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EMBRYOGENESIS AND CANCEROGENESIS

Molecular Structure and Developmental Expression of Two Zebrafish *Ankylosis Progressive Homolog* (*ankh*) Genes, *ankha* and *ankhb*[1]

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Abstract—We isolated two zebrafish *ankylosis progressive homolog (ankh)* genes, *ankha* and *ankhb*, from embryonic zebrafish. Amino acid sequences deduced from zebrafish *ankh* genes are aligned with orthologue proteins from other species, the results showed that they share high percentage of identities (74–82%). Whole-mount in situ hybridization experiments showed that *ankha* and *ankhb* are maternal inherited genes which can be detected at 1-cell stage embryos and express in the entire animal pole from 6 hours post-fertilization (hpf) to 12 hpf. At the later stages (from 24 hpf to 3 dpf), expression of *ankha* was restricted in head region. In contrast, transcripts of *ankhb* were observed in head, gut, and pharyngeal arches. In conclusion, the present studies not only help us to comparatively analyze *ankh* genes across species, but also provide useful information about expressions during early embryogenesis that will help in further investigations of functional studies of Ankh in the future.

Keyword: *ankha* and *ankhb*, zebrafish, embryogenesis

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INTRODUCTION

Inorganic pyrophosphate (PPi) exists in extracellular fluids (ex: synovial fluid, blood plasma, urine) and plays important physiological roles during bone calcification and mineralization, such as blockage of calcification (Ryan 2001). Deficient PPi promotes pathologic mineralization with basic calcium phosphate (BCP) crystals whereas excess PPi causes calcium pyrophosphate dihydrate (CPPD) crystals accumulation (Altman et al., 1973; Silcox and McCarty, 1974; Ryan 2001). Accumulation of both BCP and CPPD crystals causes serious pathological defects, especially on articular tissue (Ho et al., 2000; Rutsch et al., 2001). Thus, balance of PPi concentration is a crucial factor to keep normal function of bone and joints.

Extracellular/intracellular PPi shuttling is controlled by a membrane protein, ankylosis progressive homolog (Ankh), which is encoded by *ankh* gene (Ho et al., 2000). The molecular structure of *ankh* genes has been determined in frog (Nürnberg et al., 2001), chicken (Wang et al., 2005) and mammals (Hakim et al., 1986; Hughes et al., 1995; Ho et al., 2000; Zimin et al., 2009). In zebrafish, two *ankh* genes, *ankha* and *ankhb*, are reported (Ho et al., 2000; Strausberg et al., 2002), but their expression information during early embryogenesis are still limited.

As it might be expected, mutation on *ankh* gene induces calcification disorder or other pathological defects. For example, craniometaphyseal dysplasis (CMD), a rare inherited disorder of bone in human, is due to *ANKH* mutation (Nürnberg et al., 2001). The clinical signs and symptoms of CMD include overgrowth and sclerosis of the craniofacial bones and abnormal modeling of the metaphyses of the tubular bones (Nürnberg et al., 2001). In mice, inactivation of ANK leads to generalized, progressive form of arthritis accompanied by mineral deposition, formation of bony outgrowths, and joint destruction (Ho et al., 2000). In zebrafish, two *ankh* genes, *ankha* and *ankhb*, are identified but mutation in zebrafish *ankh* genes has not discovered thus far.

To elucidate the physiological functions of *ank* genes during early embryogenesis, it is worthy to analyze comparatively *ank* genes across species. Here, we report the spadiotemporal expressions of two zebrafish *ank homolog* (*ankh*) genes by whole mount in situ hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) experiments. This gene expression data will provide more insight into the functional studies of the lower vertebrate *ankh* genes.

[1] The article is published in the original.

MATERIALS AND METHODS

Fish Embryos Staging

Mature zebrafish (AB strain) were raised at the zebrafish facility of the Life Sciences Development Center, Tamkang University. The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark, in an aquarium supplied with freshwater and aeration (Chen et al., 2009; Wang et al., 2009a). Embryos were produced using standard procedures (Westerfield, 1995) and were staged according to standard criteria: hours postfertilization, hpf; or days postfertilization (dpf; Kimmel et al., 1995).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We corrected 100 embryos per stage to extract their total RNA. RNA isolation and first-stand cDNA synthesis procedures were according to the previous report with minor modification (Chen et al., 2001; Wang et al., 2009b; Lai et al., 2011). Primer sets (ankha-F: 5'-GGGAGCCCTTGTGCGATTCACT-3', ankha-R: 5'-TGGCATGATGCAGAGCTCTGCGA-3'; ankhb-F: 5'-GAACAATGGAGAAGCCGTCAGCA-3', ankhb-R: 5'-ACGACCATACAGAGCACCGCT-3'; and β-actin-F: 5'-GTCCCGTACGCCTCTGGTCG-3', β-actin-R: 5'-GCCGGACTCATCGTACTCCTG-3') were designed based on the sequences encoding of putative zebrafish *ankha*, *ankhb*, and β-actin.

Database Searches and Phylogenetic Analysis

Database searches were carried out using the Blast program at the National Center for Biotechnology Information (Altschul et al., 1997). According to above procedures, cDNA clones encode Ankha and Ankhb were cloned and amplified from embryonic zebrafish mRNA. The presumptive Ankha and Ankhb amino acid sequences were determined with the Wisconsin Sequence Analysis Package v. 10.0 (GCG). The Gap program of that package was used for pair comparisons, and the Pileup and Prettybox programs used for multiple comparisons. ExPASy ProtParam tool (http://expasy.org/tools/protparam.html) was used to predict the pI and MW of Ankh. The Clustalw molecular evolution genetic program was used for our phylogenic tree analysis (http://www.ebi.ac.uk/clustalw/).

Whole Mount in situ Hybridization, Cryosection and Images

The procedures for whole mount in situ hybridization, and cryosection have been described previously (Pai and Chen, 2010; Peng et al., 2010; Lee et al., 2011), except that *ankha* and *ankhb* (this study) were used as probes. They were digoxigenin (DIG)-labeled, after we cloned their partial DNA fragment. For image analysis, all embryos were observed under a microscope (DM 2500, Leica, Germany) equipped with Nomarski differential interference contrast optics (Kramer Scientific) and a digital camera (Cannon, Japan).

RESULTS AND DISCUSSION

Comparison of Deduced Amino Acid Sequences

By searching GenBank, we found two putative zebrafish ankh sequences (*ankha*: NM_001030259, and *ankhb*: NM_194370). The deduced amino acid sequence of zebrafish Ankha revealed a 496-amino acid polypeptide, whereas the deduced amino acid sequence of zebrafish Ankhb revealed a 501-amino acid polypeptide (Fig. 1). The zebrafish Ankha/Ankhb polypeptide shares sequence identities of 74–82% of the reported Ankh of human, bovine, mouse, rat, chicken, *Xenopus*, medaka and fugu. In addition, we used the Clustalw program to determine the phylogenic similarities between zebrafish Ankha/Ankhb and that of other known species. The phylogenic tree generated by the program showed that zebrafish Ankha/Ankhb (79%) was more closely related to medaka and fugu's Ankh than those from higher vertebrates (data not shown). Moreover, *ankha/ankhb* gene transcripts from selected vertebrates and their molecular features are summarized in table. These data reveal that most Ankh proteins have alkaline pIs (7.61–8.27), but medaka Ankh and zebrafish Ankha/Ankhb proteins share acidic pIs (6.42–6.97).

Developmental Expression of Zebrafish ankha and ankhb

Next, we determined the developmental expressions of zebrafish *ankha* and *ankhb* by RT-PCR, and results revealed that endogenous *ankha* expressed from 6 hpf to 7 dpf, and *ankhb* expressed from 1-cell (0 hpf) to 7 dpf (Fig. 2). Although RT-PCR products of *ankha* were undetectable at 1-cell (0 hpf), faint signals were detected by nested RT-PCR analysis (data not shown). These observations indicated that zebrafish *ankha* and *ankhb* are maternal inherited genes.

Spatiotemporal Expression of Zebrafish ankha Transcripts during Early Development

To determine the spatiotemporal expression patterns of *ankha* during early development, we performed whole mount in situ hybridization using a *ankha* antisense DIG-labeled riboprobe. Zebrafish *ankha* transcripts were first detected from 1-cell stage to cleavage period (Figs. 3a, 3b), and extended their expression from the gastrula period to the early segmentation stages (Figs. 3c, 3d). At 24-hpf, 36-hpf, 2-dpf, and 3-dpf, the zebrafish *ankha* transcripts were restricted to trunk and head regions (Figs. 3e–3h). Interestingly, zebrafish *ankha* transcripts were down-regulated to a

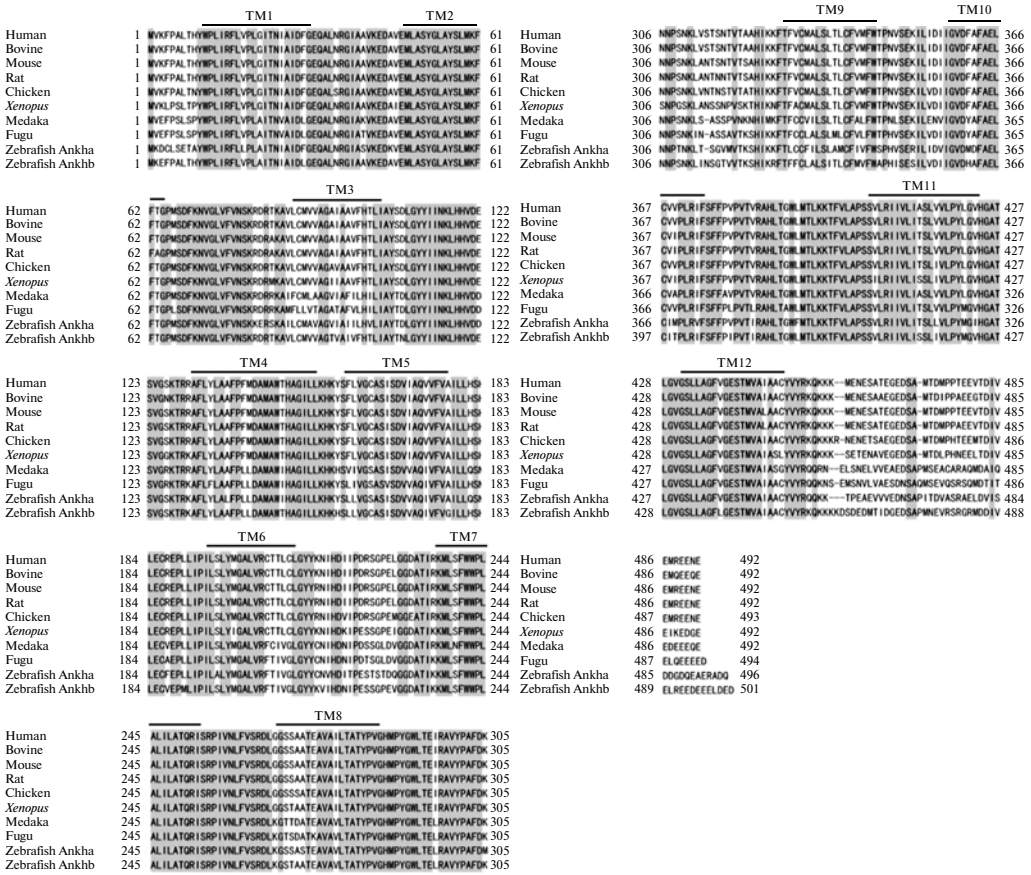

Fig. 1. Comparison of the deduced amino acid sequence of zebrafish Ankha/Ankhb with those of other known species. The information was obtained from the GenBank nucleotide sequence database and Ensembl with the following accession numbers: human (NM_054027.4), bovine (NM_001109793), mouse (NM_020332), rat (NM_053714), chicken (NM_001012562), *Xenopus* (NM_001090455), medaka (ENSORLG00000011729), fugu (ENSTRUG00000008542) and zebrafish Ankh. Amino acid residues similar to those of the zebrafish Ankha/Ankhb are presented in black. TM: transmembrane domain.

very faint level at 5-dpf (Fig. 3i), but the expressions appeared again at retina at 7-dpf (arrow, Fig. 3j). On the basis of these observations, we conclude that zebrafish *ankha* is a maternally inherited gene, restricting its expression in head and trunk at early embryonic stages.

Summary of *ankh* genes from selected vertebrates

Species, gene names	Coding region, aa	Mw, kDa	pI	GenBank accession number	References
Human *ANKH*	492	54.2	8.00	NM_054027.4	Hughes et al., 1995
Bos *ankh*	492	54.2	7.61	NM_001109793	Zimin et al., 2009
Mouse *Ank*	492	54.3	8.01	NM_020332	Hakim et al., 1986
Rat *Ankh*	492	54.3	8.01	NM_053714	Ho et al., 2000
Chicken *ankh*	493	54.5	8.27	NM_001012562	Wang et al., 2005
Xenopus ankh	492	54.0	8.02	NM_001090455	Nürnberg et al., 2001
Medaka *ankh*	492	54.2	6.42	ENSORLG00000011729	Ensembl
Fugu *ankh*	494	54.5	7.64	ENSTRUG00000008542	Ensembl
Zebrafish *ankha*	496	54.6	6.42	NM_001030259	NCBI; this study
Zebrafish *ankhb*	501	55.4	6.97	NM_194370	Ho et al., 2000; this study

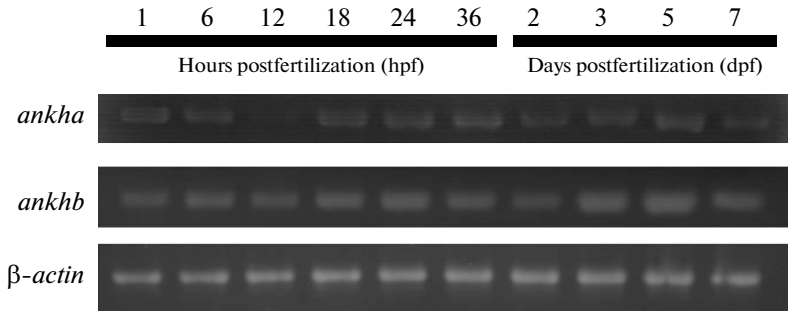

Fig. 2. RT-PCR analysis of *ankha* and *ankhb* gene transcripts, using total RNA extracted from the embryos of different developmental stages. Top panel: *ankha*; middle panel: *ankhb*; and bottom panel: loading control (β-*actin*). Stage of each sample is indicated on the top.

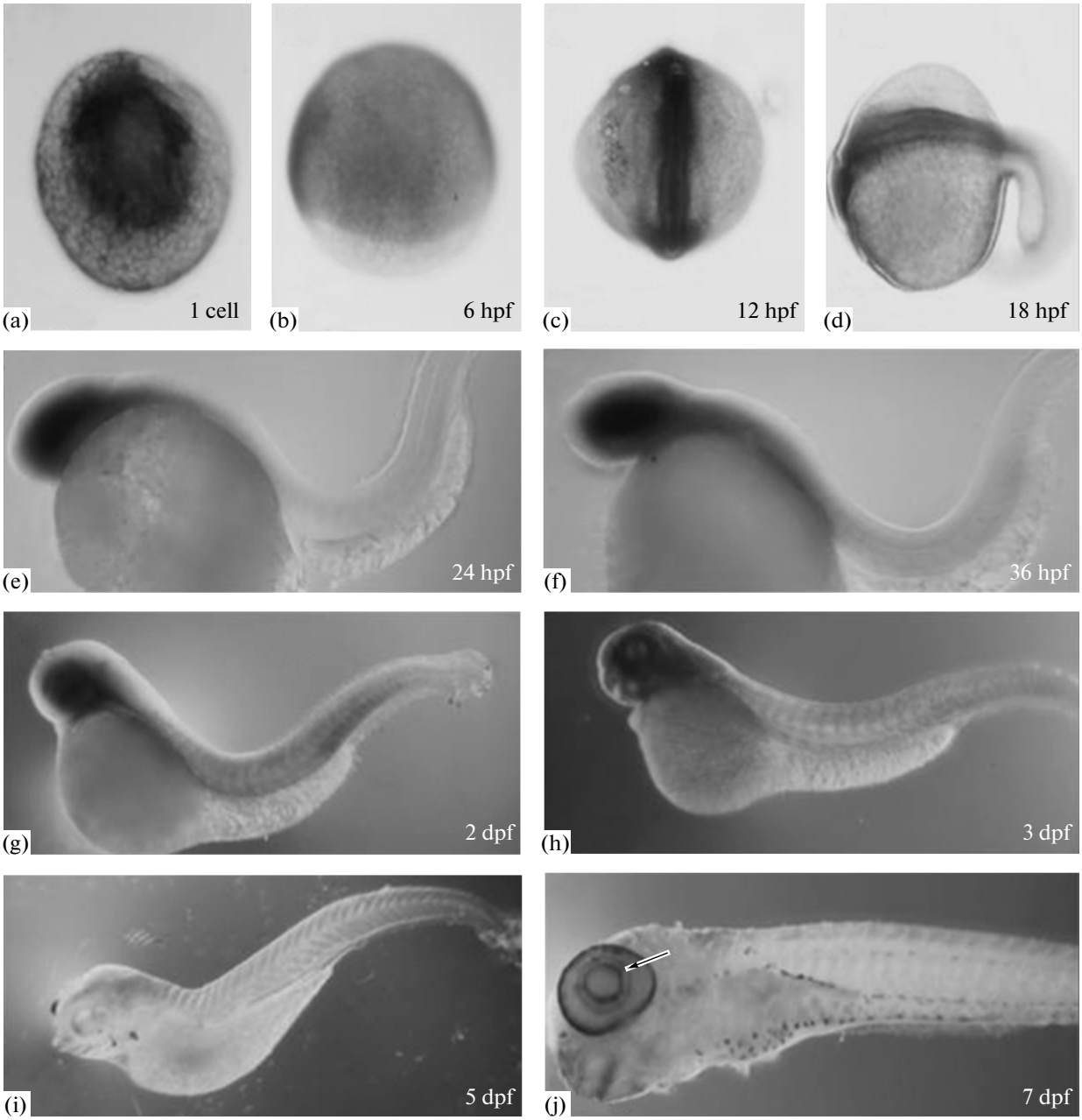

Fig. 3. *ankha* expression during early embryonic stages. (a) One-cell, top view, (b) at 6-hpf stage, lateral view, (c) at 12-hpf and (d) 18-hpf, top view, (e) at 24-hpf and (f) 36-hpf, lateral view, (g–j) at 2-, 3-, 5- and 7-dpf, lateral view.

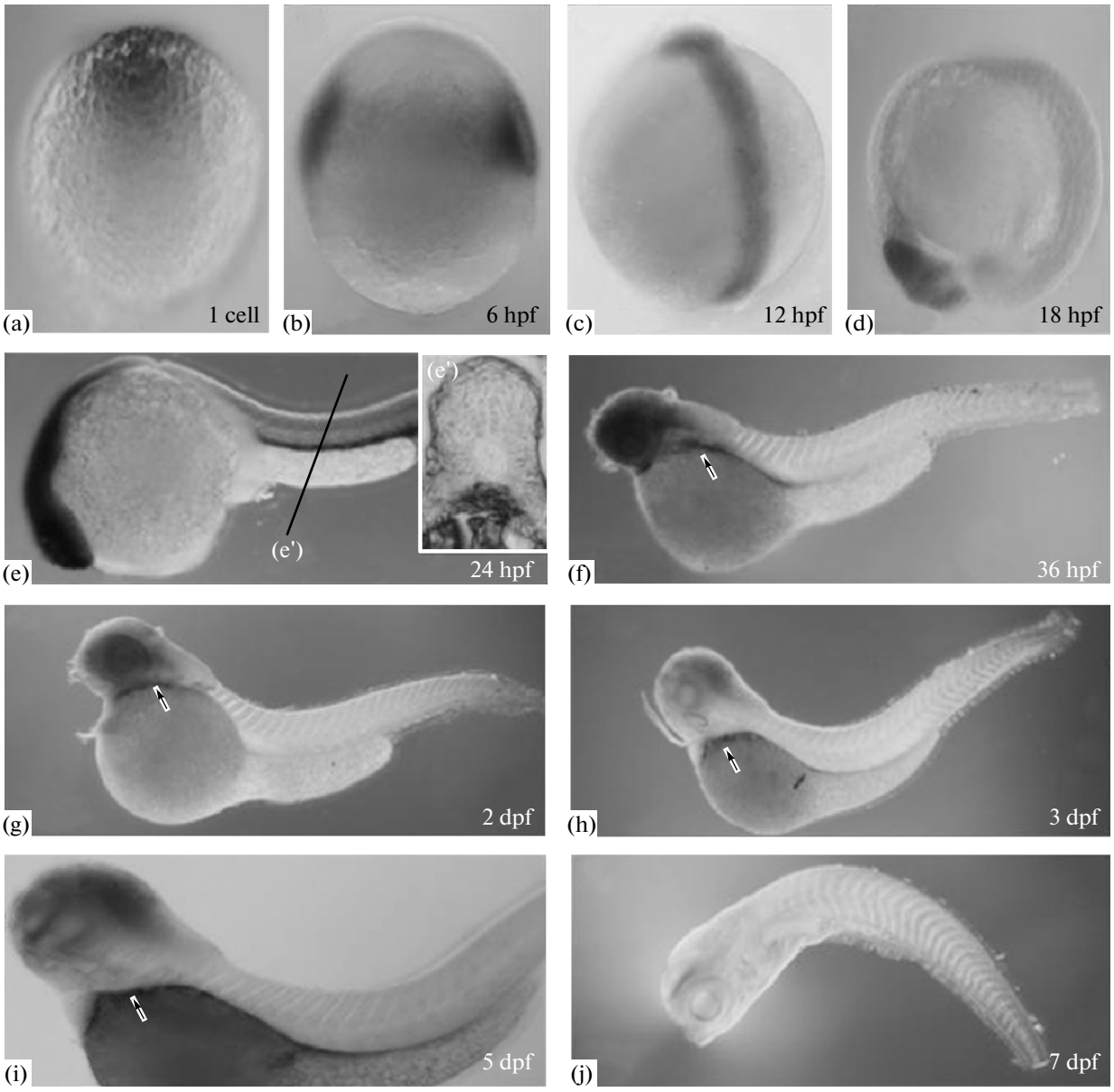

Fig. 4. *ankhb* expression during early embryonic stages. (a) One-cell, top view, (b) at 6-hpf stage, lateral view, (c) at 12-hpf, top view, (d) at 18-hpf, (e) 24-hpf and (f) 36-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (e'). (g–j) At 2-, 3-, 5- and 7-dpf, lateral view.

Spatiotemporal Expression of Zebrafish ankhb Transcripts during Early Development

Again, the spatiotemporal expression patterns of *ankhb* during early development were examined by whole mount in situ hybridization. Results showed that zebrafish *ankhb* transcripts were first observed from 1-cell stage to cleavage period and their expression extended from the gastrula period to the early segmentation stages (Figs. 4a–4c). By 18-hpf, the zebrafish *ankhb* transcripts were detected in somite, and in the head region (Fig. 4d). By 24-hpf, *ankhb* transcripts strongly expressed in the entire head region and in the guts, and that were further confirmed by cryosectioning (Figs. 4e, 4e'). Specially, we found that the expressions of zebrafish *ankhb* were strongly detected in pharyngeal arches at 36-hpf, 2-, 3- and 5-dpf (arrows, Figs. 4f–4i). By 7-dpf, no obvious signals were observed (Fig. 4j). On the basis of these observations, we conclude that zebrafish *ankhb* is a maternally inherited gene, restricting its expression in head, gut as well as pharyngeal arches.

Comparison of ankh Gene Expression Patterns between Zebrafish and Mouse

Since *ank* is a evolutionary conserved gene among different vertebrate species, it would be interesting to

compare their expression patterns between mouse and zebrafish. Though strong *ank* expression were detected in the developing mouse limbs, the *ank* mRNAs were also detected in many non-skeletal tissues of adult mouse, including heart, brain, liver, spleen, lung, muscle, and kidney (Ho et al., 2000). Inactivation of *ankh* not only leads to skeletal defects in mice and human, but also results to increased calcification in kidneys of adult mice (Storm and Kingsley, 1996; Ho et al., 2000). These observations suggest that *ankh* plays an important role in both skeletal and non-skeletal tissues. We have shown that *ankha/ankhb* transcripts were detected strongly in the presumptive head region (skeletal cell-rich), gut (non-skeletal soft tissue) and pharyngeal arches (skeletal cell-rich) of developing zebrafish embryos. These expression data are consistent with that of mouse *ANK*. Taken together, we suggest that zebrafish Ankha/Ankhb might play similar roles compare to that of mouse Ank.

In conclusion, this study highlights the distinct expression pattern of two structurally related zebrafish *ankha* and *ankhb* genes. They are both maternally inherited genes. Expression of *ankha* is mainly restricted in head region whereas *ankhb* is restricted in head region, gut and pharyngeal arches. This information may provide more insight into the molecular structure and expression patterns of the lower vertebrate *ank* genes.

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