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## RESEARCH ARTICLE

# Molecular structure and developmental expression of zebrafish *atp2a* genes

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## Abstract

We isolated two *atp2a* genes, *atp2a1* and *atp2a2a*, from embryonic zebrafish. Amino acid sequences deduced from zebrafish *atp2a* genes are aligned with orthologue proteins from other species, the results showed that they share high percentage of identities (82%–94%) and acidic pIs (5.03–5.33). Whole mount *in situ* hybridization experiments showed that *atp2a1* and *atp2a2a* 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 (48–96 hpf), expression of *atp2a1* was restricted in head and trunk muscles as well as in some neurons. In contrast to the strongly expression of *atp2a1* in head muscle, expression of *atp2a2a* was detected in head muscle in a fainter manner. In addition, transcripts of *atp2a2a* were observed in the developing heart during early cardiogenesis. The present studies not only help us to comparatively analyze *atp2a* genes across species, but also provide useful information about expressions during early embryogenesis that will help in further investigations of functional studies of Atp2a in the future.

**Keywords** Calcium; Cardiogenesis; Muscle; SERCA; Zebrafish

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## Introduction

Calcium ( $\text{Ca}^{2+}$ ) plays many important physiological roles, such as neuron transmission, blood coagulation, heart beat, and muscle contraction (Berridge et al., 1998; MacLennan, 2000; Varga-Szabo et al., 2009). Cytosolic  $\text{Ca}^{2+}$  concentrations are delicately regulated by many membrane receptors and/or  $\text{Ca}^{2+}$  pump. For example, in muscle cells, intracellular  $\text{Ca}^{2+}$  concentration is regulated by (i) activation of ryanodine receptors (RyRs) and inositol triphosphate receptors (IP3) in the sarcoplasmic reticulum (SR) membrane to release  $\text{Ca}^{2+}$  from the SR to the cytosol; and (ii) activation of SR (or ER)  $\text{Ca}^{2+}$ -ATPase (SERCA/ATP2A), a  $\text{Ca}^{2+}$  pump, to retrieve cytosolic  $\text{Ca}^{2+}$  from the cytosol to the SR lumen (Berridge et al., 1998; Fill and Copello, 2002; Mikoshiba, 2007; Berridge, 2009). Increased cytosolic  $\text{Ca}^{2+}$  activates troponin C that initiates actin/myosin sliding, thus contracting the muscles; instead, reduced cytosolic  $\text{Ca}^{2+}$  relaxes the muscles (McLennan et al., 1997; Gordon et al., 2000).

*ATP2A* family genes encode all kinds of the SERCA  $\text{Ca}^{2+}$ -ATPases, including SERCA1(ATP2A1), SERCA2(ATP2A2), and SERCA3(ATP2A3). Those enzymes catalyze the hydrolysis of ATP coupled with the translocation of  $\text{Ca}^{2+}$  from the cytosol to the SR lumen, and played many physiological roles, especially muscular excitation and contraction. The molecular structure of *ATP2A* family genes has been determined extensively in fly (Magyar et al., 1995), sea urchin (Gunaratne and Vacquier, 2006), chicken (Machuca et al., 1999) and mammals (Brandl et al., 1986; Lytton and MacLennan, 1988; Wu and Lytton, 1993; Schleeff et al., 1996). In zebrafish, there are at least four *atp2a* genes, such as *atp2a1*, *atp2a2a*, *atp2a2b*, and *atp2a3* were reported (Lo et al., 2003; Hirata et al., 2004; Ebert et al., 2005), but their expression information during early embryogenesis are still limited.

As it might be expected, any defect in the above  $\text{Ca}^{2+}$  signaling pathways induces motor disorder or other pathological defects. For example, Brody disease, a rare inherited disorder of skeletal muscle function in human, is due to *SERCA1* muta-

tion (Odermatt et al., 1996). The clinical signs and symptoms of Brody disease include exercise-induced impairment of muscle relaxation, stiffening and cramps (Brody, 1969; MacLennan, 2000). In mice, inactivation of SERCA leads to neonatal lethality because their diaphragm functions are impaired (Pan et al., 2003). In zebrafish, mutation in *atp2a1* leads to muscle relaxation defect which is similar to human *SERCA1*-deficient phenotypes (Hirata et al., 2004; Olson et al., 2010). These observations indicate that biological functions of SERCA1 are conserved between mammals and zebrafish.

On the other hand, mutations in *SERCA2* (*ATP2A2*) causes Darier disease, an autosomal-dominant skin disorder in human (Sakuntabhai et al., 1999). The symptoms of Darier disease are loss of adhesion between epidermal cells (acantholysis) and abnormal keratinization (Sakuntabhai et al., 1999). In mice, the ablation of *ATP2A2* was lethal, but heterozygous *SERCA2*<sup>-/-</sup> mice manifested impaired cardiac contractility and delayed cardiomyocyte relaxation (Periasamy et al., 1999). These observations suggest that the biological functions of SERCA2 between human and mice might be diverse. To elucidate the physiological functions of *atp2a* genes during early embryogenesis, it is worthy to analyze comparatively *atp2a* genes across species. Here, we report the spatiotemporal expressions of two zebrafish *atp2a* genes. *atp2a1* was characterized as fast-muscle-specific, whereas *atp2a2a* was found to be expressed in fast-muscle and heart during early embryogenesis. This information may provide more insight into the molecular structure and expression patterns of the lower vertebrate *atp2a* genes.

## Materials and Methods

### 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). cDNA clones encode *Ata2a1* and *Atp2a2a* were purchased from Open Biosystem (USA). The presumptive *Ata2a1* and *Atp2a2a* 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 *Ata2a1* and *Atp2a2a*. The Clustalw molecular evolution genetic program was used for our phylogenetic tree analysis (<http://www.ebi.ac.uk/clustalw/>).

### 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 hr light and 10 hr dark, in an aquarium supplied

with freshwater and aeration (Chen et al., 2009a; 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 collected 100 embryos per stage to extract their total RNA. RNA isolation and first-strand cDNA synthesis procedures were according to the previous report with minor modification (Chen et al., 2000; 2001; Wang et al., 2009b). Primer sets (*ATP2A1*-F: 5'-ACCACCAACCAGATGTGTGCACA-3', *ATP2A1*-R: 5'-AACTGGACCAGTCAGGGGCA-3'; *ATP2A2A*-F: 5'-TGCCACCGCTCTAGGCTTCAAC-3', *ATP2A2A*-R: 5'-TGAGCAGCTCGTCCAAGAGGATG-3'; and  $\beta$ -actin-F: 5'-GTCCCGTACGCCTCTGGTCG-3',  $\beta$ -actin-R: 5'-GCCGGACTCATCGTACTCCTG-3') were designed based on the sequences encoding of putative zebrafish *atp2a1*, *atp2a2a*, and  $\beta$ -actin.

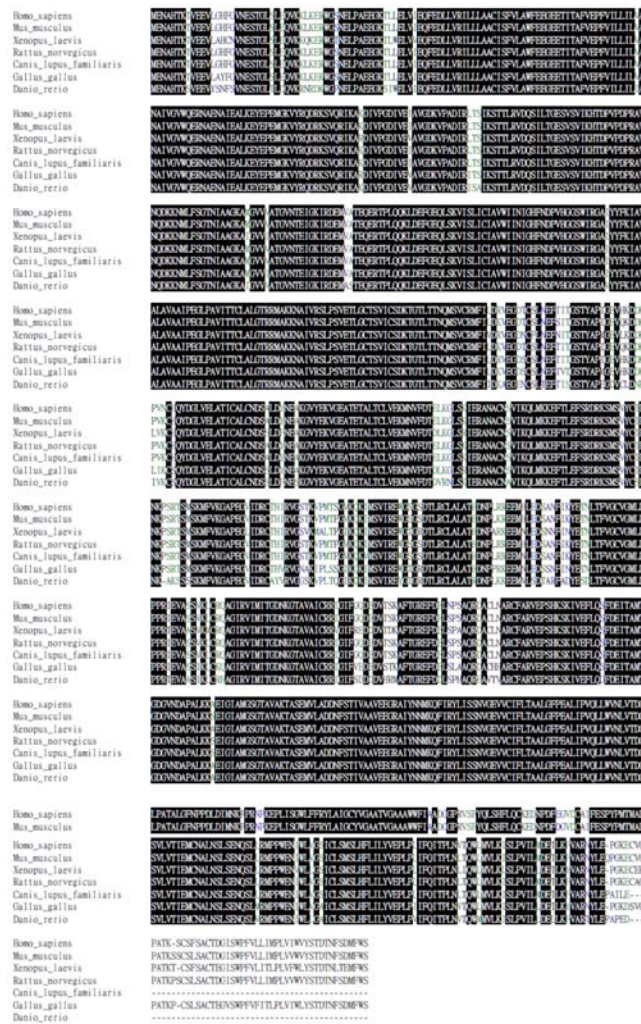
### Whole mount *in situ* hybridization, cryosection and images

The procedures for whole mount *in situ* hybridization, and cryosection have been described previously (Chen et al., 2009b; Pai and Chen, 2010; Peng et al., 2010), except that *atp2a1* and *ata2a2a* (this study) were used as probes. They were digoxigenin-labeled, after we cloned their partial DNA fragments. 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

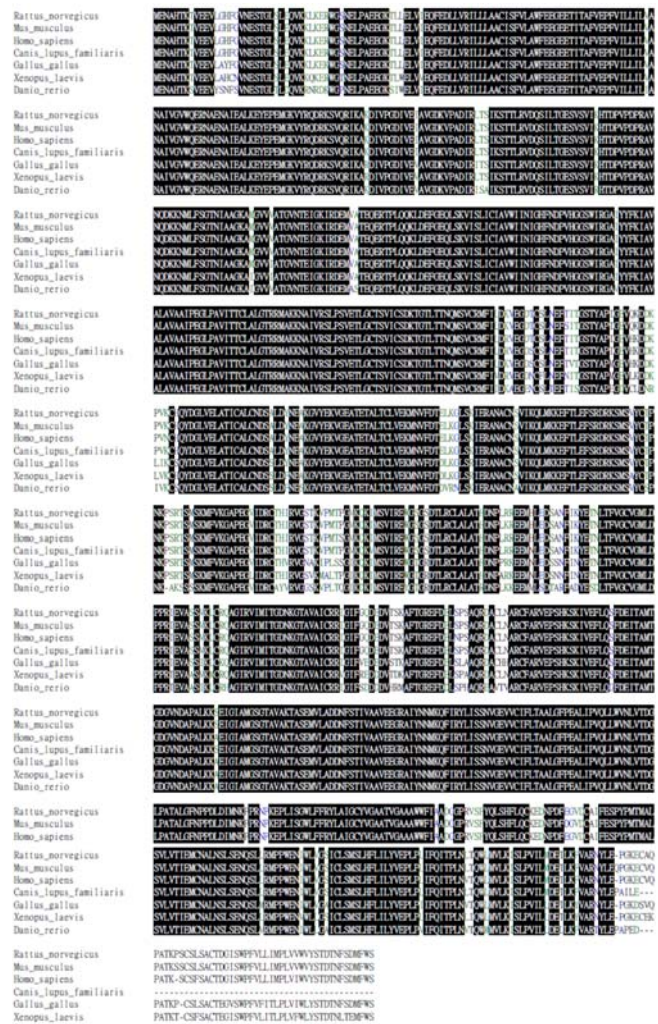
By searching GenBank, we found two putative zebrafish *atp2a* sequences (*atp2a1*: NM\_001007029, and *atp2a2a*: NM\_200965). The deduced amino acid sequence of zebrafish *Atp2a1* revealed a 994-amino acid polypeptide (Fig. 1). The zebrafish *Atp2a1* polypeptide shares sequence identities of 82-91% of the reported *Atp2a1* of human, dog, rat, mouse, chicken and *Xenopus*. On the other hand, the deduced amino acid sequence of zebrafish *Atp2a2a* revealed a 996-amino acid polypeptide (Fig. 2). The zebrafish *Atp2a2a* polypeptide shares sequence identities of 85-94% of the reported *Atp2a2* of human, dog, rat, mouse, chicken and *Xenopus*. In addition, we used the Clustalw program to determine the phylogenetic similarities between zebrafish *Atp2a1/Atp2a2a* and that of other known species. The phylogenetic tree generated by the program showed that zebrafish *Atp2a1/Atp2a2a* (76%) was more closely related to *Xenopus Atp2a1/Atp2a2a* than those from higher vertebrates and microorganisms (data not shown). Moreover, *atp2a1/*



**Figure 1.** Comparison of the deduced amino acid sequence of zebrafish Atp2a1 with those of other known species. The information was obtained from the GenBank nucleotide sequence database with the following accession numbers: human (NM\_004320), dog (XM\_855027), rat (NM\_058213), mouse (NM\_007504), chicken (NM\_205519) and *Xenopus* (NM\_001092974) Atp2a1. Amino acid residues similar to those of the zebrafish Atp2a1 are presented in black.

*atp2a2* gene transcripts from selected vertebrates and their molecular features are summarized in Table 1. These data reveal that Atp2a1 and Atp2a2 proteins have acidic pIs (Atp2a1: 5.07-5.25; Atp2a: 5.03-5.33) and are highly negative charged at pH 7.0.

**Developmental expression of zebrafish *atp2a1* and *atp2a2a***  
 Developmental expression of zebrafish *atp2a1* and *atp2a2a* transcripts were determined by RT-PCR, which revealed that endogenous *atp2a1* expressed from 18 hpf to 7 dpf, and *atp2a2a* expressed from 1-cell (0 hpf) to 7 dpf (Fig. 3). Although RT-PCR products of *atp2a1* were undetectable from 1-cell (0 hpf) to 6 hpf, weak signals were detected by nested RT-PCR analysis (data not shown). These observations in-



**Figure 2.** Comparison of the deduced amino acid sequence of zebrafish Atp2a2a with those of other known species. The information was obtained from the GenBank nucleotide sequence database with the following accession numbers: human (NM\_170665), dog (NM\_001003214), rat (NM\_001110139), mouse (NM\_001110140), chicken (XM\_415130) and *Xenopus* (NM\_001086935) Atp2a2. Amino acid residues similar to those of the zebrafish Atp2a2a are presented in black.

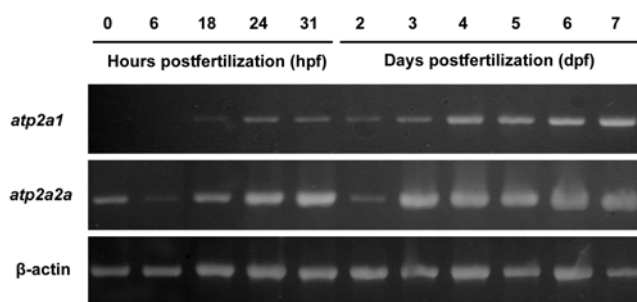
dicated that zebrafish *atp2a1* and *atp2a2a* are maternal inherited genes.

**Spatiotemporal expression of zebrafish *atp2a1* transcripts during early development**

To determine the spatiotemporal expression patterns of *atp2a1* during early development, we performed whole mount in situ hybridization using a *atp2a1* antisense DIG-labeled riboprobe. Zebrafish *atp2a1* transcripts were first detected from 1-cell stage to cleavage period (Fig. 4A, B) in a very weak manner (arrow head indicates), and extended their expression from the gastrula period to the early segmentation stages (Fig. 4C, D). At 18-, 24-, and 31-hpf, the zebrafish *atp2a1* transcripts were restricted to trunk somites (Fig. 4E, E', F, G, H). Cryosections

**Table 1.** Summary of *atp2a1/atp2a2* genes from selected vertebrates.

Gene names (species)	Coding region (aa)	Mw (kDa)	pI	GenBank accession number	References
<i>atp2a1</i>					
Human	994	109.3	5.08	NM_004320	Brandl et al., 1986
Dog	993	109.2	5.07	XM_855027	NCBI
Rat	994	109.4	5.13	NM_058213	Wu and Lytton, 1993
Mouse	994	109.4	5.13	NM_007504	Schleef et al., 1996
Chicken	994	109.0	5.25	NM_205519	Karin et al., 1989
<i>Xenopus</i>	1042	115.4	5.14	NM_001092974	Klein et al., 2002
Zebrafish	994	108.8	5.10	NM_001007029	Hirata et al., 2004
<i>atp2a2</i>					
Human	1042	114.8	5.23	NM_170665	Lytton and MacLennan, 1988
Dog	997	109.7	5.26	NM_001003214	Autry and Jones, 1997
Rat	997	109.7	5.27	NM_001110139	Gunteski-Hamblin et al., 1988
Mouse	1044	114.9	5.23	NM_001110140	Hsu et al., 1993
Chicken	1042	114.8	5.23	XM_415130	NCBI
<i>Xenopus</i>	996	109.5	5.03	NM_001086935	Klein et al., 2002
Zebrafish <i>atp2a2a</i>	996	109.5	5.08	NM_200965	Ebert et al., 2005
Zebrafish <i>atp2a2b</i>	1035	113.6	5.33	NM_001030277	Lo et al., 2003

**Figure 3.** RT-PCR analysis of *atp2a1* and *atp2a2a* gene transcripts, using total RNA extracted from the embryos of different developmental stages. Top panel: *atp2a1*; middle panel: *atp2a2a*; and bottom panel: loading control ( $\beta$ -actin). Stage of each sample is indicated on the top.

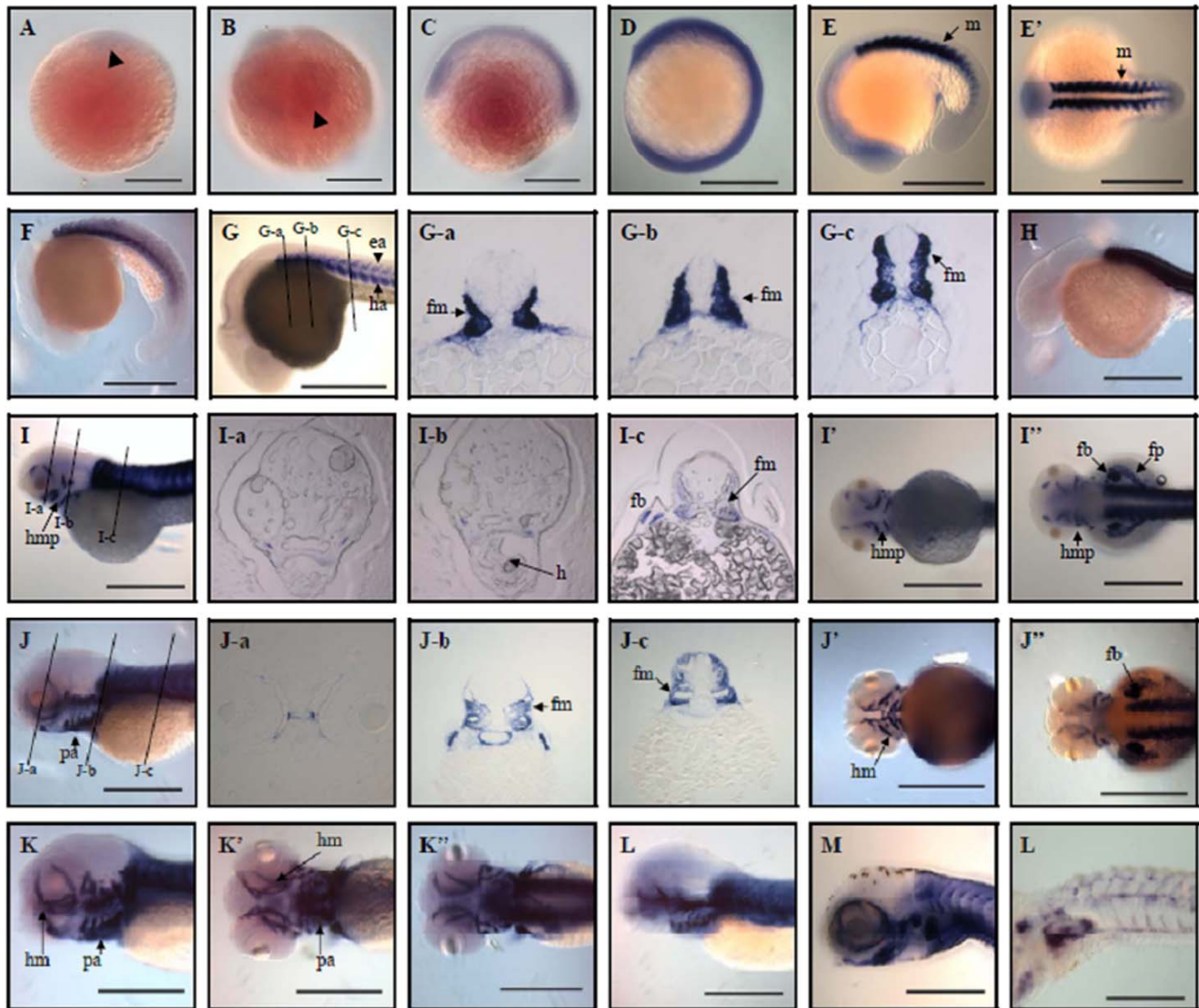
of 24-hpf embryos revealed that *atp2a1* signals distributed in the entire somite, including the presumptive epaxial and hypaxial muscle regions (Fig. 4G-a, -b, -c). By 48- and 72-hpf, zebrafish *atp2a1* expressed strongly in trunk somites, fin buds, pharyngeal arches as well as in the head muscle progenitors (Fig. 4I, I', I'', J, J', J''). Cryosections revealed *atp2a1* signals were undetected at heart regions (Fig. 4I-a, -b, -c, 4J-a, -b, -c). Expression of zebrafish *atp2a1* continued from 4-dpf to 6-dpf in the all head muscles as well as in the trunk somites (Fig. 4K, K', K'', L, M). By 7-dpf, *atp2a1* signals were detected in head muscles, pharyngeal arches and trunk somites in a fainter manner (Fig. 4N). On the other hand, *atp2a1* signals were undetectable in heart, notochord, and central neuron system. On the basis of these observations, we conclude that zebrafish *atp2a1* is a maternally inherited gene, restricting its expression in head and trunk muscles.

### Atp2a1 plays important roles on skeletal muscle contraction of vertebrates

We have shown that *atp2a1* transcripts were detected strongly in the presumptive epaxial and hypaxial muscle regions of 18-48-hpf zebrafish embryos. In mice, canonical *ATP2A1* transcripts were measured in the adults' skeletal muscles but a neonatal isoform, *SERCA1b*, is exclusively expressed in myoblasts and myotubes during embryogenesis (Zádor et al., 2007). In human, *ATP2A1* transcripts were found in the adults' skeletal muscles (Zhang et al., 1995; Loukianov et al., 1998). These observations indicate that *atp2a1* genes are not ubiquitously expressed, but are restricted in muscles among vertebrates. In zebrafish, previous study has shown that *SERCA1* is only expressed in muscle but not in spinal cord or notochord at 15 and 19 hpf (Hirata et al., 2004). Furthermore, inactivation of *atp2a1* leads to muscle contraction defects in zebrafish as well as in mice and human (Zhang et al., 1995; Odermatt et al., 1996; Pan et al., 2003; Hirata et al., 2004). Maves et al. (2007) used microarray analysis and found that *atp2a1* involved during zebrafish fast muscle differentiation. In particular, *SERCA1* null mutant mice exhibit abnormal diaphragm functions; the insufficient function and development of the diaphragm may be due to the lack of *SERCA1b* (Pan et al., 2003). Taken together, we suggest that *Atp2a1* plays important roles on skeletal muscle contraction and might play a role during vertebrates' muscle differentiation, especially in fast muscle.

### Spatiotemporal expression of zebrafish *atp2a2a* transcripts during early development

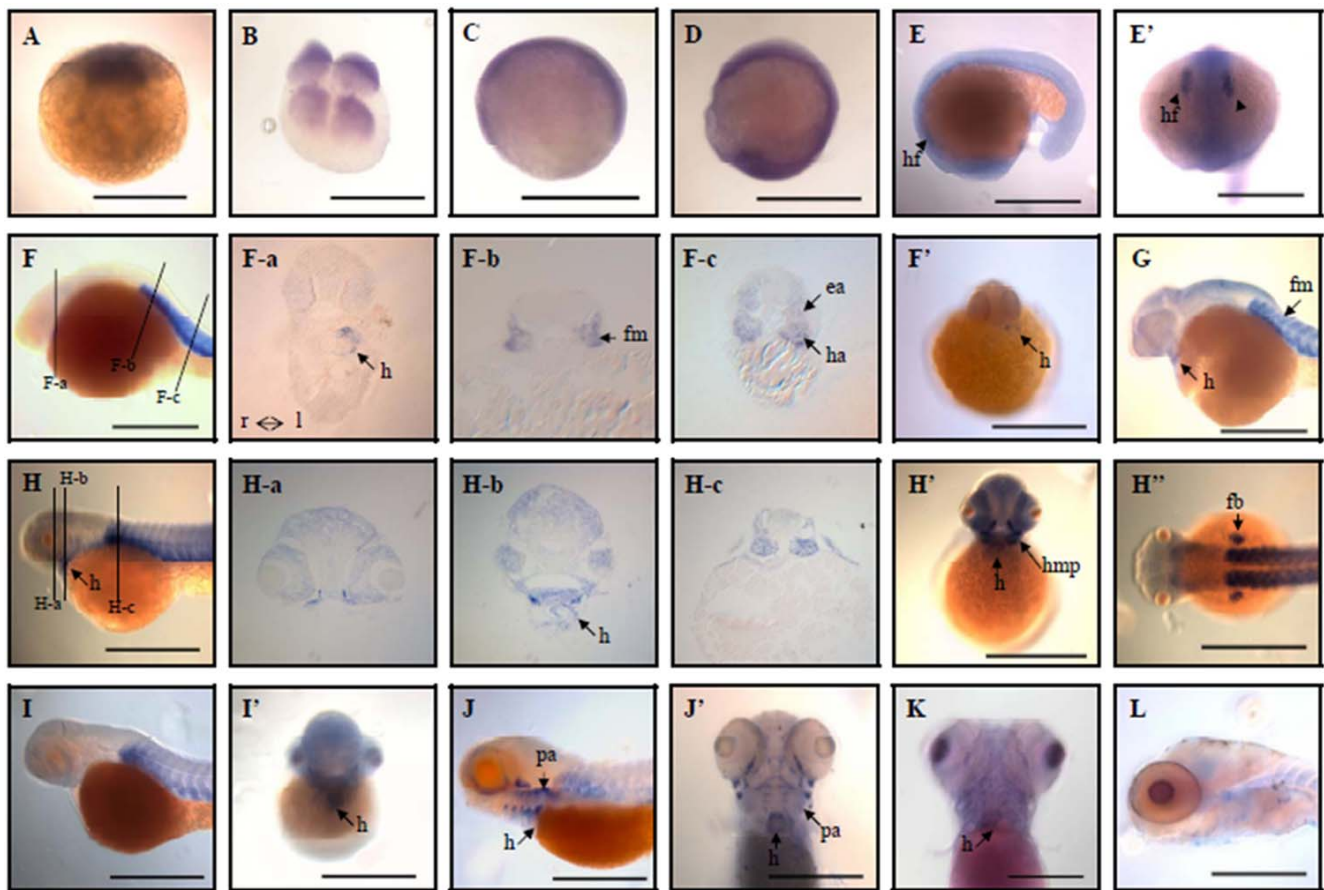
Again, the spatiotemporal expression patterns of *atp2a2a* during early development were examined by whole mount in situ



**Figure 4.** *atp2a1* expression during early embryonic stages. (A) One-cell and (B) 4-cell stages, lateral view. (C) At 6-hpf and (D) 12-hpf, lateral view. (E) At 18-hpf, lateral view, and (E') dorsal view. (F) At 20-hpf and (G) 24-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (G-a, G-b, G-c). (H) At 31-hpf and (I) 48-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (I-a, I-b, I-c). (I') At 48-hpf, ventral view, and (I'') dorsal view. (J) At 72-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (J-a, J-b, J-c). (J') At 72-hpf, ventral view, and (J'') dorsal view. (K) At 96-hpf, lateral, (K') ventral, and (K'') dorsal views. (L, M, N) At 5-7-dpf, lateral view. Scale bars, 250  $\mu$ m. (ea, epaxial muscle; fb, fin bud; fbp, fin bud precursor from somite number 4; fm, fast muscle; h, heart; ha, hypaxial muscle; hm, head muscle; hmp, head muscle progenitors; m, muscle; pa, pharyngeal arches)

hybridization. Results showed that zebrafish *atp2a2a* transcripts were first observed from 1-cell stage to cleavage period (Fig. 5A, B), and their expression extended from the gastrula period to the early segmentation stages (Fig. 5C, D). By 18-hpf, the zebrafish *atp2a2a* transcripts were detected in lateral plate mesoderm, and in the bilateral heart field (Fig. 5E, E'), and that cardiac cells expressing *atp2a2a* were fused in a single heart tube and elongated by convergent extension by 24- and 31-hpf (Fig. 5F', G). Cryosections of 24-hpf embryos revealed *atp2a2a* signals distributed in the entire somite, in-

cluding the presumptive epaxial and hypaxial muscle regions (Fig. 5F, F-a, -b, -c). By 48- and 72-hpf, zebrafish *atp2a2a* expressed strongly in trunk somites, fin buds, pharyngeal arches as well as in the heart (Fig. 5H, H', H'', I, I'). Again, cryosections revealed *atp2a2a* signals were detected in head muscle precursor, pharyngeal arches, heart regions as well as the presumptive epaxial and hypaxial muscles (Fig. 5H-a, -b, -c). Expression of zebrafish *atp2a2a* continued at 4-dpf in the all head muscles (Fig. 5J, J'). In contrast, the expression of *atp2a2a* was in a fainter manner in head and trunk region



**Figure 5.** *atp2a2a* expression during early embryonic stages. (A) One-celled and (B) 4-celled stages, lateral view. (C) At 6-hpf and (D) 12-hpf, lateral view. (E) At 18-hpf, lateral view, and (E') dorsal view. (F) At 24-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (F-a, F-b, F-c). (F') At 24-hpf, ventral view. (G) At 31-hpf and (H) 48-hpf, lateral view. Cross-sections along the plane indicated by lines were shown in (H-a, H-b, H-c). (H') At 48-hpf, ventral view, and (H'') dorsal view. (I) At 72-hpf, lateral view, and (I') ventral view. (J) At 96-hpf, lateral view, and (J') ventral view. (K) At 5-dpf, ventral view. (L) At 6-dpf, lateral view. Scale bars, 250  $\mu$ m. (ea, epaxial muscle; fb, fin bud; fm, fast muscle; h, heart; hmp, ha, hypaxial muscle; head muscle progenitors; hf, heart field; l, left direction; pa, pharyngeal arches; r, right direction.)

at 5-6 dpf (Figs. 5K, L). Specially, we found that the expression of *atp2a2a* was not significantly reduced in heart region (Fig. 5J' vs. 5K, arrows). On the basis of these observations, we conclude that zebrafish *atp2a2a* is a maternally inherited gene, restricting its expression in trunk muscles as well as in heart.

#### **Atp2a2a might play important roles during heart development of vertebrates**

In adult heart, *SERCA2* expression levels are highly associated with cardiac functions, therefore, *SERCA2* could be used as a marker for detection of cardiomyopathy (Castilho et al., 2007; Andersson et al., 2009; Dai and Rabinovitch, 2009; Kontaraki et al., 2010; Stokke et al., 2010; Summerfield et al., 2010). We have shown that *atp2a2a* transcripts were detected strongly in the developing heart of 18-96 hpf zebrafish embryos (Fig. 4). These observations conform to the previous study (Langenbacher et al., 2005) which described a

*SERCA2*-null zebrafish mutant, *tremblor* (*tre*), having heart defects such as contraction and fibrillation disorder. Furthermore, *ATP2A2*-null mutant mice were lethal, but heterozygous *SERCA2*<sup>-/-</sup> mice manifested impaired cardiac contractility and delayed cardiomyocyte relaxation (Periasamy et al., 1999). In human, it always appears that *SERCA2* is down-regulated in patients with heart failure, but for cardiomyopathy, it is more controversial. Taken together, we suggest that *Atp2a2a* might play important roles during heart development of vertebrates.

In conclusion, this study highlights the distinct expression pattern of two structurally related zebrafish *atp2a* genes, *atp2a1* and *atp2a2a*. They are both maternally inherited and muscle-restricted genes. Expression of *atp2a1* is mainly restricted in fast muscles whereas *atp2a2a* is restricted in fast muscle and heart. This information may provide more insight into the molecular structure and expression patterns of the lower vertebrate *atp2a* genes.

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