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Studies on the mechanisms involved in the expression of myosin heavy chain genes in various muscle types of torafugu *Takifugu rubripes*

（種々のトラフグ筋タイプにおけるミオシン重鎖遺伝子の発現機構に関する研究）

The molecular, genetic and cellular bases for skeletal muscle growth and regeneration have been documented in a number of vertebrate species. Formation of skeletal muscle of fish differs in several aspects when compared with mammals. These include the spatial separation of fast and slow muscle precursor cells during somite formation in embryos. The other unique feature observed in fish muscle is the increase of muscle mass during postembryonic growth by recruitment of new fibers, called hyperplasia. Fish muscle grows by stratified and mosaic hyperplasia at larval and adult stages, respectively. During stratified hyperplasia in larvae, new fibers are formed mainly at the dorsal and ventral extremes of myotome and additionally in a layer between superficial slow and deep fast fibers, whereas mosaic hyperplasia occurs throughout the whole myotome of adult fast muscle. Sarcomeric myosins including skeletal and cardiac ones are composed of two heavy chains (MYHs) and four light chains, whereas fiber characteristics are well correlated with the expression of MYH isoforms. Meanwhile, fish are known to possess highly conserved MYH multigene family, although MYH genes (*MYH*s) are much more than their higher vertebrate counterparts. However, functional implications for such a high number of MYHs have remained to be elucidated.

The present study was carried out to investigate expression patterns of sarcomeric MYHs in various adult muscles of torafugu *Takifugu rubripes*, where the total genome database is publicly available. Fiber types were then characterized by histochemical methods in adult skeletal muscles. MYHs were also cloned from embryos and larvae, and analyzed for their expression. Finally, the members of paired box protein (Pax) gene family, *Pax3* and *Pax7*, were characterized for their possible application as a marker for identification of muscle precursor cells.

1. Characterization of fiber types in adult skeletal muscles of torafugu

In the present study, myofibrillar ATPase was demonstrated in adult skeletal muscle of torafugu (body weight 290 g) by selective inhibition or activation of specific fiber groups after preincubation (2 - 3 min) either at acidic or alkaline pH. Fast muscle contained various fibers with different diameters. ATPase of fast fibers with large diameters was inactivated at pH 4.6, whereas that with small diameters was stable to this acidic pH. It was noted that the fibers with small diameters were more stable with those having

smaller diameters. Such existence of fibers with small diameters in fast muscle suggests hyperplastic growth in adult fast muscle, since it has been reported that new fibers are formed by hyperplastic process in most adult fish which grow to a large final body size like torafugu. Meanwhile, most fibers in lateralis superficialis (LS) and erector and depressor (ED) slow muscles were resistant to pH 4.6, although some large-sized fibers were found to be slightly acid-labile. In contrast, ATPase of all fast fibers of juvenile torafugu was inactivated at pH 4.6, suggesting that the existence of different fibers in fast muscle is a distinct feature of muscle growth in adult.

NADH-diaphorase staining was performed to identify oxidative fibers in skeletal muscles according to Novikoff et al. (1961). All fibers in LS and ED slow muscles were positive for NADH-diaphorase stain, suggesting that these muscles have oxidative metabolism. Importantly, fibers in LS slow muscle with large diameters adjacent to fast muscle showed lower NADH-diaphorase reaction compared with those in a superficial region with small diameters, demonstrating that the former fibers have an intermediate oxidative potential. In contrast, none of fibers in fast muscle was stained for NADH-diaphorase.

2. Expression patterns of sarcomeric myosin heavy chains in adult torafugu muscles

cDNAs encoding sarcomeric MYHs were amplified by RT-PCR using *MYH*-specific degenerate primers. In total, seven sarcomeric *MYH*s were cloned from adult fast, slow and cardiac muscles of torafugu (body weight 1 kg). The nomenclature of torafugu *MYH*s found in the present study is described following Ikeda et al. (2007) who found 20 sarcomeric *MYH*s by *in silico* approach on the total genome database. Three *MYH*s, $MYH_{M86\text{-}1}$, MYH_{M8248} and MYH_{M880}, were cloned exclusively from fast, slow and cardiac muscles, respectively, whereas two *MYH*s, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, were cloned from both fast and slow muscles and another two *MYH*s, $MYH_{M2126\text{-}2}$ and MYH_{M5}, from both slow and cardiac muscles. Evolutionary relationships of torafugu MYHs with those reported from other fish were studied by phylogenetic analysis on the deduced amino acid sequences using the neighbor-joining method. $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034} belonged to fast type as they were placed in the same clade representing fast-type MYHs from other fish on the phylogenetic tree. MYH_{M8248} and $MYH_{M2126\text{-}2}$ belonged to slow and cardiac types, respectively. MYH_{M5} and MYH_{M880} were found to have appeared in an early evolution of MYHs and thus regarded to belong to ancestral slow/cardiac type.

The frequencies of cDNA clones encoding above-mentioned *MYH*s in the cDNA clone libraries and relative mRNA levels determined by Northern blot analysis further revealed their tissue-specific expression in adult skeletal and cardiac muscles. The clones encoding fast-type $MYH_{M86\text{-}1}$ were most abundant in the cDNA clone library constructed from fast muscle. Both LS and ED slow muscles contained almost equally the clones of five *MYH*s including fast-type $MYH_{M2528\text{-}1}$ and MYH_{M1034}, slow-type MYH_{M8248}, cardiac-type $MYH_{M2126\text{-}2}$ and unique, slow/cardiac-type MYH_{M5}. Among three types of *MYH* clones from cardiac muscle, cardiac-type $MYH_{M2126\text{-}2}$ was most abundant.

In situ hybridization was performed to localize the transcripts of *MYH*s in skeletal muscles of adult torafugu (body weight 275 g). The transcripts of fast-type $MYH_{M86\text{-}1}$ were found in all fibers with

different diameters in fast muscle. Fast fibers with smaller diameters tended to have transcripts of fast-type *MYH*$_{M2528\text{-}1}$ more abundantly. Given that such fast fibers with small diameters are generated by hyperplasia, the expression of *MYH*$_{M2528\text{-}1}$ is thought to be deeply correlated with generation of these fibers and those with the smallest diameter are considered to be most newly formed.

The fibers expressing slow-type *MYH*$_{M8248}$ resided a superficial part of LS slow muscle with small diameters. Fibers expressing cardiac-type *MYH*$_{M2126\text{-}2}$ also occupied a superficial layer in LS slow muscle with small diameters. Interestingly, fast-type *MYH*$_{M2528\text{-}1}$ was expressed in fibers of LS and ED slow muscles with large diameters which showed an intermediate oxidative potential as described above, implying their possible involvement in muscle generation by hyperplasia.

The expression levels of fast-type *MYH*s were also investigated in both wild and farm-cultured torafugu individuals (body weight 0.8 - 1 kg). Among three fast-type *MYH*s, the relative mRNA levels of *MYH*$_{M2528\text{-}1}$ were significantly higher in wild than farm-cultured fish.

3. Expression patterns of myosin heavy chain genes in torafugu at embryonic and larval stages

Six sarcomeric *MYH*s were cloned from embryos and larvae of torafugu laboratory-reared at 18°C by using the same method as described for adult torafugu. These included four fast-type *MYH*s, *MYH*$_{M743}$, *MYH*$_{M86\text{-}2}$, *MYH*$_{M2528\text{-}1}$ and *MYH*$_{M1034}$, cardiac-type *MYH*$_{M2126\text{-}1}$ and slow/cardiac-type *MYH*$_{M5}$. *MYH*$_{M743}$ and *MYH*$_{M86\text{-}2}$ have been reported by Ikeda et al. (2007) using gene specific primers. Among all fast-type *MYH*s, the cDNA clone encoding *MYH*$_{M743}$ was most abundant in all clone libraries from embryos and larvae, followed by those encoding *MYH*$_{M86\text{-}2}$ in embryos [5 and 7 days post fertilization (dpf)] and *MYH*$_{M2528\text{-}1}$ in larvae (10 and 16 dpf). The cDNA clone encoding *MYH*$_{M1034}$ was marginally observed in clone libraries from larvae. While cDNA clones of slow/cardiac-type *MYH*$_{M5}$ were identified in all clone libraries from embryos and larvae, their abundance in larvae was found to be much lesser than in embryos.

RT-PCR using highly specific primers based on the 3' untranslated region nucleotide sequences of *MYH*s showed that the transcripts of *MYH*$_{M743}$ appeared in embryos at 3 dpf, whereas those of *MYH*$_{M86\text{-}2}$ in embryos at 4 dpf. These two *MYH*s continued to be expressed during embryonic and larval development, suggesting their involvement in muscle development. The transcripts of fast-type *MYH*$_{M2528\text{-}1}$ appeared in embryos at 7 dpf and continued to be expressed at successive embryonic and larval stages, as well as in adult fast and slow skeletal muscles. The transcripts of slow/cardiac-type *MYH*$_{M5}$ continued to be expressed from embryos at 3 dpf to larvae and as well in adult slow and cardiac muscles. Such expression patterns of *MYH*$_{M2528\text{-}1}$ and *MYH*$_{M5}$ in adult muscles were consistent with those described in the previous section.

Whole mount *in situ* hybridization for embryos at 4 dpf with probes specific to fast-type *MYH*$_{M86\text{-}2}$ and slow/cardiac-type *MYH*$_{M5}$ revealed that the former transcripts were localized in the whole embryonic myotome, whereas the latter transcripts were restricted to the superficial slow muscle as well as to the horizontal myoseptum. The transcripts of cardiac-type *MYH*$_{M2126\text{-}1}$ were localized adjacently to the notochord of embryos at 3 dpf.

4. Characterization of paired box protein genes as myogenic precursor cell markers in torafugu

Paired box protein (Pax) genes play pivotal roles in the formation of tissues and organs during development. This gene family encodes transcription factors characterized by the presence of paired box domain (PD), octapeptide motif and homeodomain. It has been reported that Pax3 and Pax7 regulate survival, proliferation and migration of myogenic precursor cells. In this context, *Pax3* and *Pax7* were cloned from torafugu embryos and adult fast skeletal muscle by using degenerate primers based on highly conserved amino acids in PD and homeodomain. Subsequent *in silico* analysis with the Fugu genome database (ver. 4.0) yielded two distinct genes each for *Pax3* (*Pax3a* and *Pax3b*) and *Pax7* (*Pax7a* and *Pax7b*). The 75th amino acid, glutamine (Glu75), from the N-terminus was replaced by proline in PD of Pax3b. Mammalian Pax3 and Pax7 both have alternatively spliced isoforms, differing in the presence or absence of Glu75 (Q+/Q-) in PD which affects the DNA-binding specificity (Vogan et al., 1996). One single cDNA clone encoding Pax3a had deletion of Glu75 in PD, suggesting the presence of alternatively spliced variants (Q+/Q-) for torafugu Pax3a. This was further supported by identification of two adjacent alternative 3' splice acceptor sites for torafugu which produce Pax3a Q+ (aagCAGGGA) and Q- (aagcagGGA) variants. Interestingly, torafugu *Pax7b*, but not *Pax7a*, had an insert encoding five amino acid residues (GEASS) in a C-terminal region of PD in two out of three cDNA clones. Genomic analysis showed two alternate splice donor cites at exon 4 of *Pax7b* which is responsible for forming two alternately spliced variants.

RT-PCR revealed that the transcripts of *Pax3a*, *Pax3b*, *Pax7a* and *Pax7b* were found to appear in embryos at 3 dpf and later developmental stages, suggesting their key roles during development. Interestingly, the transcripts of *Pax7b* were observed in adult skeletal muscles. Thus, *Pax3* and *Pax7* can be used to monitor muscle precursor cells.

Conclusion

Expression patterns of seven sarcomeric *MYH*s were determined in adult muscles of torafugu. While three *MYH*s were specifically expressed in either fast, slow or cardiac muscle of adult torafugu, four showed a mixed expression pattern, suggesting the functional significance of each *MYH*. Furthermore, six *MYH*s were cloned from embryos and larvae and found to be expressed sequentially during development. Fiber-type diversity was also demonstrated by *in situ* hybridization for *MYH* transcripts. This is an important step ahead in understanding fiber type diversity and associated muscle growth by hyperplasia specifically observed in larval and adult fish having an intermediate body size. Our study also greatly helps to understand functional significance of higher number of *MYH*s in fish.