論 文 の 内 容 の 要 旨

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Molecular biological studies on myomiRs and their host myosin heavy chain genes underlying fish muscle development and growth

(魚類筋肉の発生と成長過程で働く myomiR とその宿主ミオシン重鎖遺伝子に関する分子生物学的研究)

Vertebrate skeletal muscle is a heterogeneous tissue. Skeletal muscle consists of various types of muscle fibers such as slow and fast ones, where muscle fiber-type specification is crucial for the development and growth of skeletal muscle. Fish skeletal muscle is an attractive model to study the mechanisms underlying muscle fiber-type specification because slow and fast muscles are segregated in the trunk myotome. Myosin is the major contractile protein in muscle tissues, the molecule which consists of two heavy chains (myosin heavy chains, MYHs) and four light chains. MYH gene (*MYH*) is a multigene family and different expression of *MYHs* leads to the formation of different muscle fiber-types. Among *MYH* family genes, three *MYHs* named *MYH6*, *MYH7* and *MYH14* (also called *MYH7b*) have been characterized by existence of microRNA (miRNA) in their introns. These *MYH*-encoded intronic miRNAs are called as myomiRs. Emerging evidence has demonstrated that the genomic organizations and expression patterns of myomiRs and their host *MYHs* are well conserved in mammals and they form an important transcription network involved in muscle fiber-type specification. However, the functional analysis of myomiRs and their host *MYHs* is still limited in teleosts. Their genomic distribution and expression during teleost myogenesis have not been studied in detail as well. In the present study, distribution of myomiR/*MYH* loci and their expression patterns were examined with special emphasis on three representative teleosts, torafugu *Takifugu rubripes*, zebrafish *Danio rerio* and medaka *Oryzias latipes*. The transcription regulation of myomiRs/*MYHs* and their function in muscle fiber-type specification was also examined by *in vivo* reporter assay and knockdown analysis, respectively.

1. Genomic organization and expression of *MYH14*/miR-499 in teleosts

Following the completion of the human genome project, three novel sarcomeric *MYHs*, *MYH14*, *MYH15* and *MYH16* were identified. Among them, *MYH14* is unique because it contains a myomiR, miR-499. miR-499 is transcribed in a slow/cardiac muscle specific manner along with its host gene and plays a key role in muscle fiber-type specification in mammals by activating slow muscle gene program. Using available genome databases for different vertebrates, the syntenic organization of human *MYH14* and miR-499 with their orthologues was examined. In teleost genome, *MYH14*/miR-499 showed highly diverged structure. Phylogenetic analysis showed

that *MYH14* was monophyletic in the amniote lineage, including humans, chickens, and coelacanths, but was duplicated in the ray-finned fish lineage, except for the spotted gar. The miR-499s phylogenetic relationships were consistent with those of the *MYH14*s. *MYH14*/miR-499 locus was duplicated early in teleost evolution by teleost specific whole genome duplication and one of the duplicated miR-499 genes was lost in the common ancestor to cod and the Acanthopterygii, after the split from the zebrafish lineage. Additionally, *MYH14*s have seemingly been lost at independent points of teleost evolution. Interestingly, miR-499 was not located in the *MYH14* introns of certain teleost fish. An *MYH14* paralogue, lacking miR-499 exhibited an accelerated rate of evolution compared with those containing miR-499, suggesting a putative functional relationship between *MYH14* and miR-499.

To address expression of *MYH14* in teleost, *in situ* hybridization was performed in representative teleost fish species, zebrafish, torafugu and medaka. In zebrafish, total three *MYH14*/miR-499 pairs are present in its genome. Previous study (Kinoshita et al., 2011) revealed that *MYH14-1* was expressed in whole myotomal region including fast muscle area at embryonic stage but in superficial slow, intermediate, and cardiac muscles at adult stage. In this study, expressions of *MYH14-1* and *MYH14-3* were observed whereas transcripts of *MYH14-2* were not detected in both developmental and adult stages. The transcripts of *MYH14-3* were specifically expressed in slow and cardiac muscles at examined stages. Meanwhile, torafugu genome contains two *MYH14*s. One paralogue named MYH_{M5} contains miR-499, whereas the other one named MYH_{M3383} lacks the miRNA. These two *MYH14* paralogues showed different expression pattern where MYH_{M5} expression was observed in both slow and cardiac muscles in the developmental and adult stages, and MYH_{M3383} expression was restricted to adult slow muscle (Ikeda et al., 2007 and Akolkar et al., 2010). In medaka, miR-499 is present but *MYH14* is completely absent in the genome. These results indicate that teleost *MYH14*s are highly diverged in their genomic structure and expression patterns.

Expression of miR-499 was also examined in the three teleost species. Interestingly, miR-499 expression is exceptionally conserved regardless of the varied expression of their host *MYH14s*. *In situ* hybridization showed that miR-499 was not expressed in the skeletal muscle at the embryonic stage of zebrafish, torafugu and medaka. On the other hand, larvae of the three fish showed a clear expression of miR-499 in both cardiac and slow muscles. Similar to the larval stage, adult zebrafish and torafugu showed high expression of miR-499 in cardiac and slow muscles, whereas adult medaka exhibited miR-499 expression only in the cardiac muscle. In torafugu, miR-499 was also detected in the erectors-depressors (ED) muscle which exhibit characteristics of slow muscle fibers. These miR-499 expression patterns were also confirmed by next-generation sequencing of small RNA libraries. It is noted that medaka miR-499 was even expressed in the absence of its host gene *MYH14*. Comparing the flanking sequences of *MYH14*/miR-499 loci between zebrafish, torafugu and medaka revealed several highly conserved regions including an intronic sequence immediately downstream of miR-499, suggesting *cis*-regulatory elements have been functionally conserved in medaka miR-499 despite the loss of its host gene.

2. Genomic organization and expression of *MYH6/vmhc/*miR-736 in teleosts

MYH6 and *MYH7* are well-known cardiac *MYHs* in mammals and contain myomiRs, miR-208a and miR-208b, respectively within their introns. In adult mouse, *MYH6* is expressed specifically in the heart whereas *MYH7* was

expressed in slow muscle fibers. *MYH7* also showed high expression in the developing heart but is down-regulated after birth. miR-208a is co-expressed with *MYH6* and regulates the expression of *MYH7*/miR-208b and *MYH14*/miR-499 (van Rooij et al., 2009). Recent studies have revealed that miR-208b and miR-499 play redundant functions in muscle fiber-type specification by activating slow and repressing fast muscle fiber gene programs in mammals. In available teleost genome databases, *MYH7* was not detected. *MYH6* persists in cartilaginous and ray-finned fish genomes but missing any intronic miRNAs. In teleosts, known major cardiac *MYH* isoform is ventricular myosin heavy chain gene (*vmhc*) which contains an intronic miRNA, miR-736. Sequence similarity and phylogenetic analyses indicates *vmhc*/miR-736 is orthologue of *MYH6*/miR-208a. As well as *MYH14*/miR-499, syntenic and phylogenetic studies revealed that multiple orthologues of *MYH6*/*vmhc*/miR-736 are present in teleost genomes. Zebrafish genome contains a total of 10 *MYH6*/*vmhc* paralogues of which one paralogue possess miR-736 and the others lack the intronic miRNA. Torafugu and medaka genomes contain 3 and 5 *MYH6*/*vmhc* paralogues respectively, and only one paralogue in each fish contains miR-736. Kloosterman et al. (2006) reported that zebrafish miR-736 is expressed in eye and gut by northern blot analysis. Expression of miR-736 in adult torafugu and medaka was examined using next generation sequencing platform, showing trace expression in various tissues including muscle. These results indicate uncoordinated expression of miR-736 with its host gene, indicating that miR-736 is not a functional orthologue of mammalian miR-208s. Taken together with highly conserved expression of miR-499, these results suggest that the complete deletion of miR-208s function in teleost is counterbalanced by the role played by miR-499.

3. Expression regulation *MYH14*/miR-499 paralogues and functional anlysis of miR-499 in teleosts

To address mechanisms of expression regulation of diversified *MYH14* paralogues, *in vivo* reporter assay was performed by injecting reporter gene EGFP conjugating with various length of 5'-flanking sequence of zebrafish *MYH14* paralogues into zebrafish eggs. As a result, a 5710bp 5'-flanking region of *MYH14-1* and 5641bp of *MYH14-3* contained a necessary regulatory region to recapitulate their endogenous expression during embryonic and larval muscle development. *MYH14-3* promoter activated reporter gene expression specifically in slow muscle fibers, whereas the embryos of stable zebrafish transgenic line Tg:MYH14-1:5710bp-EGFP expressed EGFP in both slow and fast muscle fibers, well consistent with their endogenous expression in zebrafish. Immunohistochemistry and cyclopamine (an inhibitor of slow muscle development) treatment of the Tg:MYH14-1:5710bp-EGFP confirmed the reporter gene expression in both fast and slow muscle fibers where EGFP was concentrated especially along with the horizontal myoseptum regions. 5'-flanking region of zebrafish *MYH14-1* and its torafugu orthologue shared two distal and a single proximal conserved region. However, deletion of these conserved regions had no effect on reporter gene expression in case of zebrafish. On the other hand, additional two conserved regions were detected by comparing 5'-flanking regions of zebrafish three *MYH14* paralogues. Deletion of the two conserved regions significantly reduced the promoter activity of *MYH14-3* but had no effect on that of *MYH14-1*, indicating that *cis*-regulatory elements of *MYH14-1* and *MYH14-3* are different in accordance with differential expression between the two *MYHs*.

As observed in previous section, medaka miR-499 is transcribed despite lacking its host gene *MYH14*, suggesting

the presence of its own promoter for transcription. The present study also examined promoter activity of 5'-flanking sequence of medaka miR-499. As well as in zebrafish paralogues, various length of 5'-flanking sequence of medaka miR-499 was conjugated with EGFP reporter gene and injected into zebrafish eggs. Detail promoter analysis revealed, upstream region of -4769 ~ -1897bp from medaka miR-499, which contains several highly conserved sequences of *MYH14*/miR-499 loci between torafugu and medaka, generated reporter gene expression specifically in cardiac and fast muscle fibers in zebrafish.

Loss of function experiment of miR-499 was performed by injection of antisense miR-499 oligonucleotide into medaka and zebrafish eggs. As expected, knockdown larvae showed marked reduction of slow muscle fibers in zebrafish and medaka developmental stages. In zebrafish, such reductions were also validated by real-time PCR analysis using a transgenic line which expresses GFP in slow muscle fibers. As explained, mammalian miR-499 and miR-208b have redundant role in muscle fiber-type specification by activating slow muscle gene program. van Rooij et al. (2009) reported that knockdown of each of miR-499 and miR-208b showed no effect on mouse muscle formation, but double knockdown of the two miRNAs caused reduction of slow muscle fibers. On the other hand, in teleost, miR-499 suppression is enough to reduce the slow muscle fiber formation. Taken together with the fact of trace expression of miR-736, teleost miR-208s orthologue, miR-499 is a sole functional myomiR and thus have indispensable role in muscle fiber-type specification in teleost muscle formation.

Conclusion

Despite diversification of host *MYHs* in genomic organization and expression patterns, miR-499 expression was exceptionally conserved, indicating pivotal role of the myomiR in teleost muscle formation. Actually, knockdown analysis of miR-499 showed perturbation in slow muscle formation during zebrafish/medaka growth, indicating that a myomiR-mediated regulatory network also works in fish muscle formation although the network components will be different from that of mammals. On the other hand, diversification of host *MYHs* suggests their functional versatilities involved in diverged musculature in teleost. Fish is the most diverse vertebrate group. In response to the wide range of environmental and physiological conditions, the characteristics of fish musculature, including muscle fiber-type composition, are also highly diverse. It would be interesting to determine whether diversification in myomiRs and their host myosin genes is related to physiological and ecological variations among teleost fish species.