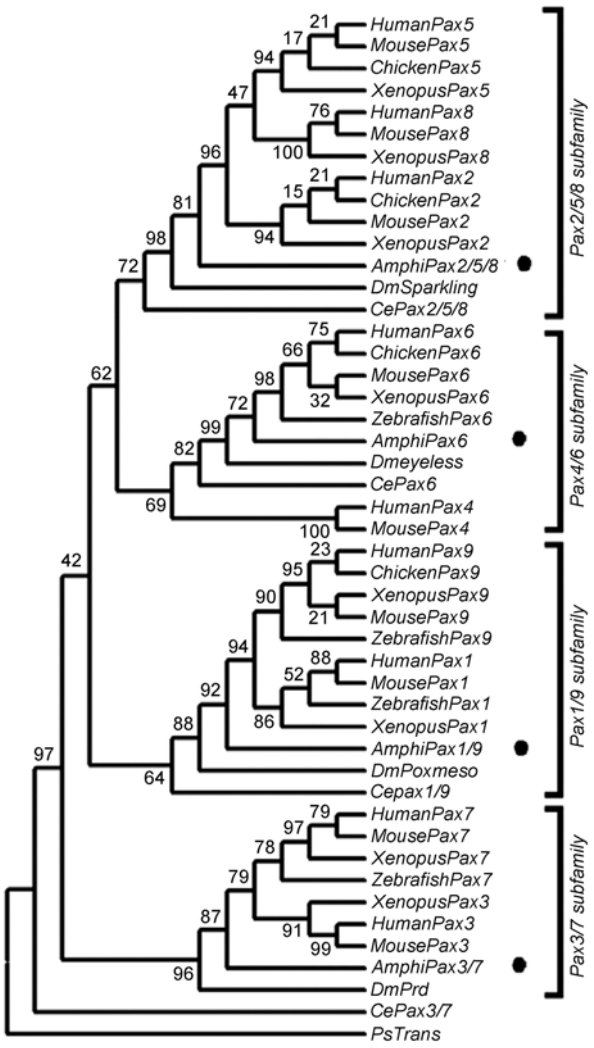

Figure 2 Phylogenetic analysis of *Pax* genes. A neighbor-joining tree based on the comparison of amino acid sequences of paired domains using the *Pseudomonas* transposase sequence as an out-group. Numbers indicate the percentage of times that a given node was supported in 1000 bootstrap replications. The four subfamilies were classified by cluster, and four amphioxus genes were denoted with black rotundities.

2.2 *AmphiPax* gene expressions at different developmental stages

Using RTqPCR, we examined the temporal expressions of four *AmphiPax* genes at 16 different developmental stages throughout the amphioxus life. The examinations generated clear dynamic expression patterns of *AmphiPax* genes at specific developmental stages.

AmphiPax1/9 was expressed throughout the life, but the expression level markedly varied and displayed 3 peaks at certain developmental stages (Figure 3). During early embryonic development, the expression was slightly detectable in the 1 h embryo and rapidly increased forming the first expression peak in the 2 h morular embryo. It decreased at the stages of the 3 h morula and blastula. After that, the gene expression rose again until the neurular embryo, peaking at about 5%. Following this high expressing phase, there was a gradual decrease from 1-d to 2-d larvae and a significant decline at 4-d larva. The low expression level remained until the late larval stage when the expression of *AmphiPax1/9* strikingly rose again. After the late larval stage, the expression continuously increased and reached a third peak at mid-juvenile. This high expression level was maintained in the late juvenile and adult, and no significant difference between male and female adults was detected.

The expression of *AmphiPax2/5/8* displayed marked changes and presented 3 peaks at specific developmental stages, but was slightly different from that of *AmphiPax1/9* (Figure 3). In early embryonic development, no clear signal of *AmphiPax2/5/8* was observed until the blastula embryo. Soon after that, the expression level significantly increased, and hit the first expression peak at the gastrula stage. After a slight decrease at the neurula, the expression amount gradually increased and reached a second expression peak at the 2-d larva, before evidently decreasing at the 4-d and 15-d larvae. As development progressed, the gene expression levels showed a distinct rise at the late larva and early juvenile, followed by a sharp decrease at the mid-juvenile. Subsequently, a gradual decrease occurred during the later de-

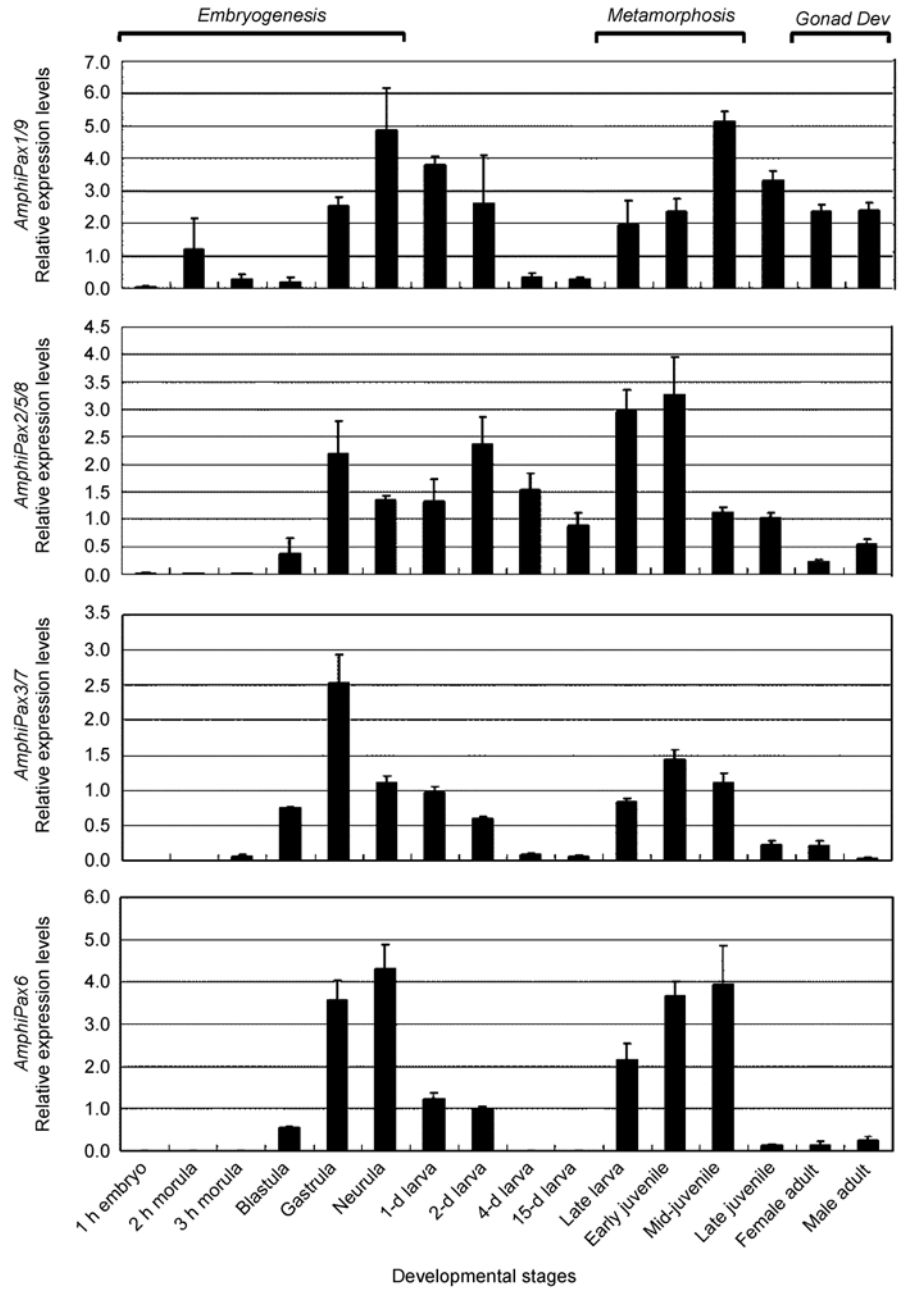

Figure 3 Temporal expression patterns of *AmphiPax* genes. Relative expression levels of four *AmphiPax* genes at 16 different developmental stages throughout the amphioxus life cycle were evaluated by real-time quantitative PCR. The three developmental phases, namely embryogenesis, metamorphosis and gonad development (Gonad Dev), are indicated above the histogram.

velopment stages. Although the signal was relatively weak in adult amphioxus, the expression level in the male was more than 2-fold higher than that in the female.

AmphiPax3/7 displayed an undulatory expression pattern during development (Figure 3). The expression of this gene was first detected at the 3 h embryo with a very weak signal. Soon after, it was clearly detected at the blastula embryo and quickly reached its highest level at the gastrula embryo. After a significant fall at the neurula embryo, the expression continuously declined and bottomed out at the 15-d larva. Unlike this low expression phase, the gene expression increased again at the late larva and attained a second peak at the metamorphic juvenile. A little lower, but nonetheless distinguishable expression appeared at the mid-juvenile when the metamorphosis was completed. Subsequently, the gene expression rapidly decreased at the later juvenile. *AmphiPax3/7* was expressed much more in female adults than in males.

Similar to *AmphiPax3/7*, *AmphiPax6* also displayed two separated expression peaks at embryogenesis and

metamorphosis (Figure 3). The initial expression of *AmphiPax6* occurred at the blastula stage, and the expression level significantly increased and peaked at the neurula stage. A similar trend was also observed at later larval development, *i.e.* the expression level gradually diminished until it reached a very low level at the 4-d and 15-d larvae. Strikingly, the gene expression jumped up at the late larva, continuously increased at the early juvenile, and finally attained the highest peak at the mid-juvenile. However, it sharply dropped at the late-juvenile and maintained low expression levels during the later developmental stages.

2.3 *AmphiPax* gene expressions in different organs

Spatially, RTqPCR revealed that *AmphiPax1/9* was intensively expressed in the pharynx area and weakly in the post-anal tail area, digestive area and anterior area of juveniles (Figure 4A). Further examination of the gene expression in eight dissected organs of adult amphioxus indicated that the pharyngeal gill (PhG) had a maximum expression level of approximately 20%. However, the expression amounts in the metapleural fold (MeF), intestine and segmental muscles (SeM) ranged between 3% and 5%, and that in the hepatic diverticulum (HeD), notochord (Nt), ovary and testis was less than 1% (Figure 4B).

The detection of *AmphiPax2/5/8* expression in four areas was also shown to be much higher in the pharynx area, followed by the anterior, post-anal tail areas and digestive area (Figure 4A). In adults, the highest expression organ was PhG. Less but nonetheless evident expressions were detected in the Nt, SeM, MeF, testis and intestine, whereas no signals of the gene expression were observed in HeD and ovary (Figure 4B).

AmphiPax3/7 showed the highest expression peak in the digestive area, and lower ones in the post-anal tail and ante-

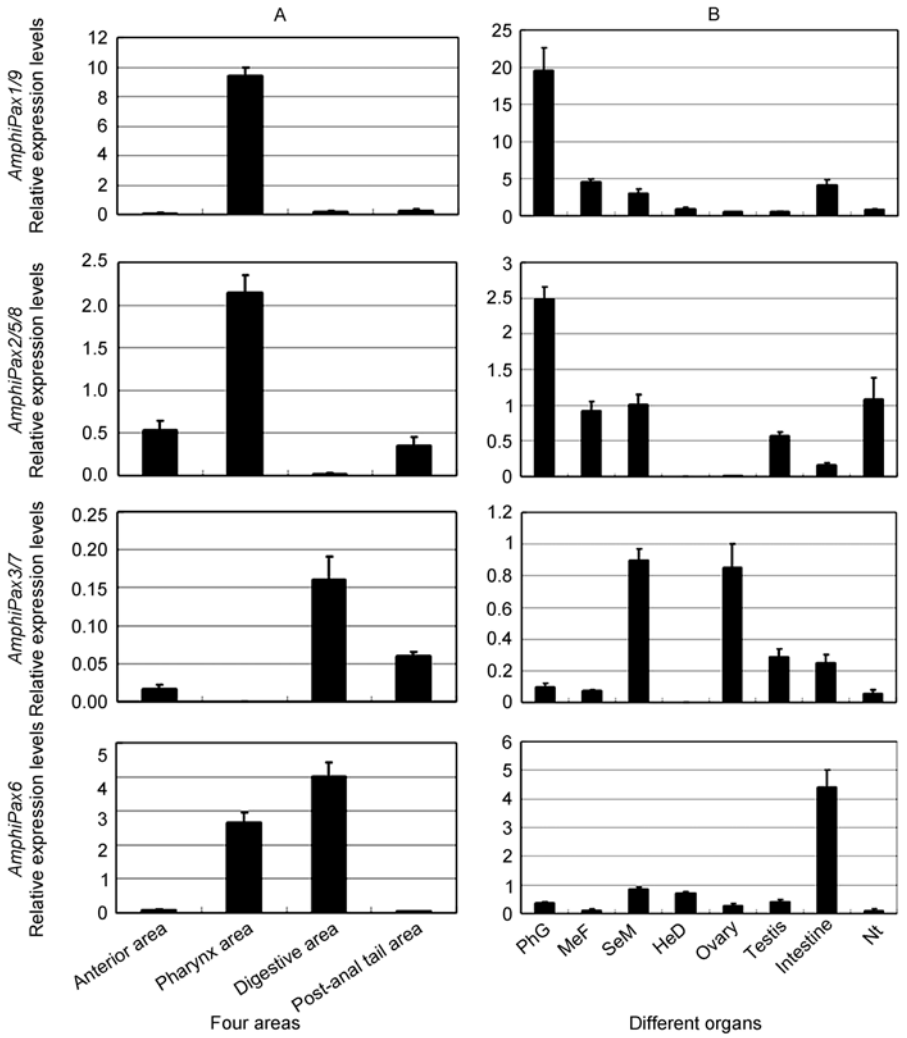

Figure 4 Spatial expression patterns of *AmphiPax* genes. A, Relative expression levels of the genes in four areas at the metamorphic juvenile: anterior area, pharyngeal area, digestive area, tail area. B, Eight different organs of adult amphioxus: PhG, pharyngeal gill; MeF, metapleural fold; SeM, segmental muscles; HeD, hepatic diverticulum; Ovary; Testis; Intestine; Nt, notochord.

rior areas, but no signal in the pharynx area (Figure 4A). Among the eight detected organs, SeM showed the highest expression of *AmphiPax3/7* and the ovary somewhat lower. The gene expression was also observed in the testis, intestine, PhG, MeF and NT, but not in HeD (Figure 4B).

The highest expression level of *AmphiPax6* was detected in the digestive area and slightly lower in the pharynx area. By contrast, the gene expression level was quite low in the anterior and post-anal tail areas (Figure 4A). In adults, this gene showed an extraordinarily high expression in the intestine, somewhat less in SeM and HeD, and slight expressions in other tissues (Figure 4B).

Considering the highest expression level of *AmphiPax1/9* among the above four genes, we performed *in situ* hybridization to further assay the gene expression in different tissues. At the early development stage, ISH results indicated that *AmphiPax1/9* was expressed in the anterior pharyngeal area (p) and the posterior end of the larva (Figure 5A), which is similar to previous reports [10,14]. The sections of the adult pharyngeal area also displayed purple ISH staining in cells around the gill bar (GB) and zone 2 of the endostyle (Figure 5E–H). Immunohistochemical staining of AmphiPax1/9 protein also showed signals in the mesenchymal cells of MeF (Figure 5C and D). However, the gonads and HeD did not clearly show the expression signal (Figure 5I). These expression patterns indicated that *AmphiPax1/9* was expressed in the mesenchyme and was a key factor in PhG development throughout the life cycle of amphioxus.

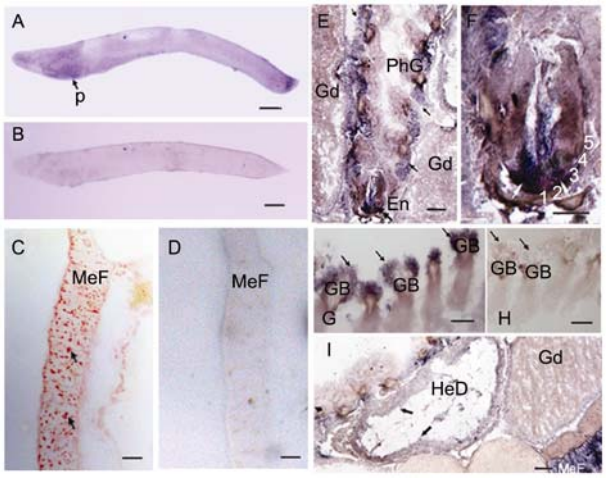

Figure 5 Expression patterns of *AmphiPax1/9* detected by *in situ* hybridization. Anterior of the entire mounts was towards the left. A, Lateral view of the embryo at the latter neurula. *AmphiPax1/9* expression was observed in the pharyngeal area (p) and the posterior end area of the embryo. B, Embryo hybridized with sense probes. C–I, Expression patterns of *AmphiPax1/9* at adult amphioxus. C, *AmphiPax1/9* immunostaining reveals the signal in the mesenchymal cells represented in the dermal fibroblasts of MeF, compared with the control (D). E, The pharyngeal gill area. Arrows indicate the expression in the pharyngeal gill (PhG). Tandem arrows indicate the expression of *AmphiPax1/9* in the endostyle (En). F, Higher magnification of the endostyle which was classified with zone 1–5. The purple signal was presented in zone 2 with a white arrow. G, Higher magnification of the pharyngeal gill (PhG) area showing the purple signal (arrows) for *AmphiPax1/9* within cells surrounding the gill bar (GB). H, Higher magnification of a control section treated with a sense probe. There was no purple label (arrows) in the cells surrounding the gill bars (GB). I, Higher magnification of the hepatic diverticulum (HeD) area. No signal was shown in the hepatic diverticulum (arrows) and gonads (Gd). Scale bars: A and B=25 μm. C–I=50 μm.

3 Discussion

3.1 The expression of *AmphiPax* genes in amphioxus

Transcriptional regulators are often variably expressed at different developmental stages relating to certain effects [21]. The dynamic expression patterns of *AmphiPax* genes are attributed to their different roles at different developmental stages or organs/tissues.

During the early development of amphioxus, all *AmphiPax* genes are highly expressed from the gastrula to the neurula stage, suggesting that the genes were strongly activated when several significant changes (including the development of the neural plate, somite and pharynx) were occurring [22,23]. Notably, the highest expression of *AmphiPax2/5/8* at the 2-d larva was related to the organogenesis of the endostyle [11]. After this period, the larvae retained the body plan relatively steadily with 3 gill slits in the pharynx area from 4-d to 15-d larvae, and the expressions of *AmphiPax* genes decreased and maintained low levels in the stages.

However, the expression levels of *AmphiPax* genes significantly increased again up to a relatively high level after 15 d when the larvae underwent metamorphosis. There were several fundamental changes occurring to establish the more nearly symmetrical body plan of adults, including (a) gill slits, (b) primary pigment spots, (c) hepatic diverticulum, (d) atrium or metapleural fold, (e) mouth and buccal cirri and (f) the number of SeM reaching the maximum [18,19]. The high gene expressions in some of those tissues during the stage indicated the essential functions of those transcription factors during organogenesis.

By the late juvenile, the organs were well developed and very similar to those of adult individuals. Within this slow development stage, the expression of *AmphiPax* genes decreased once again. During subsequent gonad development, *AmphiPax2/5/8* was more highly expressed in males than in females, while *AmphiPax3/7* presented stronger signals in females than in males (Figure 3), consistent with the data gained from tissue RTqPCR analysis (Figure 4B). Thus, we presume from those observations that *AmphiPax2/5/8* and *AmphiPax3/7* play different roles in testical and ovarian development.

3.2 Conserved expression patterns of *AmphiPax* and vertebrate homologues

Comparison of orthologous gene expression patterns between cephalochordates and vertebrates yields much insight

into the evolution of the chordate body plan and organogenesis. Thus we further compare the expression patterns of *AmphiPax* genes to those of vertebrate homologues in the present study.

3.2.1 *The expression of the Pax1/9 subfamily*

The results of *AmphiPax1/9* immunostaining showed the signals in mesenchymal cells represented by dermal fibroblasts in adult amphioxus (Figure 5C and D). In vertebrates, expressions of *Pax1* and *Pax9* seem to co-opt more areas along the rise of the mesenchyme. Peters *et al*. [24] reported that mammalian *Pax9* was expressed in the mesenchyme including craniofacial, teeth, and limb development. Wallin *et al*. [25] found that *Pax1* transcripts were confined to the mesenchyme of the intervertebral disc. The present results demonstrated that *AmphiPax1/9* was expressed in the intestine, a homolog to the esophagus (stomach, small and large intestine) where vertebrate *Pax9* was expressed [26]. The pharynx expression of *AmphiPax1/9* was maintained to the overlapping expression of *Pax1* and *Pax9* in the pharyngeal pouches (PhP) of vertebrates [27].

3.2.2 *Conserved roles of Pax2/5/8 subfamily genes*

It was documented that the vertebrate *Pax8* gene was expressed in the adult thyroid indicating the association with the organic function [28]. Hiruta *et al*. [29] demonstrated the expression of *AmphiPax2/5/8* in the adult amphioxus endostyle, a homolog to the thyroid of vertebrates. Our examinations revealed that *AmphiPax2/5/8* was highly expressed in both the pharynx area of metamorphic juveniles and in PhG during the adulthood of amphioxus, which further supported the previous observations. We found that *AmphiPax2/5/8* was expressed in the testis and tail. Similarly in vertebrates, early discoveries disclosed the expression of *Pax2* in the vas deferens and epididymis [30,31], and the expression of *Pax5* in both the testis and chordoneural hinge region of the tail [32,33]. Integratively considering all of these observations, we presume that *AmphiPax2/5/8* is a multi-function gene in amphioxus and it is subfunctionalized after gene duplication in vertebrates. Each daughter gene in the vertebrate conserves partial roles of their parental gene in the ancestor (Figure 6).

3.2.3 *Pax3/7 subfamily genes in the myotome and intestine*

In vertebrates, *Pax3* was not expressed in adult muscle although it was crucial for the skeletal muscle development in embryos [34,35] and, by contrast, *Pax7* was expressed in adult satellite cells [36]. Our data showed the expression of *AmphiPax3/7* seemed to be mostly expressed in the embryogenesis and metamorphosis stages, and maintained the highest expression level in adult muscle (Figure 4B). Therefore,

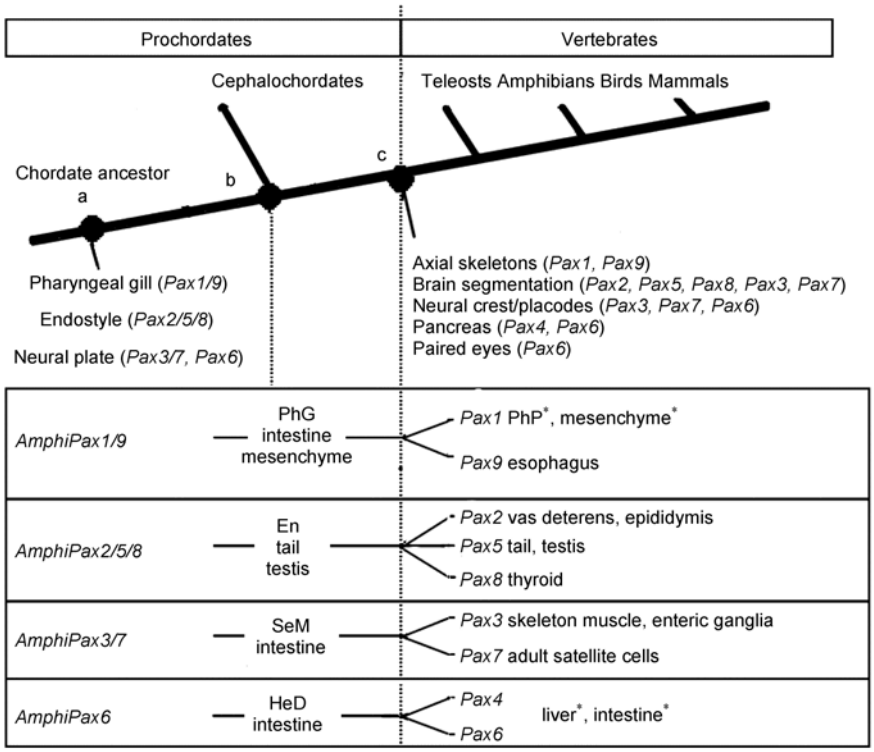

Figure 6 *Pax* genes associated with the character states for the evolution of chordate developmental functions. Because the argument has been mentioned between urochordates and cephalochordates [44], we did not present urochordates in this species-tree. Branch lengths are not drawn to scale. The cladogram indicates the probable timing for acquisition of the morphological characteristics of chordates (event 'a', 'b' and 'c') relating to *Pax* genes marked in parentheses (above), and the evolution of the *Pax* role is indicated (below). Asterisks indicate the expression patterns are overlapping between subfamily members. PhG, pharyngeal gill; PhP, pharyngeal pouches; En, endostyle; SeM, segmental muscles; HeD, hepatic diverticulum.

we assume that the ancestral genes of *Pax3* and *Pax7*, like *AmphiPax3/7*, possibly possess functions both in myogenesis during the embryonic stage and in adult muscle tissue. This diplex function is respectively assigned into *Pax3* and *Pax7* in vertebrates.

Our data also indicated that *AmphiPax3/7* was clearly expressed in the intestine and ovary of adult amphioxus. The former expression was consistent with *Pax3* in the enteric ganglia formation [37] and *Pax7* in the intestine [38] of vertebrates. The gene function in the ovary might be lost in vertebrates.

3.2.4 Expression of the Pax4/6 subfamily in liver and intestine

It has been reported that vertebrate *Pax4* and *Pax6* genes were expressed in the developing pancreas and intestine [39], as well as in the liver of *Xenopus* tadpoles [40]. Similarly, our results indicated that *AmphiPax6* was significantly expressed in the amphioxus intestine and HeD (Figures 4 and 5). It is reasonable to conclude that the ancient functions of the *Pax4/6* subfamily are associated with the intestine and HeD and that these functions have been inherited in vertebrates. After gene duplication, the members of the subfamily are endowed with new functions, *e.g.* pancreas development.

From the above discussion, we summarize a scenario concerning the ancestral role of the protochordate *Pax* genes and the conserved function of the vertebrate *Pax* genes in Figure 6. Because both cephalochordate *AmphiPax* and urochordate *Pax* homologs were expressed in the PhG, EN and neural plate [29,41–43], it was possible that *Pax* genes possessed those common expression patterns of an early chordate ancestor (event 'a') and that *AmphiPax* genes were also expressed in cephalochordates (event 'b'): *AmphiPax1/9* in the mesenchyme, *AmphiPax2/5/8* in the tail and testis, *AmphiPax3/7* in the SeM and intestine, and *AmphiPax6* in the HeD and intestine. Therefore, our study of the *Pax* gene family in the amphioxus life cycle provides more information concerning the comparable expression pattern between amphioxus and vertebrates, supporting the hypothesis that duplicated *Pax* genes maintained most of their ancestral functions but also acquired various novel functions along with the complexity of the organ systems (event 'c') after the divergence of protochordates and vertebrates.

The authors would like to express their sincere appreciation to Professor Zhang HongWei of Shandong University for her help with ISH technology. The authors are also grateful to Ms. Lin JingWen for her excellent technical assistance and to Mr. Li Guang and Sun Yi for their helpful discussions. This work was supported by the National Natural Science Foundation of China (*Grant No. 30830023*), *the National High Technology Research and Development Program of China* (*Grant No. 2008AA092602*) *and the Specialized Research Fund for the Doctoral Program of Higher Education* (*Grant No. 20070384041*).

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