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The molecular structures and expression patterns of zebrafish troponin I genes

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ABSTRACT

Troponin I (TnnI), a constituent of the troponin complex located on the thin filament, provides a calcium-sensitive switch for striated muscle contraction. Cardiac TnnI is, therefore, a highly sensitive and specific marker of myocardial injury in acute coronary syndromes. The *TnnI* gene, which has been identified in birds and mammals, encodes the isoforms expressed in cardiac muscle, fast skeletal muscle and slow skeletal muscle. However, very little is known about the *TnnI* gene in lower vertebrates. Here, we cloned and characterized the molecular structures and expression patterns of three types of zebrafish *tnni* genes: *tnni1, tnni2* and *tnni-HC* (heart and craniofacial). Based on the unrooted radial gene tree analysis of the TnnI gene among vertebrates, the zebrafish TnnI1 and TnnI2 we cloned were homologous of the slow muscle TnnI1 and fast muscle TnnI2 of other vertebrates, respectively. In addition, reverse transcription-polymerase chain reaction (RT-PCR) and whole-mount *in situ* hybridization demonstrated that zebrafish *tnni1* and *tnni2* transcripts were not detectable in the somites until 16 h post-fertilization (hpf), after which they were identified as slow- and fast-muscle-specific, respectively. Interestingly, *tnni-HC*, a novel *tnni* isoform of zebrafish was expressed exclusively in heart during early cardiogenesis at 16 hpf, but then extended its expression in craniofacial muscle after 48 hpf. Thus, using zebrafish as our system model, it is suggested that the results, as noted above, may provide more insight into the molecular structure and expression patterns of the lower vertebrate *TnnI* gene.

1. Results and discussion

The troponin (Tn) complex provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction. It is composed of three subunits: troponin I (TnnI), troponin C (TnnC), and troponin T (TnnT). Each Tn is composed of multiple isoforms which are encoded by distinct genes and expressed in a tissue-specific manner. These isoforms are also regulated at different developmental stages. (Schiaffino et al., 1993; Parmacek and Leiden, 1991). Particularly, TnnI is a myofibrillar protein involved in the calcium regulation of contraction in cardiac and skeletal muscles (Wilkinson and Grand, 1978). It has multiple functional domains that are distinct and bind with high affinity to actin (Potter and Gergely, 1974) and TnnC (Head and Perry, 1974). The interactions of these domains are regulated by actomyosin ATPase activity in resting and contracting muscle (Wilkinson et al., 1972). TnnI also interacts functionally with other muscle proteins, including TnnT, and the specific domains that are involved in other interactions have been identified (Horwitz et al., 1979). In birds and mammals, there are three different muscle fiber-type specific isoforms: a slow-twitch type (TnnIs/TnnI1), a fast-twitch type (TnnIf/TnnI2) and a cardiac type (TnnIc/TnnI3) (Toyota and Shimada, 1981; Wade et al., 1990). Each type is encoded by a single specific gene (Guenet et al., 1996).

TnnIs is transiently expressed in the developing heart of both birds and mammals (Hastings, 1996; Sabry and Dhoot, 1989; Saggin et al., 1989; Murphy et al., 1991; Gorza et al., 1993). In mammals, the transient expression of *TnnIs* in the heart is under the control of a developmentally regulated program of gene transcription (Huang et al., 2000). In contrast, although *Xenopus TnnIs* is expressed in the somites and skeletal muscles, it is not expressed in the developing heart (Warkman and Atkinson, 2002), indicating that the developmental expression pattern of the amphibian *TnnIs* gene is quite different from the mammalian and avian *TnnIs* homolog. The lines of evidences reveal that the structure and function of TnnI gene among different species may exhibit a different and complicate manner. Therefore, the first step, it is important to elucidate the molecular structure and expression pattern of the *TnnI* gene in the fish.

As a result of the ease of manipulating gene transfer, the transparency of embryos and feasibility of mutagenesis, the zebrafish (*Danio rerio*) is an excellent model for studying the developmental and fiber-specific regulation of muscle diversity (Grunwald and Eisen, 2002). Most importantly, the processes of cardiac and skeletal muscle commitment, differentiation, and maturation are observable directly *in vivo* (Briggs, 2002). Secondly, the organization of slow-twitch and fast-twitch muscles in fish is more homogeneous than that of birds and mammals. In fish, slow- and fast-twitch

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muscles are spatially separated; slow muscle forms a superficial layer, whereas fast muscle forms a deep layer (Waterman, 1969; Koumans and Akster, 1995), which makes it easy to assay developmental regulation of muscle-fiber specificity.

Because it is still unknown about the molecular structures and expression patterns of zebrafish *tnni* gene during embryonic development, we initiated the present study. Here, we report the cloning of three distinct fiber-types of zebrafish *tnni* genes. *tnni1* and *tnni2* were characterized as slow- and fast-muscle-specific, respectively. In addition, a novel *tnni* gene isoform, *tnni-HC*, was found to be exclusively expressed in heart during early cardiogenesis at 16 hours of post-fertilization (hpf). Interestingly enough, we found that *tnni-HC* was also expressed in craniofacial muscles after 48 hpf.

1.1. Identification and characterization of three types of zebrafish tnni gene

To clone the full-length of zebrafish *tnni* cDNAs, we used two degenerated primers, *tnni*-DF and *tnni*-DR, and obtained three RT-PCR produces with the molecular masses of 180-, 189- and 184-bp. After we performed BLAST search, three zebrafish EST clones corresponding Accession no. EH612065, EE686691 and CV126661, were found from zebrafish EST clone database. On the basis of these EST sequences, we designed specific primers to amplify the putative zebrafish *tnni* cDNAs by RACE. Consequently, three full-length cDNAs of zebrafish *tnni* were identified: *tnni1* was 930-bp containing a 543-bp open reading frame, *tnni2* was 755-bp containing a 531-bp open reading frame, and *tnni-HC* was 961-bp containing a 549-bp open reading frame.

The deduced amino acid sequence of three zebrafish TnnI were compared with other vertebrates, the data showed that the deduced amino acid sequence of zebrafish TnnI1 shared 59–62%, 48–53%, 55–57% and 49–51% identities with vertebrates TnnIs (slow-twitch muscle fibers), TnnIf (fast-twitch muscle fibers), TnnIc (cardiac muscle) and *Ciona* TnnI (heart TnnI, and body well TnnI), respectively (Table 1). Meanwhile, the deduced amino acid sequence of zebrafish TnnI2 shared 55%, 59–69%, 53–57% and 53% identities with vertebrates TnnIs, TnnIf, TnnIc and invertebrates TnnI, respectively (Table 1). These evidences indicate that zebrafish TnnI1 and TnnI2 was quite similar to TnnIs and TnnIf, respectively. However, the zebrafish TnnI-HC shared 63–81% identities with vertebrates TnnIs.

It has been reported that TnnI consists of 181–211 deduced amino acid residues and that the cardiac isoform TnnIc is the largest because it contains an extra 30-amino-acid at the N-terminus (Perry, 1999). When the deduced amino acid residues of the three types of zebrafish TnnI were aligned with the counterpart of other vertebrates and, we found that there are some highly conserved domains such as C-domain of TnnC binding site, TnnT-binding site, Actin/TnnC-tropomyosin binding site, N-domain of TnnC-binding site and Actin–tropomyosin binding site (Fig. 1). However, not one of the TnnI zebrafish genes contained a 30-amino-acid N-terminal amino acid extension domain, a phenomenon that otherwise distinguishes the typical TnnIc isoform from the other TnnI isoforms of known species (Hastings et al., 1991). We compared the N-terminal amino acid sequences of zebrafish TnnI genes with those known TnnIc polypeptides of *Ciona* (AAL27686), *Xenopus* (AAA65727), chicken (NM_213570), mice (NM_009406) and humans (NM_000363).We found that the N-terminal amino acid sequence of zebrafish TnnI did not resemble the counterpart of vertebrate TnnIc such as (1) a Glu-rich domain located at the N-terminus, which was prominent in the TnnIc of *Ciona* and *Xenopus*, but neither in bird TnnIc nor mammal TnnIc; (2) a Pro-rich/hydrophobic/basic motif, and (3) an AXEXH motif (MacLean et al., 1997) (Supplementary data). These evidences indicate that three types of zebrafish TnnI sequence lacked N-terminal sequence that was conserved among vertebrate TnnIc sequences.

According to ZFIN database, there were five *tnni2* genes such as *tnni2a.1* (NM_001007365), *tnni2a.2* (NM_201093), *tnni2a.3* (NM_205575), *tnni2b.1* (NM_001017587) and *tnni2b.2* (NM_001003423). When these five *tnni2* genes were compared with three types of zebrafish *tnni* described in this study, results showed that these five *tnni2* genes and zebrafish *tnni2* gene are categorized as fast muscle fiber-type. The amino acid similarity between zebrafish TnnI2 and other five TnnI2 ranged 64–81%.

1.2. Phylogenetic analysis of TnnI

To examine the evolutionary relationship between teleost and other species TnnI, we constructed a phylogenetic tree based on the deduced amino acid residues of the three types of zebrafish TnnI (Fig. 2). Results showed that zebrafish TnnI1 and TnnI2 were clustered with their tetrapod counterparts into two distinct mono-

Table 1
Identity of zebrafish TnnI1, TnnI2, and TnnI-HC with other vertebrates and invertebrates.

TnnI	Species (GenBank No.)	Zebrafish TnnI1 (NM_001002101) (Identity %)	Zebrafish TnnI2 (NM_001009901) (Identity %)	Zebrafish TnnI-HC (NM_001008613) (Identity %)
TnnIs	*Xenopus laevis* (AAL86906)	62	55	63
	Rattus norvegicus (NP_058880)	59	55	81
	Mus musculus (NP_067442)	59	55	81
	Homo sapiens (AAA61228)	59	55	79
TnnIf	*Xenopus laevis* (AAL86905)	53	64	63
	Rattus norvegicus (P27768)	48	60	58
	Mus musculus (NP_033431)	49	60	59
	Homo sapiens (AAH32148)	48	59	57
	Salmo salar (NM_001123661)	49	69	54
	Clupea harengus (U20112)	50	67	51
TnnIc	*Xenopus laevis* (AAA65727)	56	57	64
	Rattus norvegicus (NM_017144)	55	53	60
	Mus musculus (NM_009406)	55	53	59
	Homo sapiens (NM_000363)	57	55	62
TnnI-heart	*Ciona intestinalis* (AAN87358)	50	53	57
TnnI-body well	*Ciona intestinalis* (AAL27686)	51	53	57

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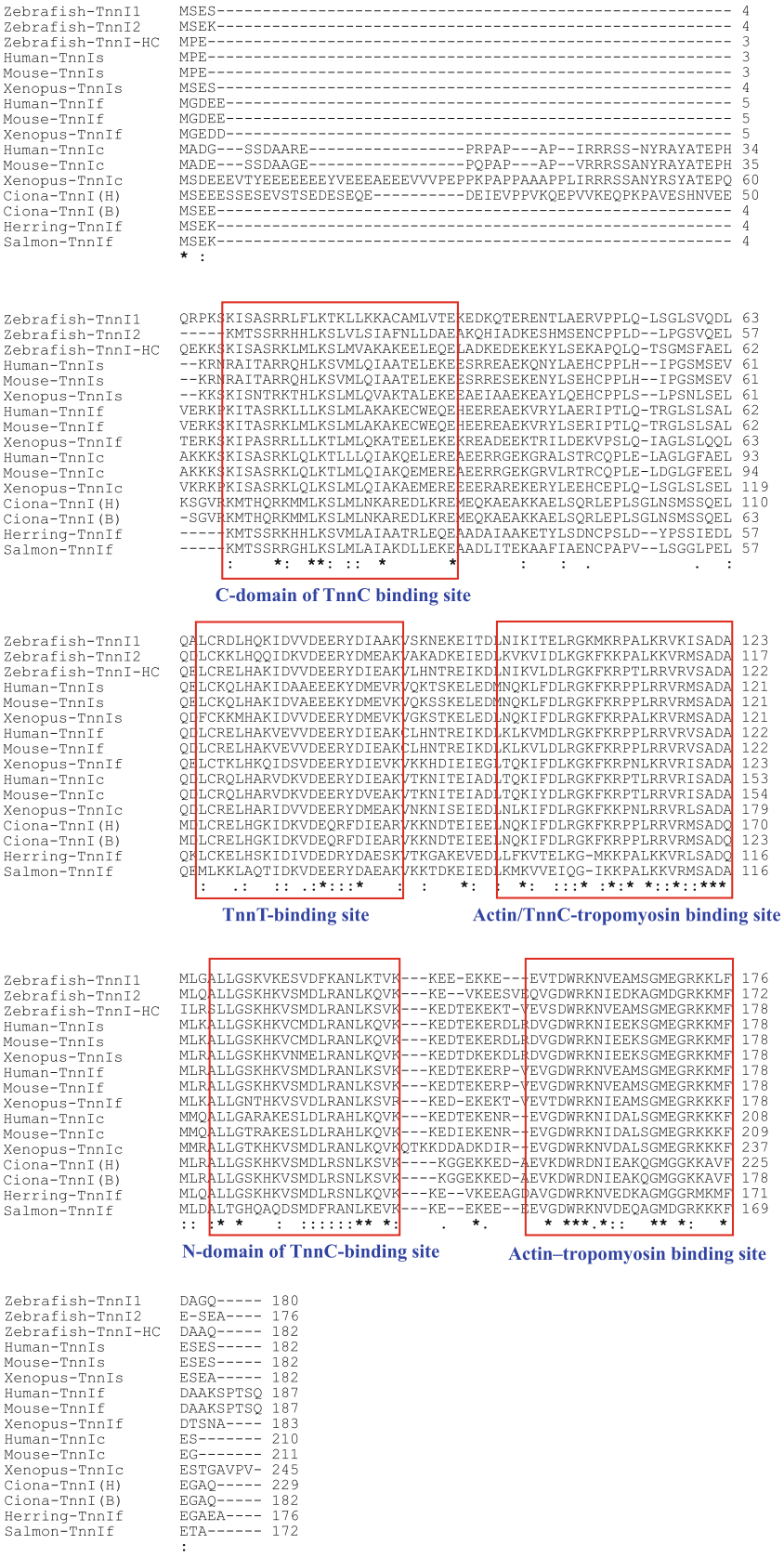

Fig. 1. The deduced amino acid sequences of three types of zebrafish TnnI compared with other TnnI from vertebrates. The alignment of amino acid sequences of three types TnnI by CLUSTALW (1.83). The conserved regions are boxed with red, including (1) C-domain of TnnC binding site, (2) TnnT-binding site, (3) Actin/TnnC-tropomyosin binding site, (4) N-domain of TnnC-binding site, (5) Actin–tropomyosin binding site. Asterisks, two dots and one dot were indicated that amino acid residues were 100%, 75%, 50% conserved among all species, respectively.

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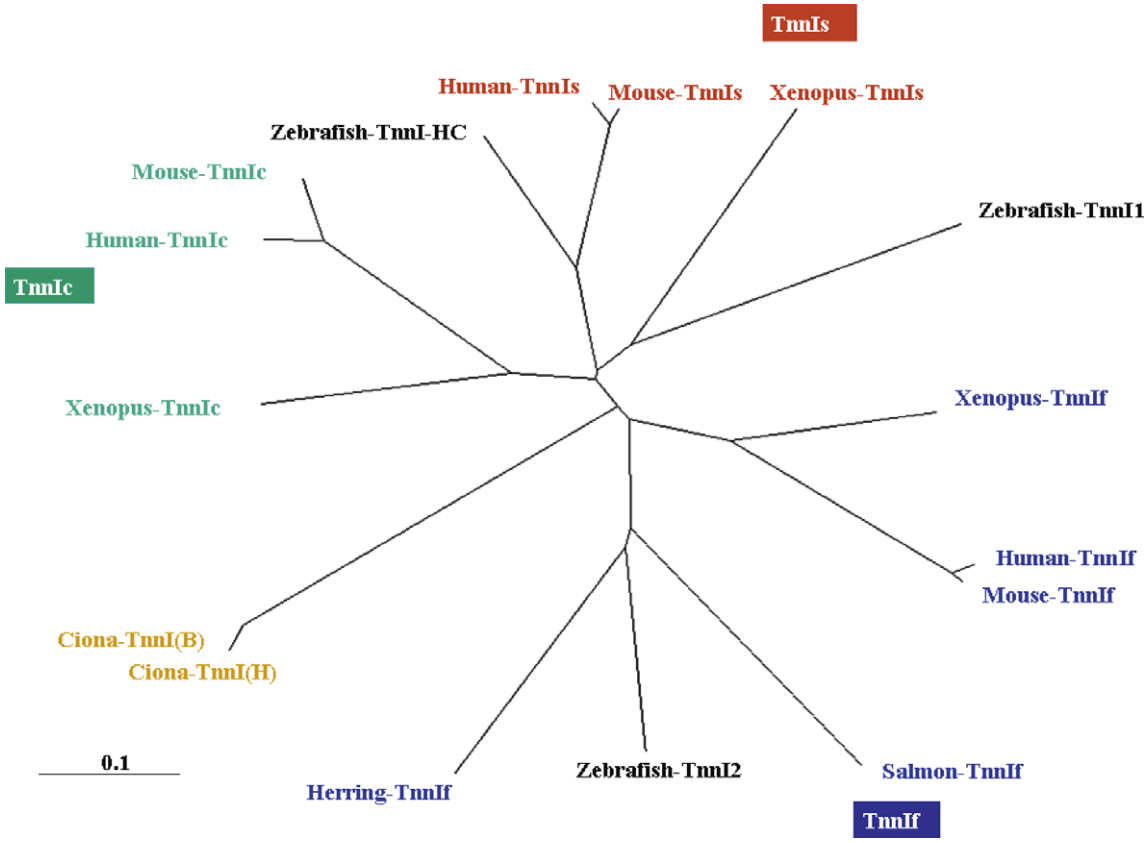

Fig. 2. An unrooted radial gene tree of TnnI among vertebrates. The gene tree was constructed with the neighbor-joining method (Pearson et al., 1999), using 1000 bootstrap values. The marker length of 0.1 corresponds to 10% sequence difference. The TnnIs, TnnIf, TnnIc clades, and *Ciona* were marked in red, blue, green, and brown, respectively. See Section 2 for details on the sources of TnnI. This phylogenic tree indicates that Zebrafish TnnI1 and TnnI2 clustered with their tetrapod counterparts into two distinct monophyletic groups. Especially, Zebrafish TnnI-HC was belonged to the tetrapod TnnIs monophyletic group, and The *Ciona* TnnI was unique monophyletic groups.

phyletic groups. Interestingly, zebrafish TnnI-HC was belonged to the tetrapod TnnIs monophyletic group. Further study of this unusual characteristic should provide additional insight into the molecular structure of TnnI genes.

1.3. Temporal expression of tnni in zebrafish

We used specific primers to detect the temporal expressions of the three types of zebrafish *tnni* gene transcripts by RT-PCR. Total RNAs were extracted from zebrafish embryos at 10 stages, ranging from 1-, 4-, 8-, 10-, 12-, 14-, 15-, 16-, 24- and 48-hpf. As shown in Fig. 3, we were able to detect all three types of zebrafish *tnni* gene at 16 hpf through 48 hpf (Fig. 3), indicating that zebrafish *tnni* gene transcripts are neither maternally expressed, nor a very early embryonic marker.

1.4. Spatial expression of tnni1 in zebrafish embryos

The deduced amino acid sequence of zebrafish TnnI1 is quite similar to that of quail (Hastings, 1996), mice (Barton et al., 2000) and humans (Wade et al., 1990). In mammals and birds, *TnnIs* transcripts are transiently expressed in the developing heart during the early embryogenic stage. At later stages, *TnnIs* are expressed exclusively in skeletal muscle (Trimmer et al., 1989). Unlike the *TnnIs* in mammals and birds, the amphibian *TnnIs* gene is expressed in the somites and skeletal muscles, but not expressed in the developing heart (Warkman and Atkinson, 2002). To examine the spatial expression of zebrafish *tnni1*, whole-mount *in situ* hybridization (WISH) was performed on zebrafish embryos collected from 20 to 96 hpf. The data showed that zebrafish *tnni1* transcripts were detected specifically in somites at 20 and 24 hpf

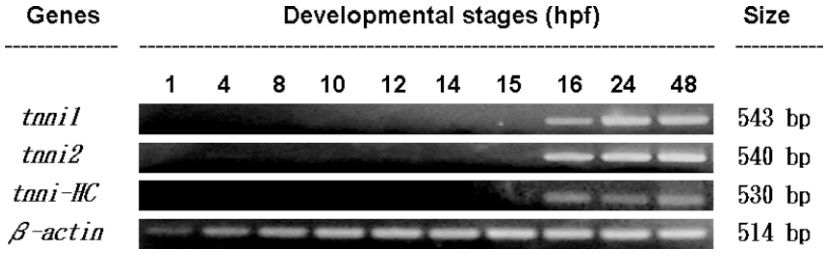

Fig. 3. Using reverse transcriptase-polymerase chain reaction (RT-PCR) to detect the temporal expression patterns of zebrafish *tnni* genes during the early development. Total RNA was isolated from different developmental stages (hours of post-fertilization; hpf) as indicated. Specific primers were designed for detecting the existence of *tnni1*, *tnni2*, and *tnni-HC* transcripts. The expected molecular size of each DNA fragment after RT-PCR amplification was indicated on the right. Three types of *tnni* genes started to be transcribed in 16 hpf. The β-*actin* transcript was used as an internal control.

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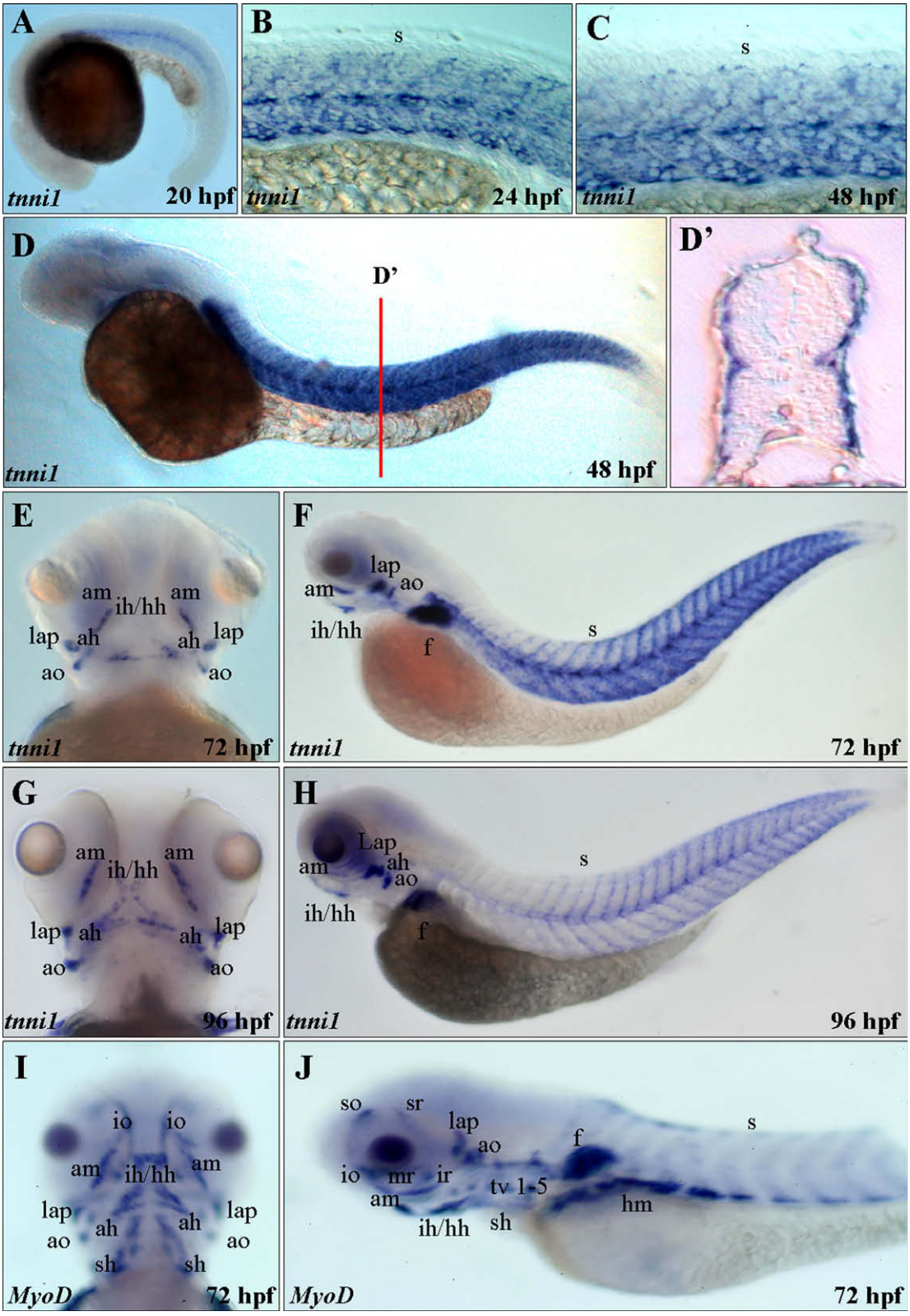

Fig. 4. The expression pattern of *tnni1* transcript during the development of zebrafish embryos. Embryos at different stages as indicated were collected and hybridized with *tnni1* using whole-mount *in situ* hybridization. Panels A, B, C, D, F and H were lateral views and anterior of embryo to the left; panel E and G were dorsal view and anterior of embryo to the top. *tnni1* was expressed in the somite at 20–48 hpf, but it was expressed in craniofacial muscle and fin at 72–96 hpf. Panel D and D′ were whole-mount *in situ* hybridization and transverse section (red line) at 48 hpf Embryos. Panel D′ was indicated that *tnni1* gene was slow-muscle-specific. Panels I and J were hybridized with *MyoD*, specific marker for craniofacial muscles and truck muscle at 72 hpf Embryos. am, adductor mandibulae; lap, levator arcus palatini; ao, adductor operculi; ah, adductor hyoideus; ih, interhyoideus; hh, hyohyoideus; s, somite; f, fin bud; tv 1–5, transversus ventralis 1–5.

(Fig. 4A and B) during early somitogenesis and were also detectable at 48 hpf (Fig. 4C and D). Histochemical section of trunk was also performed to confirm the expression of zebrafish *tnni1* at cell level. This analysis showed that zebrafish *tnni1* is expressed in a slow-

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Table 2
The comparison of expression in the craniofacial muscle and other regions among the three zebrafish *tnni* genes.

	Zebrafish *tnni1*	Zebrafish *tnni2*	Zebrafish *tnni-HC*
16–48 hpf	S	S	H
72 hpf	S, f, lap, ah, ao, ih, hh, am	S, f, am, sh, hm	H, so, sr, io, ir, mr, lr, ih, hh, sh, hm, imp, ima
96 hpf	S, f, lap, ah, ao, ih, hh, am	S, f, am, sh, hm, lap, ao	H, so, sr, io, ir, mr, lr, ih, hh, sh, hm, imp, ima

ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; mr, medial rectus; sh, sternohyoideus; so, superior oblique; sr, superior rectus; S, somite; H, heart; f, fin bud.

twitch muscle-fiber-specific manner until 48 hpf (Fig. 4D′). Interestingly enough, similar to expression patterns of mammals and amphibian *Tnnl1* (Zhu et al., 1995; Warkman and Atkinson, 2002), zebrafish *tnni1* was specifically and uniformly detected in truck somites from 20 to 96 hpf (Fig. 4F and H), In addition, *tnni1* was up-regulated in fin buds and jaw and head muscles, such as

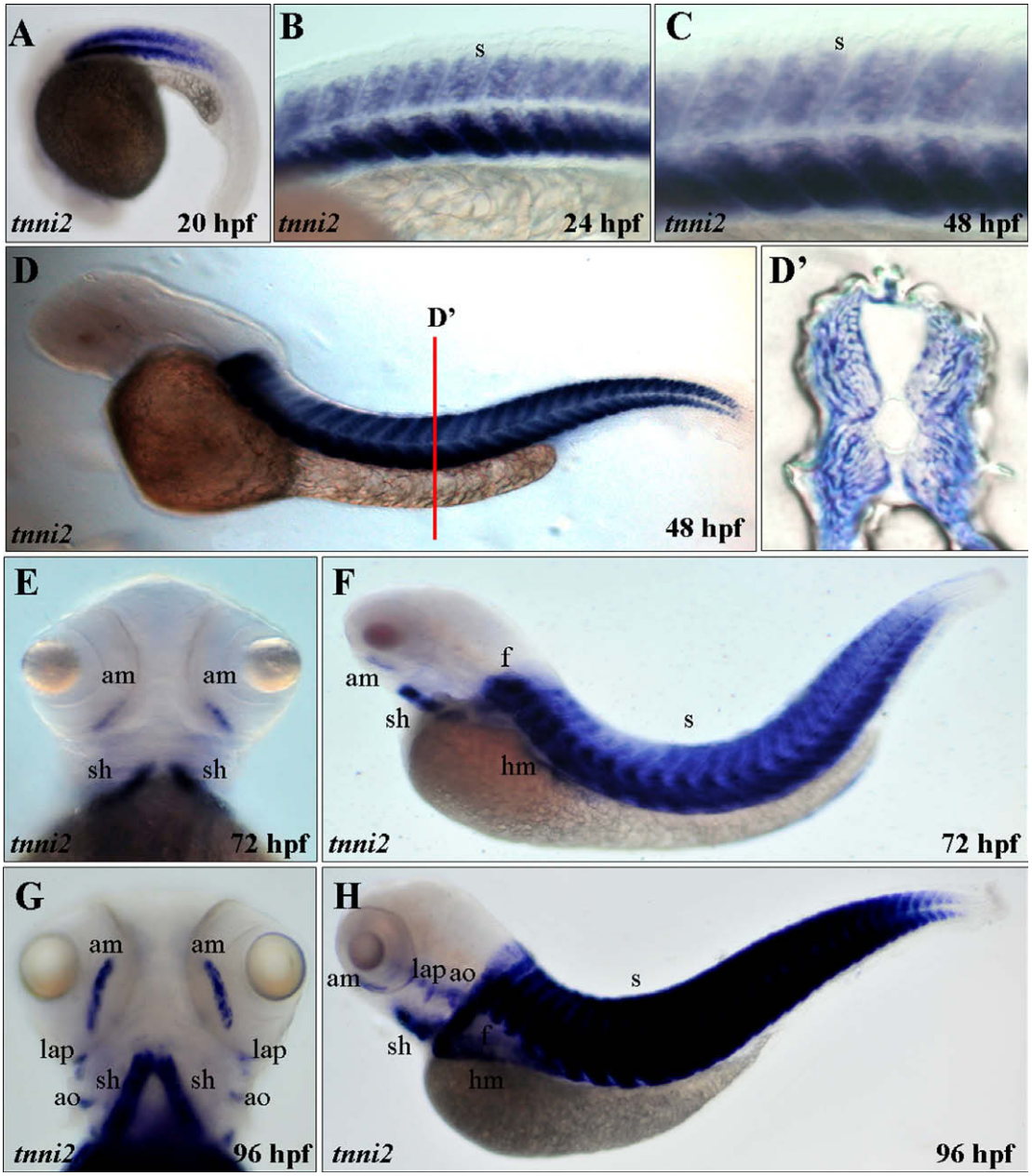

Fig. 5. The expression pattern of *tnni2* transcript during the development of zebrafish embryos. Embryos at different stages as indicated were collected and hybridized with *tnni2* using whole-mount *in situ* hybridization. Panels A, B, C, D, F and H were lateral views and anterior of embryo to the left; and panel E and G were dorsal view and anterior of embryo to the top. *tnni2* was expressed in the somite at 20–48 hpf, then it was expressed in craniofacial muscle and fin at 72–96 hpf. Panel D and D′ were whole-mount *in situ* hybridization and transverse section (red line) at 48 hpf Embryos. Panel D′ was indicated that *tnni2* gene was fast-muscle-specific. am, adductor mandibulae; lap, levator arcus palatini; ao, adductor operculi; hm, hapaxial muscles; sh, sternohyoideus; s, somite; f, fin bud.

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adductor mandibulae (am), levator arcus palatine (lap) adductor operculi (ao), adductor hyoideus (ah), interhyoideus (ih), hyohyoideus (hh) after 48 hpf (Fig. 4F and H and Table 2). This evidence indicates that the expression of zebrafish *tnni1* was different from that of mammals and birds, but similar to amphibian counterparts.

1.5. Spatial expression of tnni2 in zebrafish embryos

The amino acid sequence of zebrafish TnnI2 exhibited a high degree of identity to that of quail (Baldwin et al., 1985), mice (Koppe et al., 1989), humans (Zhu et al., 1994), amphibian (Warkman and Atkinson, 2002) and fish (Hodgson et al., 1996 and Jackman et al., 1998). In mammals, the expression of *TnnI2* is in skeletal muscle (Trimmer et al., 1989), but it is not expressed in heart. In order to study the spatial expression pattern of zebrafish *tnni2*, we performed WISH for zebrafish embryos collected from 20 to 96 hpf. Results showed that the zebrafish *tnni2* transcript was specifically detected in somites at 20 hpf (Fig. 5A and B), and it was expressed continuously up to 48 hpf (Fig. 5C and D). Histochemical examination revealed that zebrafish *tnni2* transcript displayed clearly in fast-twitch muscle fibers (Fig. 5D′). Similar to the expression patterns of zebrafish *tnni1* during early embryogenesis, zebrafish *tnni2* not only was specifically and uniformly detected in truck somites from 20 to 96 hpf (Fig. 5F and H), However, unlike *tnni1* expression in craniofacial muscle, *tnni2* was expressed in sternohyoideus (sh), but *tnni2* was absent in ih, hh, and ah (Fig. 5E and G and Table 2). These data suggest that the spatial expression of zebrafish *tnni2* was similar to that of mammals and other vertebrates.

1.6. Spatial expression of tnni-HC in zebrafish embryos

In heart development, the slow-twitch muscle fiber isoform of vertebrates, *TnnIs*, is predominantly expressed in hearts during embryogenesis. However, *TnnIs* is completely replaced by *TnnIc*, which is a cardiac-specific isoform (Huang et al., 2000). To examine the spatial expression pattern of the zebrafish *tnni-HC* isoform we found in this study, we carried out WISH for zebrafish embryos from 16 to 96 hpf. Results showed that the *tnni-HC* transcript was first detected in the lateral plated mesoderm (Fig. 6A) at 16 hpf, and then it was expressed in a single heart cone at 18 hpf (Fig. 6B). From 24 hpf onward, the *tnni-HC* transcript was expressed in the heart tube (Fig. 6C). It was specifically and uniformly detected in both atrium and ventricle from 48 to 96 hpf (Fig. 6D–G). We also noticed that *tnni-HC* was detected in craniofacial muscles, such as, lap, ah, ih, hh, sh, hm, medial rectus (mr), lateral rectus (lr), superior oblique (so), inferior oblique (io), superior rectus (sr), inferior rectus (ir) intermandibularis posterior (imp), intermandibularis anterior (ima) after 48 hpf (Fig. 6E,F and G and Table 2). These data indicates that *tnni-HC* was expressed in early heart development stage and craniofacial muscles development stage. To further examine how *tnni-HC* was expressed in the zebrafish adult heart, we performed ISH on the histological section of zebrafish adult heart using *tnni-HC* RNA as a probe. Results showed that the *tnni-HC* transcript was detected in the ventricle trabeculae of adult heart (Fig. 7C). These data indicated that *tnni-HC* was present in early heart development and continues its expression until adulthood. Thus, it appears that the heart of zebrafish does not undergo the developmental switch from *TnnIs* to *TnnIc* that is characteristic of the higher vertebrates. Instead, zebrafish express only *tnni-HC*, which is a unique isoform that is totally different from what is known in mammals, amphibians or avian homologues. Moreover, phylogenetic analysis also reveals that TnnI-HC was not belonged to the tetrapod TnnIc monophyletic group. This finding gives further evidence that zebrafish may not have a cardiac-specific isoform, but replace by a slow muscle isoform, *tnni-HC*. These hypotheses merit further investigation.

1.7. Conclusion

In this study, we cloned and characterized the molecular structures and expression patterns of three types of zebrafish *tnni* genes: *tnni1, tnni2* and *tnni-HC*. In comparing our zebrafish system model to higher vertebrates, we found that the molecular structures and expression patterns of *TnnI* orthologous genes among

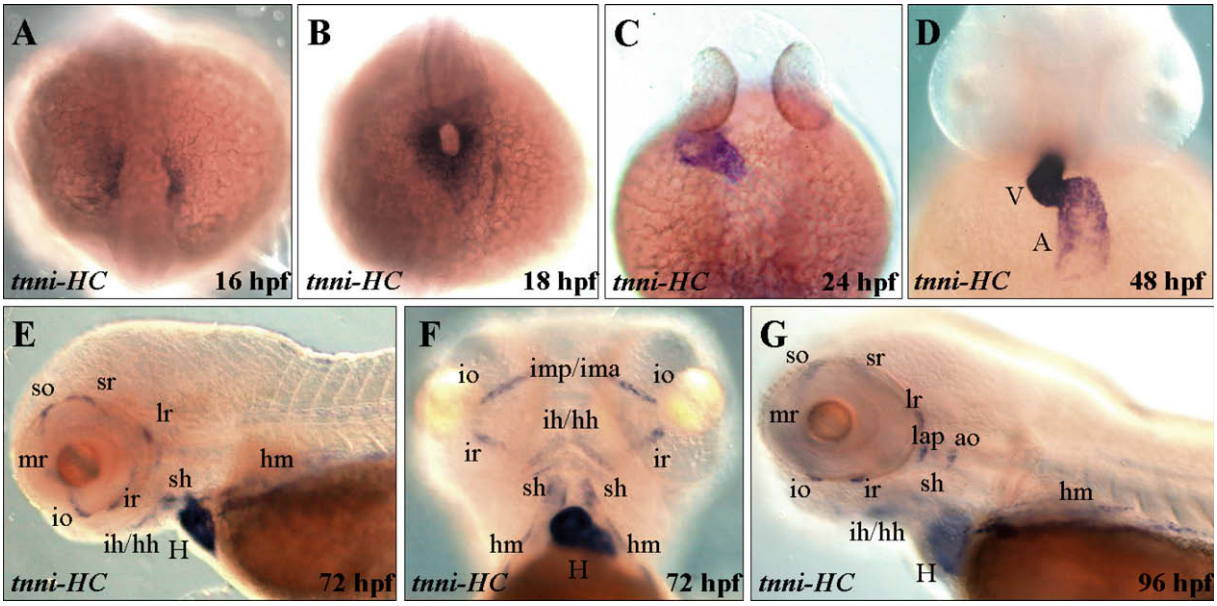

Fig. 6. The expression pattern of *tnni-HC* transcript during the development of zebrafish embryos. Embryos at different stages as indicated were collected and hybridized with *tnni-HC* using whole-mount *in situ* hybridization. Panels A, B, C, D and F were dorsal view, anterior of embryo to the top; and panels E and G were lateral views, anterior of embryo to the left. *tnni-HC* was expressed in the heart exclusively during early cardiogenesis at 16–48 hpf, but it was not only in the craniofacial muscle but also in the heart after 48 hpf. so, superior oblique; io, inferior oblique; sr, superior rectus; ir, inferior rectus; lr, lateral rectus; mr, medial rectus; sh, sternohyoideus; lap, levator arcus palatini; ao, adductor hyoideus; imp, intermandibularis posterior; ima, intermandibularis anterior; ih, interhyoideus; hh, hyohyoideus; hm, hapaxial muscles; V, ventricle; A, atrium.

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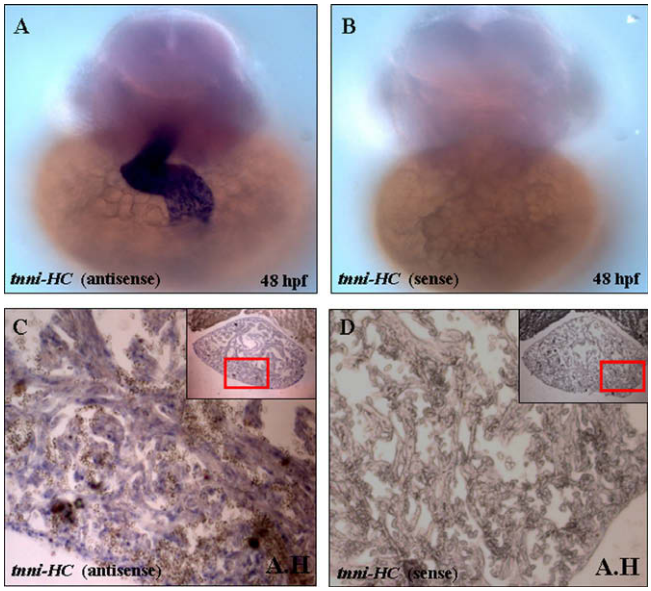

Fig. 7. The *tnni-HC* transcript was expressed in the adult heart of zebrafish. Embryos at 48 hpf were collected and hybridized with either antisense *tnni-HC* probe or sense *tnni-HC* probe using whole-mount *in situ* hybridization. Strong signal was observed in the heart of 48-hpf embryos when antisense probe was used (A), but no signal was observed for using sense probe (B). Adult heart was sectioned and also hybridized with either antisense probe or sense probe of *tnni-HC*. Like *tnni-HC* expression in embryos, positive signal was observed in the adult heart when antisense probe was used (C), but no signal was observed for using sense probe (D).

zebrafish and vertebrates are partially conserved. Thus, for example, when we compared the molecular structures and expression patterns of *tnni1* and *tnni2* genes in truck and craniofacial muscles of zebrafish with those of *TnnIs* and *TnnIf*, respectively, of other vertebrates, we found that they were highly conserved. However, there were a highly difference between vertebrate *TnnIc* and zebrafish *tnni-HC*, which was a unique isoform that has not been reported in mammals, amphibians, or avian homologues. This finding is particularly important in terms of cardiac development during embryogenesis because cardiac TnnI is commonly served as a specific marker of myocardial injury in acute coronary syndromes. Therefore, the present study lays the empirical groundwork for further study required to understand the biological function and the regulatory mechanism that explains TnnI muscle fiber diversification in lower vertebrates, in general, and how the *tnni-HC* gene switches its expression from heart to craniofacial muscles, in specific. From a larger perspective, such understanding will provide more insight into the evolutionary implications of *TnnI* genes in both lower and higher vertebrates.

2. Experimental procedures

2.1. Nucleic acids extraction and cDNA library synthesis

To obtain total RNA, zebrafish embryos aged 48–72 hpf were collected and immediately stored in liquid nitrogen. The frozen embryos were homogenized with TRIzol reagent (Bio-Rad) and their total RNAs were extracted according to the manufacturer's instructions. First strand cDNA was synthesized from 3 ng of total RNA using a SuperScript II (Invitrogen).

2.2. Molecular cloning of tnni cDNAs

To clone cDNA of zebrafish *tnni*, degenerated primers were designed on the basis of the conserved polynucleotide sequences of known EST clones or cDNA of *tnni*, such as the forward primer *tnni*-DF, G(T/G/C)(A/C/G)T(A/C/G)TC(G/T/A)GC(A/C/T)GA (T/C)GC (C/T) ATG, and a reverse primer *tnni*-DR, TTCTT(C/G/T)C(G/T) GCC(T/C) TCCAT(A/T/G)CC. Thirty-five cycles of PCR amplification were performed by EXTaq DNA polymerase (Takara). Each cycle consisted of denaturation for 40 s at 94 °C, 1 min of annealing at 57 °C, and 30 s of extension at 72 °C. The last extension step was extended for 10 min at 72 °C. All PCR fragments were ligated with pGEM T-Easy vector (Promega) and transformed into *Escherichia coli* DH5α and sequenced.

2.3. Rapid amplifcation of cDNA ends (RACE)

To get the full-length cDNA, RACE was used by following the procedures described by Chen et al. (2001). The 5′- and 3′-RACE were performed with zebrafish-EST-specific primers. For 3′-RACE, *tnni1*-F (ATGTCTGAGTCCCAGAGACC), *tnni2*-F (ATGTCAGAAAAAAA GAGGAC), *tnni-HC*-F (ATGCCCGAGCAAGAGAAAAA) were used; whereas, for 5′-RACE, *tnni1*-R (TTATTGTCCAGCATCAAACA), *tnni2*-R (TTAAGCCTCGGACTCAAACA), *tnni-HC*-R (TTATTGTGCT-GCATCAA ACA) were used. After amplification, the PCR produces were subcloned and sequenced as described above.

2.4. Bioinformatics analysis of TnnI sequences

The EST database from NCBI (http://www.ncbi.nlm.nih.gov/) was used to search for sequence annotations indicative of possible homology to zebrafish *tnni*. Nucleotide sequences were translated by using the sequence available through the BCM Search Launcher interface (http://searchlauncher.bcm.tmc.edu). Multiple sequence alignment of the deduced amino acid sequences of TnnI was performed using ClustalW (Thompson et al., 1994), and phylogenetic trees were constructed by using the neighbor-joining method (Pearson et al., 1999) through the EMBL–EBI interface (http://www.ebi.ac.uk/Tools/clustalw/). The accession numbers of sequences used in Fig. 2 and Table 1 are GenBank Human-TnnIs: AAA61228, Rat-TnnIs: NP_058880, Mus-TnnIs: NP_067442, Xenopus-TnnIs: AAL86906, Human-TnnIf: AAH32148, Rat-TnnIf: P27768, Mus-TnnIf: NP_033431, Xenopus-TnnIf: AAL86905, Human-TnnIc: NM_ 000363, Rat-TnnIc: NM_017144, Mus-TnnIc: NM_009406, Xenopus-TnnIc: AAA65727, Ciona-(Heart): AAN87358, Ciona-(Body well): AAL27686, Salmo-TnnIf: NM_001123661, Herring-TnnIf: U20112.

2.5. RT-PCR analysis

To detect the spatial and temporal expressions of zebrafish *tnni* isoforms, RT-PCR was performed by using the different specific primers as described in Section 2.2 above. The total RNAs were extracted from embryos at 1, 4, 8, 10, 12, 14, 15, 16, 24 and 48 hpf. The primer pairs of *tnni1*-F and *tnni1*-R, *tnni2*-F and *tnni2*-R, and *tnni-HC*-F and *tnni-HC*-R were used to amplify cDNA fragments of 543, 540, and 530, respectively. In order to avoid the contamination from genomic DNA, we used RNase-free DNase I to digest the total extracted RNAs before RT-PCR was performed. Additionally, we designed primers, which are corresponding to the flanking intron 1, to serve as controls to amplify the contaminated genomic DNA such as *tnni1*-intron 1-F (GGAGAAACAGGTATGAACTATTGT-ACTATT) and *tnni1*-intron 1-R (TAGTCAAAATCTGAAATATAAAGA-TTGGTG); *tnni2*-intron 1-F (AGATGTCAGAGTAAGTATTCAGATGT-TTTG) and *tnni2*-intron 1-R (CATCTTTTTTCTGTTCATGAACATGA-GTTC); and *tnni-HC*-intron 1-F (AGCACTACAAGGTAAGTTC-ATTCTGTTTGC) and *tnni-HC*-intron 1-R (TGCTTCAACCTAAGGG-ATAAAAATA AAAGT). We used the amplification of zebrafish β-*actin* (Kelly and Reversade, 1997) to serve as an RNA quality control in each tissue sample.

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2.6. WISH

WISH of whole embryos was performed by using digoxigenin (DIG)-labeled riboprobes of *tnni1*, *tnni2* and *tnni-HC*. We followed the procedures as described by Thisse and Thisse (2008). Stained embryos were placed in 100% glycerol and evaluated with a differential interference contrast microscope (DMR, Leica) with a color digital camera (COOLPIX 996, Nikon) attached. For histological examination, some stained embryos were embedded in optimum cutting temperature compound and sectioned at 10-nm intervals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gep.2009.02.001.

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