論文の内容の要旨

水圏生物科学専攻
平成 23 年度博士課程進学
氏 名　ＡＫシャクール アーメド
指導教員名　浅川　修一

Studies on the transcriptional regulatory mechanisms of gene expression mediating indeterminate muscle growth in teleost

（魚類筋肉の示す終生成長と遺伝子発現の転写調節機構に関する研究）

Skeletal muscle comprises a large portion of the mass of vertebrates. The bulk of vertebrate growth, therefore, depends on an increase in skeletal muscle mass during a species's lifespan. Skeletal musclesdisplay two types of growth patterns, hypertrophy and hyperplasia. The former is characterized by an increase in the size of existing muscle fibers (myocytes) while the latter results inan increase in the number of muscle fibers. In mammals, however, the contribution ofhyperplasia to muscle growth is quite small in the postnatal period, resulting in limited growth and a definitive body size. Production of new muscle fibersafter the neonatal period in mammals is observed only in the regeneration of injured muscle. Conversely, in teleost skeletal muscles, both hyperplasiaand hypertrophyoccur throughout theorganism'slifespan. This 'indeterminate' muscle growth is aquite important phenomenon that dictates teleost growth.However, the molecular mechanisms responsible for the indeterminate muscle growth found in teleosts are completely unknown.

Myosin heavy chain (MYH) is a subunit of myosin, the most abundant protein in skeletal muscle. Many isoforms of MYH exist, and their variation in expression is the primary determinant of thedifferential physiological properties of muscle fibers. Our previous studies identified a MYH gene (*MYH*), $MYH_{M2528\text{-}1}$, in the torafugu (*Takifugurubripes*) genome with specific expression in neonatal muscle fibers produced by muscle hyperplasia at the larval, juvenile and adult stages. Therefore, understanding the mechanisms of $MYH_{M2528\text{-}1}$ transcription regulation will provide a basis to dissect the molecular network involved in the production of neonatal muscle fibers by indeterminate muscle growth.

Here, we examined the torafugu$MYH_{M2528\text{-}1}$ promoter via an *in vivo* reporter assay using zebrafish and demonstrated its role in the activation of gene expression specifically in neonatal muscle fibers producedby larval andpost-larval muscle hyperplasia amongst different fish species.

1. Gene expression mediatingindeterminate muscle growth inteleosts

$MYH_{M2528\text{-}1}$ is highly expressed in fast and slow muscle fibers of torafugu embryos and larvae. When the 2100-bp fragment of $MYH_{M2528\text{-}1}$ was fused to the reporter gene, *EGFP* encoding enhanced green fluorescence protein, the construct could induce muscle-specific expression of EGFP in zebrafish embryos.

In most cases, the EGFPexpression was detected at 1 day post fertilization (dpf) in the somite formation stage. After hatching (2dpf), EGFP continued to be expressed in the whole myotome of larvae as well as in craniofacial muscles. To confirm the specific activity of the*MYH*$_{M2528\text{-}1}$ promoter in post-embryonic muscle hyperplasia, a stable transgenic zebrafish linewas established using the P2100 construct and temporal and spatial EGFP expression was analyzed. Similar to the*in vivo* reporter assay, stable transgenic zebrafish line displayed EGFP expression at 1 dpf. After hatching at 2dpf, EGFP continued to be expressed in the wholemyotomal region of the larva. Observation with a fluorescent microscope also confirmed that stable transgenic zebrafish line expressed EGFP in thewhole myotomal region and craniofacial muscles at 3 dpf. On the other hand, immunohistochemistry of stable transgenic zebrafish line at the larval stage (3dpf) to clarify the type and position ofEGFP-positive muscle fibers which was consistent with the zebrafish embryos microinjected the 2100 bp construct. After the larval stage, fast and slow muscles of teleosts still show different hyperplastic growth patterns. In this regard, immunohistochemistry was performed to clarify the position of EGFP-positive muscle fibers at post-larval (juvenile) and adult stages of stable transgenic zebrafish line. At the early juvenile stage (20 dpf, 10mm SL), EGFP expression in fast muscle was observed in small diameter fibers between large existing muscle fibers. In the lateralis slow and elector-depressor (ED) slow muscles of both the early and late juvenile stages of zebrafish development, EGFP expression was observed near the septum of slow and fast muscles. As well, consistent with the difference in the growth pattern between slow and fast muscles, promoter activity was not observed in fast muscle but in slow muscle at the late juvenile (40 dpf, 17mm SL) and adult stage (60dpf, 25mm SL), respectively.Taken together with the EGFP expression pattern in the larvaeand juveniles of stable transgenic zebrafish line, the 2100 bp 5'-flanking region of *MYH*$_{M2528\text{-}1}$ contains essential *cis*-regulatory sequences for myogenesis that are conserved among torafugu and zebrafish. Therefore, 2100bp 5′-flanking region of torafugu*MYH*$_{M2528\text{-}1}$ is the minimal promoter necessary to induce gene expression in zebrafish skeletal muscle.

At 10 somite stage of stable transgenic line embryodid not show any EGFP in the adaxial cells using immunohistochemistry meaning that it is not involved in primary myogenesis. As well, hedgehog signaling is important for slow muscle development in vertebrates. To further characterize EGFP expression in slow muscle fibers, stable transgenic line zebrafish embryos, carrying the transgene *MYH*$_{M2528\text{-}1}$:*EGFP*, were treated with cyclopamine (2.0, 4.0,6.0 and 10 μg/ml), a well-known hedgehog signaling inhibitor. After cyclopamine treatment, stable transgenic line larvae showed very little change of EGFP expression compared to the control wild embryos treated with only ethanol. However, the EGFP expressing fiber was detected at only dorsal extreme of the myotome and is secondary slow muscle fiber, indicating that *MYH*$_{M2528\text{-}1}$ is involved in by secondary myogenesis. Using the MatInspector and TFsearch programs, the 2100bp 5'-flanking region from the start codon of *MYH*$_{M2528\text{-}1}$ was found to contain putative transcription factor-binding sites for two MyoD, four myocyte enhancer element 2 (MEF2), two paired box 3 (Pax3), and three nuclear factor of activated T-cell (NFAT) binding sites, all of which have been implicated in the expressional regulation of muscle-specific genes during development. The reduced promoter activity for the successive deletion of 5' flanking region from P2100 indicating that any or combination of these *cis*-elements might be involved in the transcriptional regulation of *MYH*$_{M2528\text{-}1}$.

2. Transcriptional regulation offast and slow muscle-specific $MYH_{M2528\text{-}1}$expression

To identify the key regulatory region for the hyperplasicmuscle-specific expression in indeterminate muscle growth in teleost, a deletion analysis was performed within the 2.1-kb 5'-flanking region of $MYH_{M2528\text{-}1}$.Using the MatInspector and TFsearch programs, the 2100-bp 5'-flanking region from the start codon of $MYH_{M2528\text{-}1}$was found to contain putative transcription factor-binding sites for myogenic determining factor (MyoD), paired box protein (Pax3), myocyte enhancing factor 2 (MEF2) and nuclear factor of activated T cell (NFAT), all of which have been implicated in the expressional regulation of hyperplasic muscle-specific genes during development. To determine the role of these MyoD binding sites, we deleted these sites individually or in various combinations. All deletion mutant constructs were microinjected into zebrafish embryos for transient expression analysis. Deleting multiple MyoD binding sites significantly reduced EGFP expression percentage compared with the non-deletion construct (P2100). The reduction of such EGFP expression in MyoD binding sites deletion constructs were also confirmed by using real-time PCR analysis. On the other hand, immunohistochemistry of MyoD binding sites deleted larvae showed reduced EGFP expression. As well, to conclude the role of two putative Pax3 binding sites, we deleted these sites individually or in various combinations. Here, deleting all two Pax3 binding sites showed in a mark reduction of high-EGFP expressing embryos. After that reduction of such EGFP expression were also confirmed by using real-time PCR analysis. Furthermore, immunohistochemistry of all deleted Pax3 binding sites construct showed EGFP only in fast muscle. Also, deleting multiple MEF2 binding sites significantly reduced EGFP expression percentages in muscle fibers. Moreover, fluorescent optics showed that deleting all four MEF2 binding sites resulted in significant reduction of EGFP expression in skeletal muscle and reduced promoter activity. In addition, EGFP-expressing embryos were also categorized into very high (>200), high (200-100), medium (50-100), low (10-50) and very low (<10) groups. Here, each deleted MEF2 binding sites construct microinjected into zebrafish embryo demonstrated highest number of embryo which included in low groups. As well these result has also been validated by RT-PCR experiment while showed reduced EGFP expression. Too, EGFP was detected only in slow muscle which was detected by immunohistochemistry of all MEF2 mutated zebrafish larvae at 3dpf stages.

Moreover with the aim to determine their expression which of the NFAT binding sites is functional in the transcriptional regulation of $MYH_{M2528\text{-}1}$, we carried out experiments using deleted promoter constructs in which one site, multiple sites in combination, or all of the NFAT binding sites were deleted from P2100 constructs. Embryos injected with the deletion constructs of either site NFAT_1, NFAT_2, and NFAT_3 significantly reduced EGFP expression percentages in muscle fibers compared to the P2100 injected embryos, and elimination of multiple and all the sites of NFAT elements have more marked effects. The deletion of NFAT binding sites also reduced the number of EGFP expressing muscle fibers and found to be highest number of embryo express EGFP uphold low category group (<10). Subsequently, reduction of such EGFP expression were also confirmed by using real-time PCR analysis. On the other hand, all deletion larvae showed EGFPonly in slow muscle by immunohistrochemistry. Therefore, deletion mutation analyses suggested that MyoD, Pax3, MEF2 and NFAT, binding sites are crucial for $MYH_{M2528\text{-}1}$promoter activity in indeterminate muscle growthin teleost. However, the deletion of all MyoD-, Pax3-, MEF3- or

NFAT-binding sites failed to eliminate $MYH_{M2528\text{-}1}$expression completely. Taken together, it is indicated that multiple transcription factors including MyoD, Pax3, MEF2 and NFAT participate in the expression of $MYH_{M2528\text{-}1}$.

Conclusion

This studypresents a piece of information about the transcriptional regulatory mechanisms involved in myosin heavy chain gene expression mediating indeterminate muscle growth in teleost. This study is the first to report the specific promoter involved in teleost indeterminate muscle growth.Although there are intriguing characteristicsof teleost indeterminate muscle growth, little is known about the underlying causative molecular mechanisms.A better understanding of the cis-elements responsible for the transcriptional regulation of hyperplasticexpression in indeterminate muscle growth in teleostwould provide a basic insight into how these myotomal muscle mass are established and maintained in fish. In this study, the *cis*-regulatory elements are determined by deletions and mutations analyses in zebrafish embryos. Therefore, further studies ofthe $MYH_{M2528\text{-}1}$ promoter such as interactome analysis on the promoter will provide new insightsnot only into skeletal muscle biology but also into vertebrate growth and aging.