Expression and function on embryonic development of lissencephaly-1 genes in zebrafish

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Lissencephaly is a severe disease characterized by brain malformation. The main causative gene of lissencephaly is LIS1. Mutation or deletion of LIS1 leads to proliferation and migration deficiency of neurons in brain development. However, little is known about its biological function in embryonic development. In this article, we identified the expression patterns of zebrafish LIS1 gene and investigated its function in embryonic development. We demonstrated that zebrafish consisted of two LIS1 genes, LIS1a and LIS1b. Bioinformatics analysis revealed that LIS1 genes were conserved in evolution both in protein sequences and genomic structures. The expression patterns of zebrafish LIS1a and LIS1b showed that both transcripts were ubiquitously expressed at all embryonic developmental stages and in adult tissues examined. At the protein level, the LIS1 products mainly exist in brain tissue and in embryos at early stages as shown by western blotting analysis. The whole-mount immunostaining data showed that LIS1 proteins were distributed all over the embryos from 1-cell stage to 5 day post-fertilization. Knockdown of LIS1 protein expression through morpholino antisense oligonucleotides resulted in many developmental deficiencies in zebrafish, including brain malformation, circulation abnormality, and body curl. Taken together, our study suggested that zebrafish LIS1 plays a very important role in embryonic development.

Keywords LIS1; expression; embryonic development; zebrafish

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Introduction

Lissencephaly is a rare brain malformation disease caused by defects in neuronal migration [1]. Classical lissencephaly has two major types: Miller–Dieker syndrome (MDS) and isolated lissencephaly sequence (ILS). All MDS and some cases of ILS have haploinsufficiency at human chromosome 17p13.3, from where LIS1 (Lissencephaly-1) gene was isolated [2]. Mutation or deletion of LIS1 leads to proliferation and migration deficiency of neurons in brain development [3]. Thus, LIS1 is the main causative gene responsible for classical lissencephaly.

Human LIS1, which is highly expressed in brain, encodes a protein of 45 kDa in size. LIS1 protein contains a Lis-H domain at its N-terminus, followed by a coiled-coil region and seven WD repeats. LIS1 functions as a dimer in vivo. Lis-H domain and the coiled-coil region are believed to be important for LIS1 dimerization, stability, and localization [4,5], while WD repeats are required for protein interactions, through which LIS1 interacts with a variety of proteins, such as tubulin [6], dynein [7], dynactin [7], CLIP-170 [8], DCX [9], and Nudel [10], conferring its influences on neuronal migration and mitotic cell division. LIS1 was also identified as a subunit of platelet-activation factor (PAF) acetylhydrolase [11], which regulates the physiological activity of PAF.

Although human LIS1 is a brain disease-caused gene, it is much conserved in evolution. The LIS1 gene has also been identified in other species besides human, such as budding yeast [12], Aspergillus nidulans [13], nematode worm [14], fruit fly [15], chick [16], and mouse [17]. Zebrafish (Danio rerio) LIS1 genes have also been cloned, named LIS1a and LIS1b, and a function of zebrafish LIS1 in positioning cell nucleus in photoreceptors was found [18]. However, a basic understanding of LIS1 genes is lacking in zebrafish. Because zebrafish has emerged as an important vertebrate model system in development, owing to its unique advantages,
such as production of large amounts of transparent eggs and rapid and external embryonic development [19], it will be interesting to investigate the function of LIS1 genes during embryonic development in zebrafish.

In this article, we identified the LIS1 genes in zebrafish, analyzed their expression patterns and investigated their functions on embryonic development. Our results showed that both transcripts of zebrafish LIS1a and LIS1b were expressed at all embryonic developmental stages and in adult tissues examined. The LIS1 proteins were mainly detected in brain tissue and early developmental embryos. The whole-mount immunostaining data showed that LIS1 proteins were distributed all over the embryos from 1-cell stage to 5 day post-fertilization (dpf). Cellular localizations of zebrafish LIS1 proteins were shown in the cell cytoplasm. Knockdown of zebrafish LIS1 genes led to embryonic developmental deficiency. Our results suggested that zebrafish LIS1 genes are necessary for embryonic development.

Materials and Methods

Zebrafish and cell lines
Zebrafish were provided by the Lab of Germ Cell and Embryonic Developmental Biology in the Shanghai Institutes for Biological Sciences (Shanghai, China) and maintained at 28.5°C on a cycle of 14 h of light and 10 h of darkness. Fish breeding and embryo manipulation were conducted according to established protocols [20]. Human HEK-293T and HeLa cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were maintained in our laboratory. These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL).

Extraction of RNA and RT–PCR
Total RNA was extracted from zebrafish embryos and tissues using Trizol reagent (Invitrogen, Carlsbad, USA) as described previously [21]. RNA preparation was digested with DNase I to eliminate possible genomic DNA contamination. RT reactions were performed using the SuperScript II RT–PCR kit (Gibco BRL) according to the manufacturer’s recommendations. To examine the expression patterns of zebrafish LIS1a and LIS1b, RT–PCR was carried out to amplify the fragments of LIS1a (150 bp) and LIS1b (200 bp) with primers listed in Table 1 (lisRT_F and lisRT_R). PCR conditions were as follows: 94°C for 2 min; 30 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 20 s; 72°C for a 5 min extension. An 893-bp fragment of zebrafish β-actin (the sequence at GenBank under accession number AF057040) was used as a control and amplified with primers listed in Table 1, under the conditions: 94°C for 2 min; 30 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 20 s; 72°C for a 5 min extension. An 893-bp fragment of zebrafish β-actin (the sequence at GenBank under accession number AF057040) was used as a control and amplified with primers listed in Table 1 (lisRT_F and lisRT_R). PCR conditions were as follows: 94°C for 2 min; 30 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 20 s; 72°C for a 5 min extension. An 893-bp fragment of zebrafish β-actin (the sequence at GenBank under accession number AF057040) was used as a control and amplified with primers listed in Table 1, under the conditions: 94°C for 2 min; 30 cycles of 94°C for 20 s, 50°C for 30 s, 72°C for 1 min; 72°C for a 5 min extension. PCR products were separated on 12% polyacrylamide gel for LIS1a and LIS1b followed with silver staining, or 1% agarose gel for β-actin and stained with Goldview (SBS, Beijing, China).

Plasmid construction
To generate plasmids pEGFP-C1-LIS1a and pEGFP-C1-LIS1b, the full length of coding sequences of zebrafish LIS1a (GenBank accession No. NM_201346) and LIS1b (GenBank accession No. NM_201345) were amplified from zebrafish liver cDNA using PyroBest polymerase (TaKaRa, Dalian, China). Touchdown PCR was used to clone zebrafish LIS1a with primers lis1a_EF and lis1a_XR (Table 1) and cycling condition was an initial step of 94°C for 2 min, followed by 10 cycles of 94°C for 30 s, annealing temperatures starting at 60°C for 30 s, decreasing by 0.5°C per cycle, and 72°C for 90 s for extension. This step was followed by 25 cycles.

| Table 1 Oligonucleotides primers used in the study |
|----------------|----------------|
| Gene      | Primer sequence (5’→3’)   |
| lis1_RT   | lisRT_F: AAAGTGTTGGGAAATGTCGCTGAT  |
|           | lisRT_R: CCAGAATAATTCAATAAAAGGACA  |
| actin     | actin_F: CAACGGCTCCGGGTATGTG  |
|           | actin_R: TGCCAGGGTACATGGTGG |
| lis1a     | lis1a_EF: AGAATTCAATGGTGCTGTCACAGAGC   |
|           | lis1a_XR: ACTCGAGGGCACTCCCACACTTTGACC |
| lis1b     | lis1b_F: CTGTCCCGTCCTTCTGCAA  |
|           | lis1b_R: TTTGGAAGGCCCTGGCGGG  |
|           | lis1b_EF: AAAAGATCTGAATGTCGCTGTCACAGAG |
|           | lis1b_XR: TTTTCTCGAGGGCAGATTCCCACAACCTTTT |
| lismo     | lismo_BF: GGATCCGAGCTGCTGACCACCC-TGGCA |
|           | lismo_ER: GAATTCTTGGGAAAGCTATCAGCT |

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of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. For cloning of LIS1b, primers lis1b_F and lis1b_R (Table 1) were used with first cycling conditions: a 2 min initial denaturing step at 94°C; followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s; and a final extension step at 72°C for 5 min. PCR product was used as the template for a second round PCR with primers lis1b_EF and lis1b_XR (Table 1) and the cycling condition was same as cloning of LIS1a. The PCR fragments of LIS1a and LIS1b were cut with EcoRI and Xhol and inserted into pEGFP-C1 vector (Clontech, Mountain View, USA). The two plasmids were used for subcellular localization analysis.

Plasmid pCS2-lismo-GFP was generated by PCR amplifying of the 5′-UTR sequences of zebrafish LIS1b with primers listed in Table 1 (lismo_BF and lismo_ER) and inserted into pCS2-GFP (kept in our Lab). PCR was performed with the same cycling conditions as cloning of LIS1a with Taq polymerase from BioDev (Beijing, China). The PCR fragment was ligated to pMD-18T (TaKaRa), cut with BamHI and EcoRI and inserted into pCS2-GFP. The plasmid was used for RNA transcription and analysis of morpholino efficiency.

**Transient transfection and subcellular localization**

Cells were transiently transfected with pEGFP-C1 vector or pEGFP-C1-LIS1 plasmids. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, HEK-293T or HeLa cells were seeded into 24-well plates, and 0.4 μg of plasmid was mixed with 0.6 ml transfection agent per well and added to cells. After 24 h transfection, HEK-293T cells were collected for western blotting analysis; HeLa cells were washed three times with PBS and then fixed for 10 min in chilled methanol. The nuclei were stained with Hoechst 33258 for 5 min. After several washes, coverslips were sealed and the cells were examined under a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

**Preparation of antibody**

Rabbit polyclonal antibody against the N-terminus of human LIS1 peptide (residue 1–55 amino acids) fused to N-terminal GST tag was prepared in our laboratory. The specificity of the antibody was examined by western blotting analysis.

**Protein extraction and western blotting analysis**

For the preparation of proteins from embryos, zebrafish embryos of different developmental stages were dechorionated and cells were scattered by pipette aspiration. After centrifuged at 6000 g for 1 min, supernatants were removed and cell pellets were dissolved in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 20% glycerol, and 0.2 mg/ml bromophenol blue). For the preparation of proteins from tissues, zebrafish tissue samples were homogenized in liquid nitrogen, lysed with RIPA buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM NaF, and 1 mM Na3VO4) containing Protease Inhibitor Cocktail (P8340; Sigma, St. Louis, USA) on ice for 30 min and centrifuged at 16,000 g for 20 min at 4°C. Supernatant fractions were collected and dissolved in appropriate SDS sample buffer. For western blotting analysis, the proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia, San Francisco, USA) and immunoblotted with the following primary antibodies: anti-LIS1 antibody (dilution 1:2000) or anti-tubulin mAb (dilution 1:500; A54 Cocktail NeoMarkers, Fremont, USA). After washing, the membranes were incubated with goat anti-rabbit or mouse IgG-HP secondary antibodies (dilution 1:2000; Santa Cruz Biotechnology, Santa Cruz, USA). Immunodetection was performed by the luminescence method using ECL reagents kit (Perfect Biotech, Shanghai, China), and the membranes were then exposed to an X-Omat film.

**Whole-mount immunochemistry**

Whole-mount immunohistochemistry was performed according to Hashimoto et al. [22]. Briefly, embryos of different developmental stages were fixed for 24 h with 4% paraformaldehyde at 4°C, dechorionated and washed with PBST (PBS with 1% Tween-20), and blocked with blocking buffer (PBST containing 10% heat inactivated goat serum). Preimmune serum or anti-LIS1 antibody (dilution 1:300 in PBST containing 1% goat serum) was added, and the embryos were incubated overnight at 4°C. After washing three times for 30 min each, the embryos were incubated with fluorescein isothiocyanate-conjugated rat anti-rabbit IgG (dilution 1:100; 111-096-144; Jackson Immunoresearch, West Grove, USA) for 10 h at 4°C. After washing three times for 15 min each, the specimens were examined using a fluorescence stereoscopic microscope (DP71; Olympus).
Capped RNA transcription, morpholino oligonucleotides, and microinjection

To produce capped lismo-GFP RNA, the pCS2-lismo-GFP plasmid was linearized by restriction enzyme NolI, followed by in vitro transcription using the SP6 mMessage mMACHINE kit (Ambion, Austin, USA). Antisense morpholino oligonucleotides (MOs) against zebrafish LIS1a (MO-LIS1a-ATG: 5'-CTCGTTGCTCCTGACGACCCAT-3', sequence was provided by Tsujikawa [18]), LIS1b (MO-LIS1b1: 5'-AGCGTTGCTCCTGACGACCCATG-3'; MO-LIS1b2: 5'-TCAGGTGTAGAGTGCACTG-3', which were designed by Gene Tools, LLC), and a standard MO control (MO-Sdc: 5'-CCTCTACTCTCATACATATTTATA-3') were synthesized by Gene Tools, LLC (Philomath, USA). The antisense MOs were microinjected into the yolk of 1- or 2-cell embryos. The lismo-GFP RNA (100 ng/μl) and MOs (4 ng/μl) were injected in embryos with the injection volume of 4 nl per embryo. Phenol red (0.05%) was co-injected as a non-toxic injector tracer. Embryos were photographed with a digital camera (DP71; Olympus).

Results

Bioinformatics analysis of zebrafish LIS1 genes

By GenBank searching, we found that LIS1 homologous genes existed in 29 species including 18 vertebrates and 11 invertebrates. To infer evolutionary relationships of zebrafish LIS1 homologues, we constructed a phylogenetic tree based on the protein sequences of LIS1 from these 29 species using the neighbor-joining method (Fig. 1). The phylogenetic tree showed that zebrafish LIS1 (D. rerio-a and D. rerio-b) were evolutionarily conserved, especially among the vertebrate species. It is interesting that the similarity of LIS1 proteins between social ameba and human, the positions of which are very distant in evolution, is still as high as about 64% identical, suggesting that LIS1 gene appeared very early in evolution and evolved in a much conserved way.

To analyze the relatedness between the zebrafish LIS1 proteins and other animal model species more detailedly, multiple amino acid sequence alignments of the human, mouse, zebrafish, fruit fly, nematode worm, and yeast were performed with ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Results showed that LIS1 proteins including LIS1a and LIS1b are more conserved in evolution [Fig. 2(A)]. Both LIS1a and LIS1b consist of three motifs: N-terminal Lis-H domain followed by a coiled-coil region and seven WD repeats. All of these motifs are highly conserved among different species. The lengths of different LIS1 proteins are also very similar except yeast, which is a little longer. The percentages of sequence identity among different species were also calculated [Fig. 2(B)], which showed that zebrafish LIS1 proteins have high identities with human and mouse (94% of LIS1a and 95% of LIS1b). LIS1b is a little more conserved than LIS1a.
Figure 2 Bioinformatics analyses of zebrafish LIS1 genes

(A) Alignment of deduced amino acid sequence of zebrafish LIS1 proteins with other five LIS1 proteins from H. sapiens, M. musculus, D. melanogaster, C. elegans, and S. cerevisiae. Dark shaded areas indicate the residues conserved in all seven sequences, gray shaded areas indicate the residues conserved in four or six sequences. Gaps introduced to maximize the alignment are indicated by dashes. The positions of the amino acid residues are listed on the right. Motifs of Lis-H domain, coiled-coil region, and seven WD repeats are shown above of alignment sequences. (B) Percentage of sequence identity among the LIS1 protein family members. (C) Gene synteny analysis of LIS1 contained chromosomes. Conserved syntenic regions between human LIS1 (chromosome 17) and zebrafish LIS1a (chromosome 21) or LIS1b (chromosome 15) are shown. The homologous genes near the LIS1 locus in the syntenic regions are also indicated. The numbers in the parenthesis indicated the positions of the genes on the chromosome. Homologous genes of zebrafish (uncapitalized) and human (capitalized) were connected with lines.
Zebrafish contains two LIS1 genes: LIS1a and LIS1b, which is different from other species. They are located on different chromosomes with LIS1a on chromosome 21 and LIS1b on chromosome 15. In order to understand the relationship of zebrafish and mammalian LIS1 genes in vertebrate genome evolution, syntenic analysis was performed between human and zebrafish. Human LIS1 was used as the anchor site; orthologous comparisons of the genes in the regions flanking the LIS1 loci of human and zebrafish genomes were performed with NCBI-BLAST. Result showed that more genes in the vicinity of human LIS1 were mapped to the neighborhood of zebrafish LIS1b locus than LIS1a locus [Fig. 2(C)]. This suggests that LIS1b may be evolved to mammalian LIS1 conservatively, while zebrafish LIS1a is diverged more rapidly and may be lost during evolution.

Expression patterns of zebrafish LIS1 transcripts

Alignment of LIS1a and LIS1b sequences showed that there were some gaps in the 3' UTR of LIS1a sequence [Fig. 3(A)]. To examine the expression of zebrafish LIS1a and LIS1b transcripts, we designed specific primers (lisRT_F and lisRT_R) to amplify sequences covering this region to produce the 150 bp fragment of zebrafish LIS1a and 200 bp fragment of LIS1b. We then analyzed the expressions of zebrafish LIS1a and LIS1b mRNA by RT–PCR at different embryonic developmental stages. We found that both zebrafish LIS1a and LIS1b were ubiquitously expressed at all developmental stages including 2 h post-fertilization (hpf) (64-cell), 10 hpf (tail bud), 24 hpf (prim-5), 48 hpf (Long pectoral fin bud), 72 hpf (Protruding mouth), and 120 hpf (swimming larva) [Fig. 3(B)]. The existence of zebrafish LIS1a and LIS1b in the 2 hpf embryonic cleavage stage, when the embryonic genome is transcriptionally inactive [23], indicates that zebrafish LIS1 messages are maternal RNA stored in the eggs. We also analyzed the expressions of zebrafish LIS1a and LIS1b in different zebrafish adult tissues including heart, brain, liver, intestine, muscle, spleen, ovary, eye, gill, fin, and testis. As shown in Fig. 3(C), zebrafish LIS1a and LIS1b were
also ubiquitously expressed in all eleven tissues examined. The concordant expression of zebrafish LIS1a and LIS1b during embryonic development and in adult tissues suggests that the two genes may be controlled by similar mechanisms of transcriptional regulation.

Expression patterns of zebrafish LIS1 proteins
To examine the expression of zebrafish LIS1 proteins at different embryonic developmental stages and adult tissues, western blotting analysis with polyclonal antibody against human LIS1 was used. The efficiency of the antibody against zebrafish LIS1 protein was examined. It showed that the antibody could recognize exogenous zebrafish LIS1a and LIS1b proteins [Fig. 3(D)]. Then we assayed the expression of LIS1 proteins in embryos. The zebrafish LIS1 proteins were mainly expressed in 6, 10, and 24 hpf embryos, but not detectable in 2 hpf, and with very low expression after 72 hpf [Fig. 3(E)]. We next analyzed the zebrafish LIS1 proteins in adult tissues such as eye, brain, liver, gill, fin, ovary, and testis. As shown in Fig. 3(F), zebrafish LIS1 proteins were expressed at high levels in brain tissue, but not detectable in other six tissues. As the zebrafish LIS1a and LIS1b mRNA could be detected in all tissues examined by RT–PCR, we interpret that the absence of LIS1 proteins in tissues may result from the low sensitivity of polyclonal antibody which might not be able to recognize the very low levels of zebrafish LIS1 proteins in the tissues.

Whole-mount immunostaining was also used to investigate the expression patterns of LIS1 proteins during embryogenesis. The results indicated that zebrafish LIS1 proteins were distributed all over the embryos from 1-cell stage to 5 dpf, as well as in the unfertilized eggs (Fig. 4). In the unfertilized eggs [Fig. 4(A–D)], LIS1 proteins were distributed evenly in the eggs [Fig. 4(B)]; with the

Figure 4 Whole-mount immunostaining of zebrafish LIS1 proteins Immunostaining of LIS1 proteins in unfertilized eggs (A–D) and fertilized embryos (E–P) with preimmune serum as negative controls (A, E, N, and P). Time points of unfertilized eggs and developmental stages of fertilized embryos are indicated. Eggs and embryos are lateral views with the animal pole up, except the 4-cell stage (G), which is apical view. Scale bar = 250 μm.
advance of cytoplasmic streaming, cytoplasm was separated from yolk, LIS1 proteins were translocated to the putative animal pole, and accumulated there gradually [Fig. 4(C,D)]. After fertilization, LIS1 proteins were accumulated at the animal pole during the 1-cell stage as in unfertilized eggs and then distributed throughout the blastomeres in the cleavage period [Fig. 4(F–H)]. Intensity of LIS1 expressions was much higher in fertilized embryos than unfertilized eggs. At the 4-cell stage, we found that LIS1 proteins were not evenly distributed, with lower expression in some areas of blastomeres [Fig. 4(G)]. Generalized distribution of LIS1 proteins persisted from shield stage to 5-somite stage; it was disturbed after 24 hpf [Fig. 4(I,J,M)]. From 24 hpf to 5 dpf, the LIS1 proteins were highly expressed in brain than other tissues [Fig. 4(K,L,O)], suggesting that LIS1 proteins are very important for brain development.

Subcellular localizations of zebrafish LIS1 proteins
To identify the subcellular localizations of zebrafish LIS1 proteins, we constructed the GFP-fused LIS1a and LIS1b expression plasmids. After transfection of human HeLa cells with pEGFP-C1 control vector, pEGFP-C1-LIS1a, or pEGFP-C1-LIS1b plasmid, cellular distributions of GFP-LIS1 proteins were examined under the fluorescence microscope. We found that zebrafish GFP-LIS1a and GFP-LIS1b were both localized in the cell cytoplasm, but none in cell nucleus, comparing with the GFP control (Fig. 5). The data demonstrated that the localizations of zebrafish LIS1 proteins were mainly in the cell cytoplasm, which were the same as that of human and mouse LIS1 proteins [7,10,24].

Knockdown of zebrafish LIS1 proteins affects embryonic development
Owing to the advantages of zebrafish over other animal system, especially the external embryonic development, we examined the effect of LIS1 on zebrafish embryonic development using antisense MO technology. Three MOs (MO-LIS1a-ATG, MO-LIS1b1, and MO-LIS1b2) were used to knockdown the expression of endogenous LIS1 proteins. The MO-LIS1a-ATG, against LIS1a, had been determined to inhibit LIS1a protein expression efficiently [18]. The efficiency of MO-LIS1b1 and MO-LIS1b2 corresponding to the 5′-UTR and the beginning of ORF of the LIS1b gene were determined. The lismo-GFP RNA mixed with different MOs tested were injected into embryos. Results showed that the expression of GFP protein was inhibited in embryos injected with the mixture of lismo-GFP and MO-LIS1b1 or lismo-GFP and MO-LIS1b2, while in embryos injected with lismo-GFP and MO-Sdc control, the expression of GFP was not inhibited, the same as lismo-GFP only [Fig. 6(A)]. This result demonstrated that the two designed LIS1b morpholinos could efficiently inhibit the expression of LIS1b.

To examine the phenotype changes of embryos after injection of MO-LIS1, six groups of MOs including individuals and combinations of MO-LIS1a-ATG, MO-LIS1b1, and MO-LIS1b2 as listed in Table 2, were injected into 1- or 2-cell embryos. We found that, compared with the control MO group, the embryos after injection of MO-LIS1 displayed many developmental defects. The abnormal phenotypes can be classified into three levels: slight, moderate, and severe degrees. The pictures showing the MO-LIS1a-ATG morphants represented the typical developmental deficient embryos at three degrees [Fig. 6(B,D)]. The embryo defects could be observed at 12 hpf with developmental delay of presumptive brain and tail bud. Midbrain–hindbrain boundary (MHB) [25], which organizes the formation of the midbrain and cerebellum, was disrupted. At 30 hpf, brain structures, including optic vesicles, were developed irregularly possibly with cell necrosis. Heartbeat was delayed and embryos moved stiffly. At 60 hpf, embryos developed small eyes, thin pigmentation, and abnormal circulation. The comparison of phenotypes between normal embryos and MO-LIS1a-ATG morphants at same stages was indicated [Fig. 6(C)]. The borders of optic

![Figure 5 Subcellular localizations of zebrafish LIS1 proteins in cells](blue-green-merge.jpg)
Figure 6 Knockdown of LIS1 proteins with antisense MOs affects embryo development

(A) Efficiency of MO-LIS1b1 and MO-LIS1b2 morpholinos. The lismo-GFP RNA was co-injected with MO-Sdc control, MO-LIS1b1, or MO-LIS1b2 into embryos. The expression levels of GFP protein reflected the ability of MO-LIS1 morpholinos to inhibit protein expression. Scale bar=1000 μm. (B) Phenotypes of MO-LIS1a-ATG morphants were observed at different time points (12, 30, and 60 hpf). The abnormal embryonic phenotypes are classified into three degrees: slight, moderate, and severe, and viewed under light microscopy. Scale bars = 500 μm. (C) Embryonic deficiencies of MO-LIS1a-ATG morphants. (a) and (a′) showed normal embryo and MO-LIS1a-ATG morphant at 16 hpf. Optic vesicle (arrow) and notochord (arrow head) of MO-LIS1a-ATG morphant was malformed with obscure border compared with the normal embryo. (b) and (b′) showed normal embryo and MO-LIS1a-ATG morphant at 30 hpf. Brain structures, such as optic vesicle (arrow) and MHB (arrow head), were severely disputed in MO-LIS1a-ATG morphant. (c), (d) and (c′), (d′) showed phenotype of normal embryo and MO-LIS1a-ATG morphant at 5 dpf. MO-LIS1a-ATG morphant developed many abnormalities such as smaller brain (arrow in c′), swelling heart (arrow head in c′), disrupted muscle (arrow in d′), and abnormal posterior intestine (arrow head in d′). Scale bars = 250 μm. (D) The slight, moderate, and severe embryos of MO-LIS1a-ATG morphants at 60 hpf. The control were included in each class for comparison and indicated with white arrowheads. Scale bar = 500 μm.
Expression and function of LIS1 in zebrafish

**Table 2 Percentage of phenotypes of different morphants at 60 hpf**

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<thead>
<tr>
<th>Type</th>
<th>MOs</th>
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<th>rate (%)</th>
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</table>

MOs, Morpholinos; n, number. *, because of the high mortality of embryos after injection of LIS1a-ATG and LIS1b2 morpholino mixture with the same dose as other groups, total number of morphants was small.

vesicles and notochord were blurry at 16 hpf morphants [Fig. 6(C), a']. The irregular brain structures with cell necrosis appeared at 30 hpf morphants [Fig. 6(C), b']. The small eyes and head, thin pigmentation, abnormal circulation, and disorganized musculature were observed at 5 dpf morphants [Fig. 6(C), c' and d']. Most embryos (88%, n = 16) displayed curled body as raf1 morphant [26] and other deficiencies, and died within 9 days. We also found that injection of morpholino combinations displayed much severe embryonic deficiencies, especially MO-LIS1a-ATG plus MO-LIS1b2 (Table 2). The ratios of abnormalities of different MO-LIS1 morphants were also shown in Table 2. Our results clearly indicate that LIS1 genes are vital for zebrafish embryonic development.

**Discussion**

LIS1 gene has been cloned in a variety of animals, including zebrafish, which was reported by Tsujikawa et al. [18], while we had also cloned zebrafish LIS1 genes. Interestingly, zebrafish contains two LIS1 genes, LIS1a (also called pafah1b1b) and LIS1b (also called pafah1b1a), which is different from other species, such as human, mouse, worm, fruit fly, and yeast. We performed bioinformatics analysis to explore the evolution status of LIS1 gene, and found that it was more conserved in evolution, especially in vertebrates. The origin of LIS1a and LIS1b appears to be the result of gene or genome duplication in fish evolution [27], as we found that the form of two LIS1 was also existed in Salmo salar (LIS1a, GenBank accession No. BT045765; LIS1b, GenBank accession No. BT045543). Although zebrafish LIS1a and LIS1b share much similarity in gene and protein sequences, we guess the evolutionary consequences of them may be different. The sequence analysis [Fig. 1(C)] and gene synteny analysis [Fig. 1(D)] suggest that LIS1b may be contributed to evolve to mammalian, while LIS1a may be degenerated in evolution, since the identity of LIS1b is a little closer to mammalian LIS1 than LIS1a. We also analyzed the genomic structures of zebrafish LIS1 genes and compared them with that of human LIS1. It showed that the genomic structure of zebrafish and human is much alike with same numbers and similar sizes of exons and introns, indicating the unique signature of zebrafish LIS1 genes in evolution.

To investigate the expression of LIS1a and LIS1b, we designed specific primers to amplify the transcripts for LIS1a and LIS1b. We found that zebrafish LIS1a and LIS1b were expressed with similar patterns, they are ubiquitously expressed in all embryonic developmental stages and adult tissues examined. However, because of the high similarity of these two proteins, it is difficult to prepare the antibody that can distinguish between the LIS1a and LIS1b. Here, an anti-human LIS1 antibody was used to identify the zebrafish LIS1 proteins, since we had determined that human LIS1 antibody can recognize the zebrafish LIS1 proteins produced by 293T cells [Fig. 3(D)]. The western blotting analysis showed that zebrafish LIS1 proteins were highly expressed in brain tissue, but not in other tissues such as eye and liver. Thus, the expression pattern of LIS1 at the protein level is different from that at the mRNA level, which maybe resulted from the methodological sensitivity. For example, with immunostaining method, zebrafish LIS1 proteins were shown at all embryonic developmental stages from 1-cell to 5 dpf (Fig. 4), while using western
blotting LIS1 proteins only existed at embryos from 6 to 24 hpf [Fig. 3(E)]. However, concerning the tissue distribution of LIS1 proteins, the higher expression of LIS1 proteins in brain reflects the most important role of LIS1 in brain development. Consistent with our results, the expression of LIS1 in chicken also showed a ubiquitous expression in embryos of different developmental stages, and a higher expression in brain than other tissues [16]. The subcellular localizations of zebrafish LIS1a and LIS1b were shown in the cytoplasm (Fig. 5), which is the same as mammalian counterparts [7,10,24]. It implies that zebrafish LIS1 proteins may have similar cellular functions as human LIS1, such as neuronal migration and mitotic cell division.

Studies in other animal systems suggest that LIS1 plays an important role in reproduction. For example, depletion of LIS1 in nematode worm led to embryo lethality and sterility [14]. Mutations of LIS1 in fruit fly resulted in partial ventralization of the eggshell and abnormal oocyte differentiation [15,28]. Knockout of LIS1 in mouse led to impairment in spermatogenesis and embryo lethality [13,29]. In addition, inactivation of a testis-specific LIS1 transcript caused male infertility [30]. However, detailed investigation of LIS1 in embryonic development is lacking. Thus, we explored the function of zebrafish LIS1 in embryonic development due to the unique advantages of zebrafish. After knockdown of the endogenous zebrafish LIS1 proteins with antisense MOs, zebrafish embryos displayed many developmental deficiencies, such as growth delay, body curl, brain malformation, small eyes, and circulation abnormality. Percentages of abnormal phenotype after injection of LIS1a and LIS1b morpholinos mixtures (LIS1a-ATG + LIS1b1 and LIS1a-ATG + LIS1b2) are higher than that of single morpholinos. This suggests that LIS1a and LIS1b may corporate to execute their functions in embryonic development. The developmental defects of zebrafish embryos may reflect the important functions of LIS1 proteins in the cellular content, such as active mitosis division and dynamic cell migration. Further investigations may be needed to find out the functions and molecular mechanisms of zebrafish LIS1a and LIS1b involved in embryonic development extensively.

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