Studies on the transcriptional regulatory mechanisms of myosin heavy chain genes expression during skeletal muscle development in fish

（魚類骨格筋形成におけるミオシン重鎖遺伝子の転写制御機構に関する研究）

Md. Asaduzzaman

モハマド　アサドザマン

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A

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By

Md. Asaduzzaman

January, 2013

DECLARATION

I, Md. Asaduzzaman, hereby declare that the thesis entitled **"Studies on the transcriptional regulatory mechanisms of myosin heavy chain genes expression during skeletal muscle development in fish"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

13th December 2013

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Laboratory of Aquatic Molecular Biology and Biotechnology,
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The University of Tokyo

DEDICATION

This thesis is dedicated to my son "Zaman Aashir Anjam"

-the best gift and inspiration of my life

Acknowledgements

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Contents

Abbreviations

ANOVA	:	Analysis of variance
AP	:	Adapter primer
ATP	:	Adenosine 5’-triphosphate
BCIP	:	5-bromo-4-chloro-3-indoyl-phosphate
bp	:	Base pair
cDNA	:	Complementary DNA
CIP	:	Calf intestinal phosphtase
Ct	:	Cycle threshold
DAPI	:	diamidine-20-phenylindole dihydrochloride
DIG	:	Digoxigenin
DNA	:	Deoxyribonucleic acid
dpf	:	Days post fertilization
ECL	:	External cell layer
ED	:	Erectors and depressors
EGFP	:	Enhanced green florescence protein
FLmax	:	Maximum fork length
HLH	:	Helix-loop helix
hpf	:	Hours post fertilization
HS	:	Horizontal septum
Hh	:	Hedgehog
LMM	:	Light meromyosin
LS	:	Lateralis superficialis
MADS	:	MCM1, AGAMOUS, DEFICIENS and SRF
MEF2	:	Myocyte enhancing factor 2
MEF3	:	Myocyte enhancing factor 3
MH	:	Mosaic hyperplasia
MPCs	:	Myogenic precursor cells
MRF	:	Myogenic regulatory factors
MYH	:	Myosin heavy chain
MyoD	:	Myoblast determining factor
Myog	:	Myogenin

NBT	:	Nitroblue tetrazolium
ORF	:	Open reading frame
PBSTw	:	Phosphate-buffered saline with 0.1% tween 20
PCR	:	Polymerase chain reaction
PFA	:	Paraformaldehyde
Pax	:	Paired box protein
Ptc	:	Patched
RACE	:	Rapid amplification of cDNA ends
RFP	:	Red fluorescence protein
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
RT-PCR	:	Reverse transcription PCR
S1	:	Subfragment 1
S2	:	Subfragment 2
SD	:	Standard deviation
SH	:	Stratified hyperplasia
SPSS	:	Statistical package for social science
SRF	:	Serum response factor
SSC	:	Saline sodium citrate
SSM	:	Suppression subtractive method
Smo	:	Smoothened
TAP	:	Tobacco acid pyrophosphatase
TBSTw	:	Tris-buffered saline with 0.1% tween 20
TEEA	:	The transient embryonic excision assay
TFsearch	:	Transcription factor search
UTR	:	Untranslated region

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Abstract

Members of the myosin heavy chain genes (*MYH*s) are expressed in a complex, sequential fashion during skeletal muscle development and growth of fish. A greater understanding of the transcriptional regulatory mechanisms of these *MYH*s expression in different muscles would provide insight into how these muscles are established and maintained in fish. Therefore, the 5'-flanking regions of different *MYH*s of fish were analyzed for the spatial and temporal regulation by using transient and stable transgenic techniques in zebrafish embryos. $MYH_{M743\text{-}2}$ is highly expressed in fast muscle fibers of torafugu embryos and larvae. Combining both transient and stable transgenic analyses, we demonstrated that the 2,075 bp 5'-flanking sequence was sufficient for its expression in skeletal, craniofacial and pectoral fin muscles (Chapter 2). We discovered that the serum response factor (SRF)-like binding sites are required for promoting $MYH_{M743\text{-}2}$ expression, whereas myoblast determining factor (MyoD) and myocyte enhancer factor-2 (MEF2) binding sites participate in the transcriptional control of $MYH_{M743\text{-}2}$ expression in fast muscle fibers (Chapter 2). Again, $MYH_{M86\text{-}2}$ exhibited restricted expression in slow muscle fibers of torafugu embryos and larvae. Combining both transient and stable transgenic approaches, we demonstrated that the 2,614 bp 5'-flanking sequence of $MYH_{M86\text{-}2}$ contains a sufficient promoter activity to drive gene expression specific to superficial slow muscle fibers (Chapter 3). The transcriptional mechanism that prevents $MYH_{M86\text{-}2}$ expression in fast muscle fibers was mediated through Sox6 binding elements (Chapter 3). It was further discovered that nuclear factor of activated T cells (NFAT) binding elements play a key role and myocyte enhancer factor-2 (MEF2) binding elements participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression (Chapter 3). The relationship of *MYH*s expression with muscle fiber formation has not been well understood in fish. Therefore, we further discovered that $MYH_{M2528\text{-}1}$ is involved in hyperplastic muscle growth of torafugu (Chapter 4). In conclusion, the transcriptional regulation of these development-dependent *MYH*s in fish consists of a complex process that is often regulated by a cohort of transcriptional factors.

List of Publications

The contents of this thesis have been published as follows:

1. **M. Asaduzzaman,** S. Kinoshita, B. S. Siddique, S. Asakawa and S. Watabe. 2011**.** Multiple *cis*-elements in the 5′-flanking region of embryonic/larval fast-type of the myosin heavy chain gene of torafugu, $MYH_{M743\text{-}2}$, function in the transcriptional regulation of its expression **Gene 489: 41-54.**
2. **M. Asaduzzaman**, S. Kinoshita, B. S. Siddique, S. Asakawa and S. Watabe. 2013. Stimulatory and inhibitory mechanisms of slow muscle-specific myosin heavy chain gene expression in fish: transient and transgenic analysis of torafugu $MYH_{M86\text{-}2}$ promoter in zebrafish embryos. **Experimental Cell Research 319: 820-837.**
3. **M. Asaduzzaman**, Dadasaheb B. Akolkar, S. Kinoshita and S. Watabe. 2013. The expression of multiple myosin heavy chain genes during skeletal muscle development of torafugu *Takifugu rubripes* embryos and larvae. **Gene 515: 144-154.**

CHAPTER 1

General Introduction

1.1 General Background

1.1.1 General features of myotomal muscle of teleost fish

In teleosts, skeletal muscle is characterized by the sequential arrangement of segmental myotomes in a complex three-dimensional structure. Each myotome contains a deeper part where muscle fibers are arranged in a helical fashion and a superficial region lying directly beneath the skin, where muscle fibers are run parallel to the body axis. This distinct arrangement of muscle fibers is related to the requirement to produce equal shortening of sacromeres at different body flexures (Alexander, 1969; Rome and Sosnicki, 1990). In mammals, most of skeletal muscles contain a mixture of various fiber types, enabling individual muscles in providing long-lasting, low-intensity contractions, together with first burst of activity. In contrast with mammals, muscle fibers types in teleost are anatomically separated into distinct locations (Fig 1-1). Fast twitch fibers locate deeply in the myotomal muscle, whereas slow twitch fibers are present at lateral superficial to the myotome (Sanger and Stoiber, 2001).

Fiber types in fish can be separated by the histochemical demonstration of ATPase (Fig. 1-1). Fast twitch fibers constitute the majority of myotomes (70-100%) and are usually larger in diameter than slow muscle fibers in teleosts (Greer-Walker and Pull, 1975; Altringham and Johnston, 1982). Fast muscle fibers have a sparse capillary network (Johnston, 1982; Egginton and Sidell, 1989; Sanger and Stoiber, 2001) and contain a low density of mitochondria (Johnston, 1982; Egginton and Sidell, 1989; Sanger and Stoiber, 2001). Fast muscle fibers fatigue and contract faster than other fiber types (Altringham and Johnston, 1982; Johnston and Salmonski, 1984; Langfeld et al., 1989). They are recruited to power rapid bursts of movement e.g. escape responses when a predator appears (Altringham et al., 1993; Altringham and Ellerby, 1999).

Slow twitch fibers are usually comparatively smaller in diameter than the other types of myotomal muscle fibers (Greer-Walker and Pull, 1975; Altringham and Johnston, 1988). Slow

muscle fibers are located in a narrow superficial strip adjacent to the lateral line known as lateralis superficialis (LS) with a wedge-shaped thickening in the region of the horizontal septum. In addition to LS slow muscle, another type of slow muscle is located at the median fins in fish belonging to the order Tetradontiformes, termed erectors and depressor (ED) slow (Fig. 1-1) (Winterbottom, 1974). In addition, slow muscle fibers are also widely found to contain high volume densities of mitochondria (Johnston, 1982; Egginton and Sidell, 1989; Sanger and Stoiber, 2001), contract slowly (Altringham and Johnston, 1982; Johnston and Salmonski, 1984; Langfeld et al., 1989), and are supplied with a dense capillary network (Egginton and Sidell, 1989; Sanger and Stoiber, 2001). All these distinct features of slow muscle fibers reflect their functional role in sustained and steady swimming activities fuelled by aerobic metabolism (Johnston et al., 1977; Rome et al., 1984; Altringham and Ellerby, 1999).

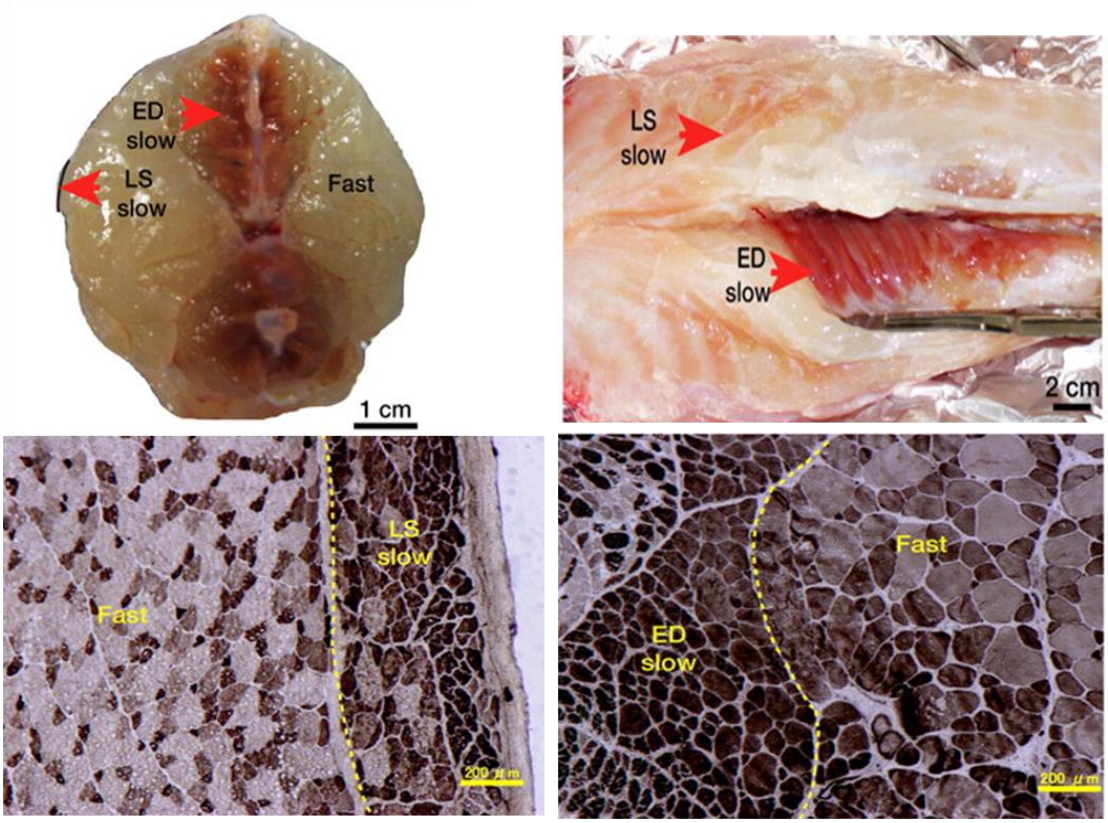

Fig. 1-1: Anatomical location of fast and slow muscle fibers in torafugu (upper two) and their differentiation by ATPase (lower two) (Adapted from Akolkar et al., 2010). LS-Lateralis superficialis; ED-Erectors and depressors.

In addition to the above major fiber types, minor ones such as intermediate or pink muscle fibers (Scapolo and Rowlerson, 1987; Gill et al., 1989), tonic fibers (Zowadowska and Kilarski, 1984; Sanger et al., 1997) and red bim fibers (van Raamsdonk et al., 1980) have been reported in fish. The intermediate, or pink muscle, is composed of intermediate twitch muscle fibers, which are usually intermediate in diameter and constitute 10%~20% of total teleost myotomal cross-sectioned area (Johnston et al., 1977; Langfeld et al., 1989). They are located between slow and fast muscles and have intermediate contraction speeds and fatigue-resistance compared to slow and fast muscle fibers (Altringham et al., 1993; Altringham and Ellerby, 1999). Like slow twitch fibers, intermediate fibers have orientation parallel to the axis of body (Scapolo and Rowlerson, 1987). The other fiber types such as red rim fibers and tonic fibers form a relatively small part of myotomal muscle and are considered to contribute less significantly to the swimming of fish.

1.1.2 Teleost myogenesis

Myogenesis refers to the process required for muscle development and growth, which involves expansion of existing muscle fibers (hypertrophy) and the generation of new muscle fibers (hyperplasia). This process is a common phenomenon to all vertebrates and consists of serial complex cell events, including the specification, activation, proliferation, differentiation, migration and fusion of the cells (Fig. 1-2). Three major phases of myogenesis have been observed in a variety of teleost species, namely embryonic myogenesis, stratified hyperplasia (SH), and mosaic hyperplasia (MH) (Rowlerson and Veggetti, 2001; Johnston, 2006). The relative timing and importance of individual phases vary considerably between species. In species with determinate growth that have a small maximum body size such as guppies (*Poecilia reticulate*) (Veggetti et al., 1993), the first two phases of myogenesis give rise to the majority of muscle fibers. On the other hand, MH gives rises to the majority of muscle fibers in species which show indeterminate growth and reach a large body size (Johnston, 2006).

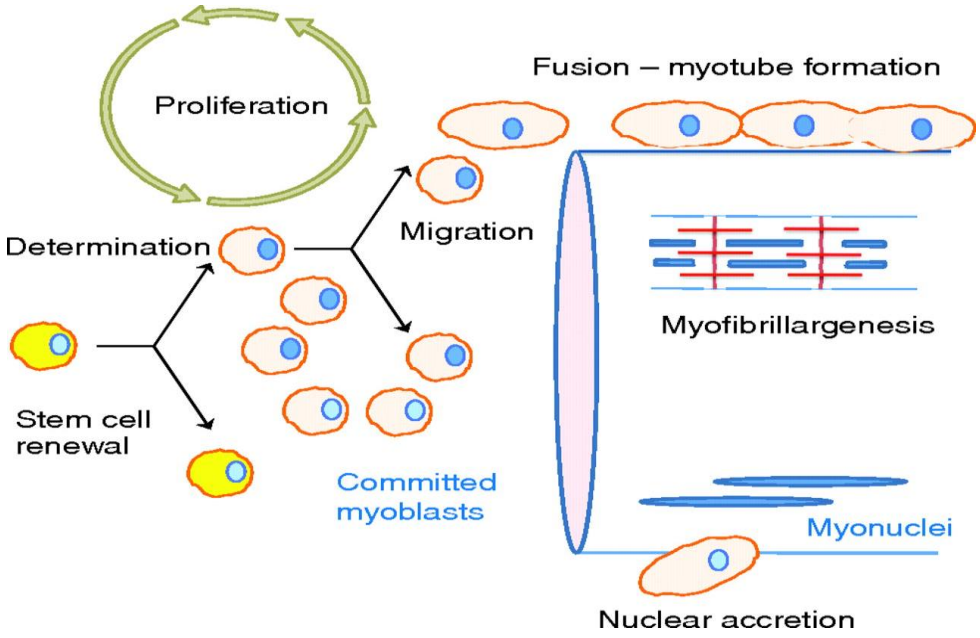

Fig. 1-2: A simple and general model for vertebrate myogenesis (Johnston et al., 2009).

Pioneering work establishing patterns of teleost embryonic muscle development was largely performed in zebrafish. During development, it was first demonstrated that the spatial separation of teleost muscle types occur in the embryo, where slow and fast muscle fibers originate from two separate subpopulations of muscle pioneers (adaxial cells and lateral presomitic cells) in the somites (Devoto et al., 1996). The adaxial and lateral presomitic cells can be distinguished in the segmental plate on the basis of their morphologies and position (Devoto et al., 1996) as well as the expression of genes such as *myod* (Weinberg et al., 1996) and *snail* (Thisse et al., 1993). Initially, adaxial cells are located at each side of the notochord prior to segmentation at the level of the horizontal septum (HS) as a epithelia-like sheet monolayer flanking each side of the notochord (Devoto et al., 1996). Adaxial cells are relatively larger and more regularly shaped than the surrounding lateral presomitic cells that surround them (Devoto et al., 1996). The specification of adaxial cells to follow the slow muscle differentiation program depends crucially on inductive signals from the notochord and floorplate mediated by members of the hedgehog protein family (Wolff et al., 2003). The acquisition of hedgehog signals by adaxial cells results in the induction of the expression of transcriptional repressor Blimp1/Prdm1 (von Hofsten et al.,

2008). Blimp1/Prdm1 inhibits both fast muscle differentiation and expression of Sox6 transcriptional repressor (von Hofsten et al., 2008), function of which is to inhibit slow muscle differentiation (Hagiwara et al., 2007, 2011; von Hofsten et al., 2008).

Recent studies further indicate that the region previously thought to contain lateral presomatic cells (Devoto et al., 1996) is composed of two distinct cell populations, lateral-anterior and lateral-posterior somite cells (Fig. 1-3A). These three distinct cell populations are rearranged through a series of whole somite rotation events in order to give rise to embryonic slow and fast muscle fibers as well as the myogenic progenitor cells (MPCs) used for subsequent myogenesis. During the whole somite rotation, lateral anterior and posterior cells are rearranged into the lateral-external and later-medial cell layer (Fig. 1-3B). At late segmentation the 90 degree somite rotation is completed leading to the formation of a distinct external cell layer (ECL) sourced from the lateral anterior somite cells (Fig. 1-3C). Additionally, the adaxial cells start to migrate both laterally and radially (Fig. 1-3C). The migrated adaxial cells eventually form a single layer of embryonic slow muscle fibers between ECL and embryonic fast muscle fibers (Fig. 1-3D). Subsequently, some cells from ECL migrate into the myotome though the slow muscle layer giving rise to fast muscle fibers in distinct zones as SH (Fig. 1-3E). It has been suggested that ECL plays an important role in other subsequent postembryonic muscle fiber production although this remains to be proven.

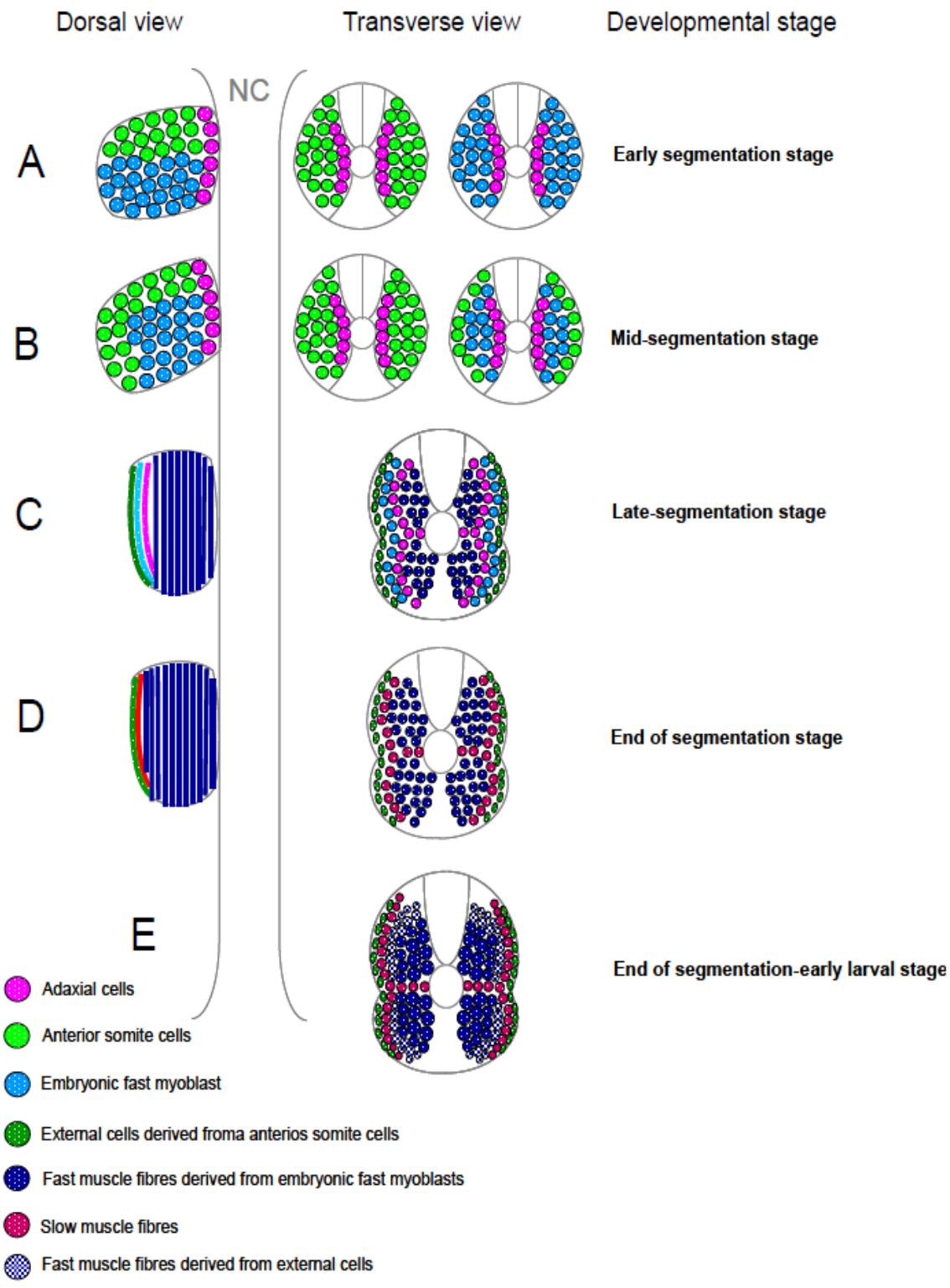

Fig. 1-3: Schematic illustration of the patterning of slow and fast muscle fibers during embryonic and early postembryonic stages (Adapted from Lee, 2010). The transverse views of somites during early and mid segmentation stage indicate the distinct origins of anterior somite cells (anterior) and embryonic fast myoblast (posterior).

Following embryonic myogenesis, new muscle fibers are continued to be added from the so-called "germinal zones" by a distinct process termed SH (Fig. 1-4). SH has been widely identified in many species (review in Rowlerson and Veggetti, 2001) and provides the major source of new muscle fibers during late embryonic and early postembryonic growth. SH gives rise to distinguishable gradients in muscle fiber diameter from the peripheral to the deep myotomes (Fig. 1-4) (Rowlerson and Veggetti, 2001). SH occurs after the completion of segmentation according to the identification of new muscle fibers by their gene expression pattern in zebrafish (Barresi et al., 2001), pearlfish (Steinbacher et al., 2006) and brown trout (Steinbacher et al., 2007). Initially, new slow muscle fibers are mostly added into the dorsal-ventral extremities of the existing single slow muscle layer (Barresi et al., 2001) whereas new fast muscle fibers are widely added at the border of the slow muscle layer, the periphery of the existing fast myotome and dosal-ventral extremities of the myotome (Steinbacher et al., 2006, 2007).

Following SH, the final phase of hyperplasia occurs through MH (Rowlerson and Veggetti, 2001). In contrast to SH, MH involves the proliferation of a population(s) of precursor cells which subsequently migrate and fuse to form myotubes on the scaffold of existing fibers to produce a mosaic of muscle fiber diameters in a myotome across section (Fig. 1-4) (Rowlerson et al., 1995). In fast muscle, muscle fiber recruitment continues until ~40% of the maximum fork length (FLmax) (Weatherley et al., 1988; Johnston et al., 2003; 2004). In teleost which are larger than 40 % FLmax, muscle fiber recruitment is stopped unless the muscle becomes damaged in which case new fiber formation is initiated to repair the injury (Rowlerson et al., 1997). Following the end of muscle fiber recruitment all subsequent growth is by fiber hypertrophy alone, which requires the accretion of additional nuclei to maintain the myonuclear domain size within certain limits (Johnston et al., 2003, 2004). In contrast, slow fiber number continues to increase with body length to the maximum body size (Johnston et al., 2004).

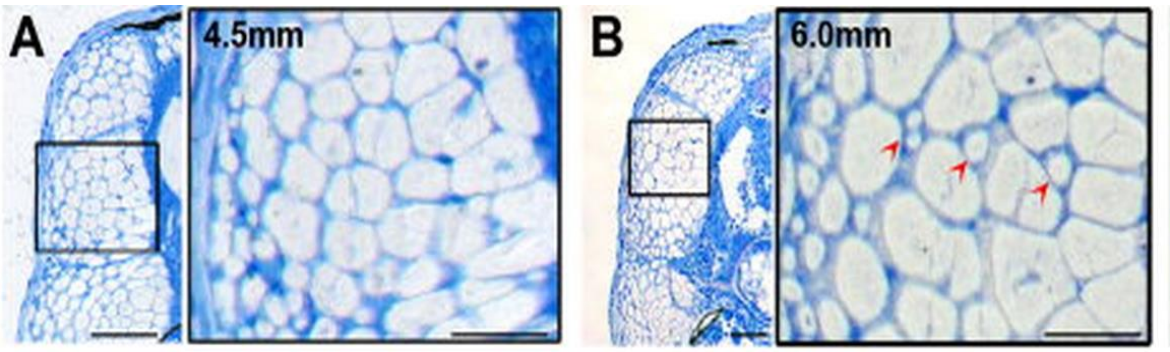

Fig. 1-4: Hyperplastic growth of trunk muscle in zebrafish larvae (Adapted from Patterson et al., 2008). A: Methylene blue stained section showed stratified hyperplastic growth. B: Methylene blue stained section showed mosaic hyperplastic growth (red arrowheads).

1.1.3 Characteristics features of myosin heavy chain

The primary function of skeletal muscle is to produce contractile force via the formation and maintenance of a highly specialized contractile apparatus. Such motile functions are basically attributed to the major muscle proteins, myosin and actin. Myosin is highly conserved, ubiquitous actin-based motor protein that drives a wide range of motile processes in eukaryotic cells. It consists of various types divided into 9–11 classes. Conventional type class II includes the extensively studied group of sarcomeric myosins, a motor protein that interacts with actin to generate the force for muscle contraction (Berg et al., 2001). Sarcomeric myosin consists of two myosin heavy chains (MYHs) of about 200 kDa and four light chains of about 20 kDa. It consists of an N-terminal half of each heavy chain folds into globular head, called subfragment-1 (S1), with two light chains (Fig. 1-5). In contrast, C-terminal halves of two heavy chains, called rod, fold into α helices necessary for thick filament formation and maintaining force (Weiss and Leinwand, 1996). Myosin is organized *in situ* into thick filaments in which the heads of the myosin molecules protrude from the thick filaments surface and form cross-bridges with actin-containing thin filaments. Each cross-bridge is thought to be composed of two myosin heads containing a site for ATP hydrolysis and an actin binding site necessary for converting chemical energy into mechanical force. S1 can be further cleaved into three fragments of 25, 50 and 20 kDa, while rod can be subdivided into subfragment 2 (S2) and light meromyosin (LMM)

fragment (Fig. 1-5). Several MYHs have been found in vertebrates, which show strong amino acid sequence homology with each other. The regions that vary in amino acid sequence between MYHs are largely confined to two external loops associated with the ATP (loop 1) and actin sites (loop 2) in S1 (Weiss et al., 1999). This diversity is thought to play a crucial role in determining speed of contraction and motor function in a particular category of muscle fibers.

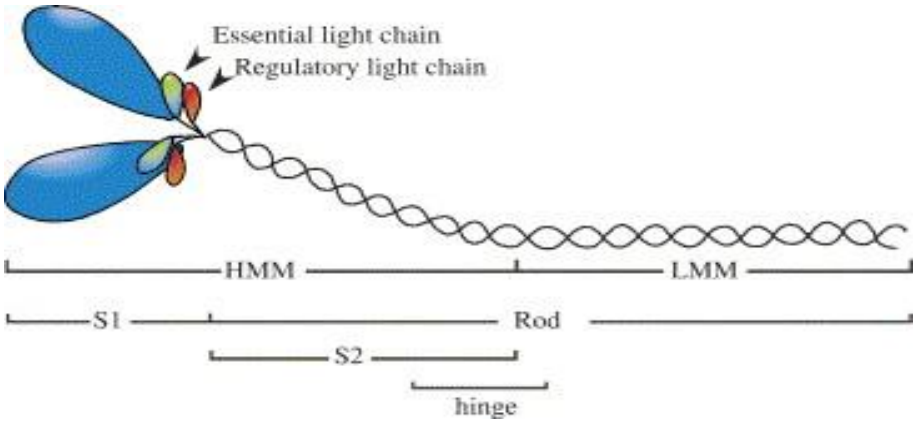

Fig. 1-5: The structure of sarcomeric myosin molecule (Adapted from Watabe and Ikeda, 2006)

1.1.4 Developmentally regulated myosin heavy chain gene expression in fish

Vertebrate genomes contain a variety of MYHs with different functional properties that are encoded by multigene family (Weiss and Leinwand, 1996). Mammalian muscles have at least 11 sarcomeric MYH genes (*MYH*s) and their development- and tissue-specific expressions contribute to the formation of various muscles with diverged muscle-fiber types such as embryonic, neonatal, fast, slow, and cardiac ones with different functional properties (Weiss et al., 1999). In contrast to mammals, fish possess highly diverged MYH multigene family where *MYH*s numbers are much more, possibly due to the additional round (3R) genome duplication (McGuigan et al., 2004). For example, 29 fast-type *MYH*s have been identified in common carp *Cyprinus carpio* (Kikuchi et al., 1999) and 20 sarcomeric *MYH*s in torafugu *Takifugu rubripes* (Ikeda et al., 2007). Studies on expressed *MYH*s have shown a complex developmental-stage-specific expression isoforms in different tissue types that continues throughout much of the life cycle in fish (Mascarello et al., 1995; Johanston et al., 1998).

*MYH*s are expressed sequentially during development in fish. Among *MYH* family members, expression patterns of multiple *MYH*s has been characterized during embryonic development of certain fish species such as zebrafish *Danio rerio* (Bryson-Richardson et al., 2005; Elworthy et al., 2008), common carp *Cyprinus carpio* (Ennion et al., 1999; Nihei et al., 2006), rainbow trout *Onchorhynchus mykiss* (Rescan et al., 2001), medaka *Oryzias latipes* (Ono et al., 2006) and torafugu *Takifugu rubripes* (Ikeda et al., 2007; Akolkar et al., 2010; Asaduzzaman et al., 2013). In zebrafish, adaxial cells are earliest to express slow-type MHY, *smyhc1*, well before their migration towards the lateral surface of myotome (Bryson-Richardson et al., 2005; Elworthy et al., 2008). Bryson-Richardson et al. (2005) also showed that adaxial cells co-express fast-type MYH, *myhc4*, well before their radial migration. Another developmentally regulated *myhz2*, embryonic and tail muscle-specific *MYH*, is expressed in protruding-mouth larvae but not detected in adult fish (Peng et al., 2002). In medaka, three genes are predominantly expressed during development; *mMYHemb1* in embryos and *mMYHL1* and *mMYHL2* in larvae (Ono et al., 2006). In rainbow trout, fast-type *MYH* is expressed in adaxial cells before the expression of slow-type *MYH* (Rescan et al, 2001). In common carp, two fast-type *MYH*s named *MYHemb1* and *MYHemb2* and their homolog *Egg22* and *Egg24* have been characterized during embryonic and larval development (Ennion et al., 1999; Nihei et al., 2006; Ikeda et al., 2010). In torafugu, our laboratory members showed detail expression patterns of *MYH*s during development and adult stages (Ikeda et al., 2007; Akolkar et al., 2010). $MYH_{M743\text{-}2}$ is found be predominantly expressed in fast muscle fibers whereas $MYH_{M86\text{-}2}$ and MYH_{M5} are found to be expressed in slow muscle fibers during embryonic and larval development (Ikeda et al., 2007; Asaduzzaman et al., 2013). These lines of information indicate that each *MYH* showed strictly regulated temporal and spatial expression pattern during development of fish, and that the formation of an individual muscle component is closely related to the temporal expression of specific *MYH*.

1.1.5 Experimental fish species: torafugu and zebrafish

Takifugu rubripes, also called as torafugu, is one of the most popular marine fish cultured in Japan and has a higher market price (Kikuchi et al., 2006). Therefore, it became increasingly important to collect adequate information on the muscle development and growth of torafugu. It is the second vertebrate to have its genome sequenced at draft level (Aparicio et al., 2002). Although only one-eighth the size of the human genome (_365 Mb), the *Takifugu* genome has a similar number of predicted gene loci, and 75% of its putative peptides have homologs in the human proteome (Aparicio et al., 2002). In addition, torafugu genome is compact, generally with short introns and a low proportion of repetitive elements (Brenner et al., 1993; Aparicio et al., 2002; Hedges and Kumar, 2002), making this organism an ideal tool for comparative genomics as well as for evolutionary research (Elgar et al., 1996; Venkatesh et al., 2000). The availability of the torafugu genome sequences greatly facilitates the identification of *MYH*s (Ikeda et al., 2007) and detailed expression analysis showed their complex expression pattern regulated in tissue- and development-specific manners (Akolkar et al., 2010; Asaduzzaman et al., 2013). However, the regulatory mechanisms involved in the spatio-temporal expression of *MYH*s have remained unknown.

Although torafugu represent excellent models for comparative genomic studies, their applications for functional genomics studies such as *in vivo* reporter analysis to understand the regulatory mechanism of a specific gene expression were found to be largely limited by the difficulties of routinely rearing and breeding them in the laboratory compared to other experimental teleost models (Alestrom et al., 2006). There are several important features of zebrafish making it an ideal experimental animal (Fig. 1-6). Differences in the appearance between male and female zebrafish are easily distinguishable (Fig. 1-6A). In the laboratory, a pair of zebrafish can produce approximately 30-50 embryos per spawning, possible in every week, throughout the whole year depending on the level of maturity (Fig. 1-6A). Zebrafish eggs are transparent and relatively large

(~0.7 mm in diameter) compared to other teleost of a similar size (Fig. 1-6A). Embryogenesis is rapid and all major organs develop within 24 hr (Fig. 1-6A). During the first 24 hours of development, embryos are completely transparent, allowing the visualization of developing organs, even deep inside living embryos. The generation time is also relatively short requiring 3-4 months (Fig. 1-6A). Therefore, it was possible to use zebrafish for the first vertebrate large-scale genetic screens which generated a large number of mutants and stable transgenic lines with various phenotypes (Fig. 1-6B). Therefore, zebrafish is an economic animal model system to generate transgenic lines and are easily accessible to transient reporter analysis for quantification. In addition, transgenic zebrafish that express fluorescent protein under the control of tissue-specific promoters are useful tools for following cell movements, visualizing dynamic gene expression patterns, and dissecting regulatory transcription elements in live embryos (Long et al., 1997; Motoike et al., 2000; Zhang and Rodaway, 2007).

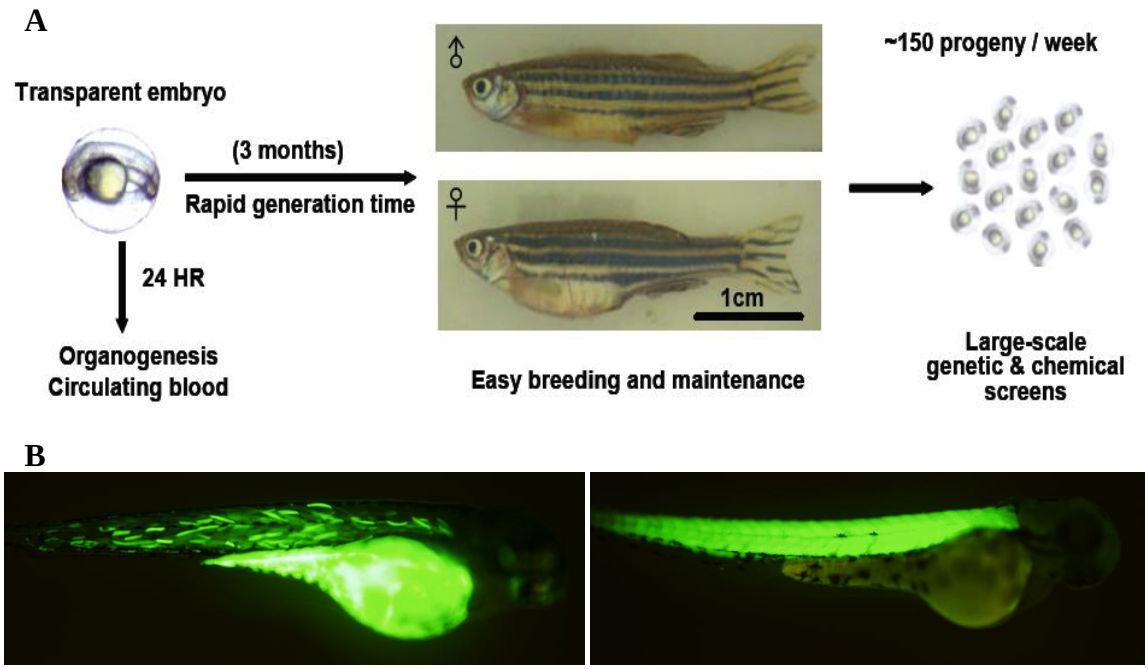

Fig. 1-6: The utilization of zebrafish as model system (modified from Lee, 2010). (A) General features of zebrafish that make them excellent laboratory models. (B) Transgenic approaches in zebrafish embryos are possible.

1.1.6 Transcriptional regulation of muscle-specific gene expression

Cell-autonomous activation of myogenesis in different regions of the embryo is controlled by a series of complex transcriptional regulatory networks that ultimately result in the expression of members of the basic helix-loop-helix domain-containing myogenic regulatory factors (MRFs), which include myogenic factor 5 (*myf5*), myogenic differentiation 1 (*Myod1*, also known as *MyoD*), *Myf6* (also known as *Mrf4*) and myogenin (*Myog*) within nascent and differentiating myoblasts. MRFs are responsible, in concert with a myriad of cofactors, for directing the expression of genes that are required to generate the contractile properties of a mature skeletal muscle cell. These genes act downstream of, or in parallel with, the paired domain and homeobox-containing transcription factors paired box gene 3 (*Pax3*) and 7 (*Pax7*) in different phases of myogenesis in the embryo and adult. Other important transcription factors in differentiation of skeletal muscle fibers are the myocyte enhancer factor 2 (MEF2) family members, which bind to an A/T-rich sequence existing in many muscle-specific promoters and enhancers (Jordan et al., 2004; Berkes and Tapscott, 2005). These are believed to underlie the expression of most, if not all, muscle-specific genes. Serum response factor (SRF), a MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor related to MEF2, also regulates skeletal, as well as cardiac and smooth muscle genes by binding a DNA sequence known as a CArG box (Norman et al., 1988; Miano, 2003). Like MEF2 and other MADS box transcription factors, SRF activates transcription by associating with a variety of signal-responsive and cell type-restricted cofactors.

Another pathway postulated as having a regulatory role in fiber type-specific gene expression is the calcineurin-NFAT (nuclear factor of activated T cells) pathways (Chin et al., 1998). Calcineurin is a phosphatase that dephosphorylates the NFAT family of transcription factors (Rao et al., 1997). Binding of calcium to a calmodulin-calcineurin complex stimulates serine/ threonine phosphatase activity of calcineurin, the major substrates of which are NFAT

transcription factors. Dephosphorylation of NFATs by calcineurin promotes their translocation from the cytoplasm to the nucleus, where they bind a cognate nucleotide recognition sequence (Rao et al., 1997) in slow-specific genes, and stimulate transcription of target genes. MEF2 is also activated by calcineurin as well as by calcium-calmodulin kinase and may activate transcription of some slow fiber-specific genes (Wu et al., 2000, 2001).

Sox6, a member of the Sox family of transcription factors, is a multi-faceted transcription factor involved in the terminal differentiation of fetal skeletal muscle during myogenesis (Higawara et al., 2007). It directly represses the transcription of multiple myosin and sarcomeric genes by binding to conserved *cis*-elements in their promoters (Quiat et al., 2011). In zebrafish and mice, Sox6 functions as a transcriptional suppressor of slow fiber-specific genes (Higawara et al., 2007; von Hofsten et al., 2008). To date, transcriptional regulatory regions have been mapped for a small number of skeletal muscle-specific *MYH*s in fish.

1.2 Objectives of the study

Teleost fish represent a good model for studying vertebrate myogenesis, since slow and fast muscle fibers are arranged in anatomically discrete layers and myotube production is not restricted to early developmental stages, reflecting the large difference between embryonic and final adult body size (Johnston et al., 2003). Muscle development of fish has been extensively investigated with zebrafish especially focusing on the expression of muscle-specific genes and those encoding transcriptional factors that regulate the expression of muscle proteins in relation to the fate of muscle formation (Xu et al., 2000; Hinits and Hughes, 2007). Myosin is the major structural and functional protein of muscle which requires specific expression of MYH isoforms during development. As in mammalian skeletal muscles, *MYH*s of fish are also expressed in a sequential fashion during development and growth.

The *Takifugu* genome has been proposed as a model for rapid characterization of vertebrate genes due to its smallest size among vertebrates (Brenner et al., 1993). Thus, the organization of *MYH*s in the torafugu genome database was investigated, revealing that it contains 20 sarcomeric *MYH*s which formed four clusters on the genome (Watabe and Ikeda, 2006; Ikeda et al., 2007). Their detailed expression analysis of torafugu *MYH*s showed their complex expression patterns regulated in tissue- and development-specific manners (Akolkar et al., 2010; Asaduzzaman et al., 2013). Although a considerable progress has been made in elucidating the molecular genetics underlying the development-dependent muscle-specific expression of *MYH*s in mammals, little is currently known regarding the molecular mechanisms governing *MYH*s expression in fish and much remain unknown or not clearly known regarding the following aspects. Whether the 5'-flanking sequences of *MYH*s of fish, which are phylogenically far from zebrafish in teleost lineage, would function similarly in zebrafish model system for functional genomics studies? What are the factors regulating the expression of *MYH*s in different muscles in fish to provide a more clear insight into how these muscles are established and maintained? Whether the same or different transcription factors regulate the fiber type-specific expression of *MYH*s in the same species of fish? Whether or not *MYH*s are involved in secondary muscle development and growth by hyperplasia in fish and how are their expressions regulated transcriptionally? The present study was carried out in order to understand the above concerns in torafugu by using zebrafish embryos for transient and transgenic analysis. The topics involved in the present study are as follows-

- transcriptional regulation of fast muscle-specific *MYH*, $MYH_{M743\text{-}2}$, during skeletal muscle development of torafugu
- transcriptional regulation of slow muscle-specific *MYH*, $MYH_{M86\text{-}2}$, during skeletal muscle development of torafugu
- expressional regulation of *MYH*s associated with hyperplastic muscle growth in fish

1.3 Outline of the thesis

This thesis is composed of general introduction (**Chapter 1**), three research chapters (**Chapters 2**, **3** and **4**) and general discussion (**Chapter 5**). **Chapter 1** briefly reviewed the general features of myotomal muscle and myogenesis of teleost fish, characteristic features of MYH, developmentally regulated expression of *MYH*s in fish, torafugu and zebrafish as model fish species for genomics and muscle development study and lastly, the transcriptional regulation of muscle-specific gene expression. The research (**Chapter 2-5**) followed a step-wise approach. The first approach (**Chapter 2**) demonstrated the transcriptional regulation of embryonic/larval fast-type torafugu *MYH*, $MYH_{M743\text{-}2}$, expression. Here, we analyzed the 2,075 bp 5'-flanking region of torafugu $MYH_{M743\text{-}2}$ to examine the spatial and temporal regulation by using transgenic and transient expression techniques in zebrafish embryos. The results of this chapter clearly demonstrated that multiple *cis*-elements (MyoD, MEF2 and SRF) in the 5'-flanking region of $MYH_{M743\text{-}2}$ function in the transcriptional control of its expression. The next step (**Chapter 3**) was stimulatory and inhibitory mechanisms of slow muscle-specific *MYH*, $MYH_{M86\text{-}2}$, expression in torafugu. In this chapter, we described an important Sox6 mediated-fast muscle-specific repression mechanism for governing slow muscle-specific expression of $MYH_{M86\text{-}2}$. We further discovered that NFAT binding elements play a key role and MEF2 binding elements participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression. The last step (**Chapter 4**) examined the expressional regulation of *MYH*s associated with hyperplastic muscle growth in fish. In this chapter, we demonstrated the involvement of $MYH_{M2528\text{-}1}$ in both SH and MH of torafugu. In this chapter, we also demonstrated that expression patterns of two zebrafish *MYH*s, *myhz1* and *myhz2*, are complementary to each other. In general discussion (**Chapter 5**), major conclusions of the previous chapters were integrated and interpreted, strength and weaknesses of the followed approaches were outlined, and suggestions for further studies were given.

CHAPTER 2

Multiple *cis*-elements in the 5′-flanking region of embryonic/larval fast-type of the myosin heavy chain gene of torafugu, MYH_{M743-2}, function in the transcriptional regulation of its expression

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Abstract

The myosin heavy chain gene, MYH_{M743-2}, is highly expressed in fast muscle fibers of torafugu embryos and larvae, suggesting its functional roles for embryonic and larval muscle development. However, the transcriptional regulatory mechanism involved in its expression remained unknown. Here, we analyzed the 2,075 bp 5'-flanking region of torafugu MYH_{M743-2} to examine the spatial and temporal regulation by using transient and stable transgenic techniques in zebrafish embryos. Combining both transient and transgenic analyses, we demonstrated that the 2,075 bp 5'-flanking sequences was sufficient for its expression in skeletal, craniofacial and pectoral fin muscles. The immunohistochemical observation revealed that the zebrafish larvae from the stable transgenic line consistently expressed enhanced green fluorescent protein (EGFP) in fast muscle fibers. Promoter deletion analyses demonstrated that the minimum 468 bp promoter region could direct MYH_{M743-2} expression in zebrafish larvae. We discovered that the serum response factor (SRF)-like binding sites are required for promoting MYH_{M743-2} expression and myoblast determining factor (MyoD) and myocyte enhancer factor-2 (MEF2) binding sites participate in the transcriptional control of MYH_{M743-2} expression in fast skeletal muscles. We further discovered that MyoD binding sites, but not MEF2, participates in the transcriptional regulation of MYH_{M743-2} expression in pectoral fin and craniofacial muscles. These results clearly demonstrated that multiple *cis*-elements in the 5'-flanking region of MYH_{M743-2} function in the transcriptional control of its expression.

2.1 Introduction

A major structural protein which is expressed during differentiation of myoblast and myotube in vertebrates is sarcomeric myosin heavy chain (MYH) encoded by highly conserved multigene family. It exists as a variety of isoforms adapted to function in individual muscles according to physiological requirements during development. These isoforms are evolved from primordial MYH genes (*MYH*s). During muscle fiber development, each *MYH* shows a complex expression pattern that continues throughout life cycle (Mascarello et al., 1995; Johnson et al., 1998). Fish are known to possess highly conserved MYH multigene family, although *MYH*s are much more than their higher vertebrate counterparts (Watabe and Ikeda, 2006; Ikeda et al., 2007). A higher number of *MYH*s expressed in fish is the result of many environmental factors such as water temperatures and physiological requirement in different developmental stages, resulting in changes of the composition of muscle-fiber type (Hirayama and Watabe, 1997; Watabe, 2002; Liang et al., 2007). Among *MYH*s family members in fish, specific *MYH*s are expressed during embryonic and larval development. Such expression patterns of *MYH*s have been reported in some fish species such as rainbow trout *Oncorhynchus mykiss* (Rescan et al., 2001), common carp *Cyprinus carpio* (Ennion et al., 1999; Nihei et al., 2006), medaka *Oryzias latipes* (Ono et al., 2006, 2010), torafugu *Takifugu rubripes* etc. (Ikeda et al., 2007), providing some basic insight into embryonic and larval muscle development. These lines of information indicate that the expression of *MYH*s contribute to the formation of specific muscle-fiber types during development of fish in response to various internal and external stimuli. However, the molecular mechanisms of *MYH*s expression in fish remained mostly unknown.

Based on the primary structure, sarcomeric *MYHs* of fish are classified into fast and slow/cardiac types (McGuigan et al., 2004; Ikeda et al., 2004, 2007), and the fast type is further grouped into adult and embryonic types (Ikeda et al., 2010). Genomic structure analysis of torafugu revealed that fast-type *MYH*s in scaffold M743 (Fugu genome database Ver 3.0) form gene cluster C and

classified into an embryonic fast type (Ikeda et al., 2007) with its orthologous genes, all of which have also been reported to be expressed predominantly during embryonic and larval development in common carp (Nihei et al., 2006), medaka (Ono et al., 2006; Liang et al., 2007; Ono et al., 2010), and zebrafish *Danio rerio* (Berdougo et al., 2003; Bryson-Richardson et al., 2005; Wallace et al., 2005). The expression patterns of sarcomeric MYH_{M743-2} in early developmental stages of torafugu were analyzed by our laboratory members. MYH_{M743-2} appeared to be expressed at the somite formation stage (about 10-14 somites) which corresponds to 3 days post fertilization (dpf) and continued through successive embryonic and larval development but not in adult skeletal muscle (Yasmin et al., 2011). The whole mount *in situ* hybridization revealed that the transcripts of MYH_{M743-2} were localized in fast muscle fibers of torafugu embryos and larvae (Ikeda et al., 2007). However, the transcriptional mechanisms in regulating the expression of MYH_{M743-2} are not well defined. Transcriptional regulatory regions have been mapped for a small number of skeletal muscle-specific genes in fish, identifying both unique and shared *cis*-acting elements (Du et al., 2003; Kobiyama et al., 2006; Chen et al., 2007; Liang et al., 2008). Therefore it is important to identify *cis*-acting elements located at various distances upstream or downstream of the promoter responsible for the expression of torafugu MYH_{M743-2}.

The early steps in skeletal muscle development are controlled by combinatorial interactions between the members of myogenic regulatory factors (MRFs) (Bergstrom et al., 2002; Berkes and Tapscott, 2005), MADS box transcription factors (Jordan et al., 2004; Berkes and Tapscott, 2005) and paired-box transcription factors (Pax3 and Pax7) (see reviews by Rawls and Olson, 1997; Buckingham, 2001). So far, the transcriptional activities of *MYH*s have been extensively investigated on the fast skeletal *MYHs* of mammals (Lakich et al., 1998; Swoap, 1998; Allan et al., 2005; Harrison et al., 2011) and fish (mainly carp and medaka) in a temperature dependent manner (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008). However, it is still far

from comprehensive understanding of the transcription factors functioning in the regulation of embryonic and larval fast-type *MYH*s expression in fish.

The purpose of the present study was to identify the *cis*-regulatory regions within the upstream promoter that induce torafugu MYH_{M743-2} promoter activity by transient and stable transgenic analysis. The transient analyses by using expression vector containing various deletion and mutation forms of the 5'-flanking region of a particular gene are useful for identifying *cis*-acting elements responsible for its expression. Here, we used zebrafish to investigate the regulatory mechanisms involved in the expression of torafugu MYH_{M743-2}. We reported previously the isolation of the upstream promoter region of torafugu MYH_{M743-2} and demonstrated that the 2.1 kb 5'-flanking region regulated developmental muscle-specific expression in zebrafish embryos (Yasmin et al., 2011).

In this study, we have characterized torafugu MYH_{M743-2} expression using both transient and transgenic analyses in zebrafish embryos. Combining both transient and stable transgenic analyses, we demonstrated that the 2,075 bp 5'-flanking sequences was critical for its muscle-specific expression in skeletal, craniofacial and pectoral fin muscles. A minimal promoter region containing SRF-like binding site was defined to direct MYH_{M743-2} expression. We discovered that the SRF-like binding sites are required for promoting MYH_{M743-2} expression in myotomal compartments and MEF2 and MyoD binding sites participate in the transcriptional regulation of MYH_{M743-2} expression in myotomal compartments with other transcriptional factors. We further discovered that MyoD binding sites also participate in the transcriptional regulation of MYH_{M743-2} expression in craniofacial and pectoral fin muscles.

2.2 Experimental procedure

2.2.1 Experimental fish and tissue sampling

Adult torafugu (Japanese pufferfish, *Takifugu rubripes*, Tetraodontidae) (body mass about 1 kg) grown in the Department of Aquatic Bioscience, The University of Tokyo, was used for the collection of different tissues such as fast, lateralis superficialis (LS) and erectors and depressors (ED) slow skeletal muscles of the trunk and cardiac muscles. Artificial fertilized eggs of torafugu were collected from Oshima Fisheries Hatchery, Nagasaki, Japan, brought to The University of Tokyo and reared at 18–20 °C in a tank at the Department of Aquatic Bioscience. About 50 embryos each from 1 to 7 days post fertilization (dpf) and an equal number of larvae from 8, 10 and 16 dpf were collected. About 50 embryos of zebrafish each at 2, 6, 20 and 36 hours post fertilization (hpf) and an equal number of larvae from 48 and 72 hpf were collected from the zebrafish rearing facility in the Department of Aquatic Bioscience. All the samples were snap-frozen in liquid nitrogen (-196 ^{o}C) and kept at -80 ^{o}C for RT-PCR.

2.2.2 Total RNA preparation and template cDNA synthesis

Total RNAs were prepared from each developmental stage of torafugu and zebrafish by using an ISOGEN solution (Nippon Gene, Tokyo, Japan) following the instruction of the manufacture. Around 50 embryos and larvae of torafugu and zebrafish were homogenized in 1 mL of Isogen. The homogenates were centrifuged at 12000 g for 10 min and supernatants were transferred to new tubes. About 0.2 mL of chloroform was added to each sample and the mixture was shaken vigorously for 20s and incubated at room temperature for 15 min. After centrifugation at 12000 g for 20 min at 4 ^{o}C, an upper aqueous layer was pipetted out for isopropanol precipitation. RNA was precipitated by the addition of 0.5 mL isopropanol and 2 μL ethachinmate (Nippon Gene, Tokyo, Japan) and kept at -80 ^{o}C for 20 minutes. RNA was pelleted by centrifugation again for 30 min, washed with 70% ethanol, briefly air-dried, and resuspended into 100 μL

diethylpyrocarbonate-treated water. To avoid any possible contamination with genomic DNAs, the RNA preparation was treated with Turbo DNA-free™ (Applied Biosystem, Foster, CA, USA) at 37 ºC for 1 h. The integrity of RNA was determined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel followed by staining with ethidium bromide. The quantity of RNA was determined by measuring optical density at 260 nm with a DU530 Life Science UV-Visible spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The isolated RNA was used immediately or stored at -80 ºC.

cDNA templates for use in RT-PCR were synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's recommendation. Briefly, 2 μg of total RNAs were mixed with 0.5 μL of 10 μM oligonucleotide (dT)-tailed primer (Table 2-1) and 1 μl of 10 mM dNTP solution and the mixture was heated at 65 ºC for 5 min and chilled on ice. Then *in vitro* transcription was carried out at 50 ºC for 60 min in a 20 μL reaction mixture containing 40 units of ribonuclease (RNase) inhibitor (Invitrogen), 1 units of Superscript™ III reverse transcriptase (Invitrogen), I μL 0.1 M dithiothreitol and 4 μL of 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$).

2.2.3 3'RACE

3'-Rapid amplification of cDNA ends (RACE)-PCR was performed to determine the nucleotide sequence in the 3'-untranslated region (3'-UTR) of MYH_{M743-2} by using a specific forward primer (3'RACE-F) and an adapter primer (AP) (Table 2-1). PCR amplifications were carried out at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min with a final extension step for 5 min, using a GeneAmp PCR system 9700 (Applied Biosystems). A single PCR reaction of 20 μL contained 1 μL each of forward and reverse primers (10 μM), 1 μL of cDNA template (dilution 1:100), 2 μL of 10× PCR buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1 U Taq DNA polymerase

(Takara, Tsu, Japan) and 13.8 μL of sterilized water. Amplified DNA fragments were subcloned into the pGEM-T vector (Promega, Madison, WI, USA) and the fragments were sequenced with an ABI 3100 genetic analyzer (Applied Biosystems) after labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Table 2-1. Nucleotide sequences of oligonucleotide primers used in different kinds of PCR

Expt.	Primer name	Nucleotide sequence (5'----------3')	Length (bp)
3' RACE	3'RACE-F	AGA AGC TCG AGT CTG ATC TGG TT	23
	Oligo (dT)	CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA $GC(T)_{17}$	52
	AP	CCA GTG AGC AGA GTG ACG	18
5'RACE	GeneRacer™	CGA CTG GAG CAC GAG GAC ACT GA	23
	5'RACE-R	CCA GGA CTT TGA CCG TGA CCT TAG C	25
RT-PCR	M743-F	TGT TCA CTT GTC CAA GTG CCG GAAG	25
	M743-R	CAA GAG CAA AAA TGT TTA TTA TGG	24
	Fugu β-actin-F	CTG TAT GCC AAC ACT GTG CTG T	22
	Fugu β-actin-R	TTA GAA ACA TTT GCG GTG GAC GAT	24
	SRF1-F	ACG GTG GTC GTA AAG GGC TAG TTT	24
	SRF1-R	TAG AGG TGT CAA TCC CGT GCC ATT	24
	SRF2-F	ATG CTG TCC AGT CAG ACC GGA GC	23
	SRF2-R	AGG TGG TGT ATC TCC GCA GTT TGT	24
	Zebrafish β-actin-F	CCC ATT GAG CAC GGT ATT GTG AC	23
	Zebrafish β-actin-R	ATT GTG AGG AGG GCA AAG TGG TAA	24

2.2.4 5'RACE

GeneRacer™ kit (Invitrogen) was used to define the transcriptional start site of MYH_{M743-2} through RNA ligase-mediated rapid amplification of 5' cDNA ends. Previously synthesized Total RNA from 4dpf torafugu embryos was used and treated with calf intestinal phosphtase (CIP).

Dephosphorylated RNA was then decapped by tobacco acid pyrophosphatase (TAP) and ligated with GeneRacer RNA oligos. After reverse transcription, the 5' cDNA end was amplified by PCR using a *$MYH_{M743\text{-}2}$* specific reverse primer (5'RACE-R) together with GeneRacer™ 5' primer included in the kit (Table 2-1). The PCR was carried out at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s and extension at 72 °C for 1 min with a final extension step for 5 min, using a GeneAmp PCR system 9700 (Applied Biosystems). A single PCR reaction of 20 μL contained 1 μL each of GeneRacer™ 5' primer and gene specific reverse primers (10 μM), 1 μL of cDNA template (dilution 1:100), 2 μL of 10× PCR buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1 U Taq DNA polymerase (Takara) and 13.8 μL of sterilized water. Amplified DNA fragments were subcloned into the pGEM-T vector (Promega) and the fragments were sequenced with an ABI 3100 genetic analyzer (Applied Biosystems).

2.2.5 RT-PCR

RT-PCR was performed to examine the expression of (1) *$MYH_{M743\text{-}2}$* to be restricted to embryonic and larval stages in torafugu and further possible expression in different tissues of adult torafugu and (2) two SRF genes (*srf1*and *srf2*) in zebrafish embryos and larvae. The synthesized cDNAs from embryos, larvae and various tissues of adult torafugu and zebrafish were used as template. PCR amplifications were carried out at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58-60 °C for 30 s and extension at 72 °C for 1 min with a final extension step for 5 min, using a Gene Amp PCR system 9700 (Applied Biosystems). A single PCR reaction of 20 μL contained 1 μL each of forward and reverse primers (10 μM), 1 μL of cDNA template (dilution 1:100), 2 μL of 10× PCR buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1 U Taq DNA polymerase (Takara) and 13.8 μL of sterilized water. The transcripts of torafugu β-actin (U38849) and zebrafish β-actin (NM_131031) genes were used as the internal control for RT-PCR (Table 2-1).

2.2.6 Bioinformatics analysis

The nucleotide sequence of $MYH_{M743\text{-}2}$ determined by 3'RACE was used to identify the genomic sequence of $MYH_{M743\text{-}2}$ by searching the torafugu genome databases assembly ver. 3.0 and 4.0 at QMLU Fugu Genomic Project. Transcription factor-binding sites in the 5'-flanking region of $MYH_{M743\text{-}2}$ were predicted by MatInspector, V7.1 (http://www.genomatix.de) and TFSEARCH program, V1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html). Genes orthologous to torafugu $MYH_{M743\text{-}2}$ were searched in zebrafish, medaka and green spotted pufferfish (*Tetraodon nigroviridis*) genomes using ensemble genome browser (http://www.ensembl.org). The homology search for the 5'-flanking region between torafugu $MYH_{M743\text{-}2}$ and green spotted pufferfish *MYH13* was carried out by rVISTA plot analysis through the Vista server (http://genome.lbl.gov /vista/index.shtml).

2.2.7 Generation of a non-deletion and a series of 5'-deletion constructs

Genomic DNA was isolated from the dorsal fin of adult torafugu by using DNeasy Tissue Kits (QIAGEN Sciences, Maryland, USA). The pT2AL200R150G (Tol2) vector (courtesy of Dr. Kawakami) containing *EGFP* was used as a reporter plasmid. The Tol2 vector was linearized by using *XhoI* and *BamHI* restriction enzymes. To identify the minimal promoter, a series of 5'-deletion constructs within the 2,075-bp fragment was generated by PCR using torafugu genomic DNA as a template. First, PCR primers were designed and converted into In-Fusion® Primers that share 16 bases of identity with the sequence at the ends of the linearized cloning pT2AL200R150G (Tol2) vector by using online In-Fusion® Primers design tool (http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do). A total of 10 forward primers and one reverse primer (Table 2-2) were designed to amplify one non-deletion (2,075 bp) and a series of 5'-distal deletion regions. These primers were then used to amplify the 5'-distal deletion of $MYH_{M743\text{-}2}$ by PCR and the reaction product was purified using QIAquick Spin Columns (QIAGEN Sciences).

All of these purified PCR products with 5'-distal deletion were sub-cloned individually into the *BamHI-XhoI* site of Tol2 vector by In-Fusion® Advantage PCR Cloning Kit (Clontech) following the manufacturer protocol and then transformed into JM109 competent cells. Positive colonies were selected by colony PCR and their sequences were confirmed by ABI3100 genetic analyzer (Applied Biosystems). The plasmid DNA for microinjection was isolated from each deletion construct using GenElute™ plasmid Mini-prep Kit (Sigma-Aldrich). These constructs were P2075, P1482, P1006, P819, P600, P500, P468, P448, P425 and P340, in which numbers refer to the nucleotide positions upstream of the MYH_{M743-2} translation start codon. P2075 was generated as a non-deletion control and the rest deletion constructs contained a deleted MYH_{M743-2} promoter region, *EGFP*, and SV40 polyA signal.

Table 2-2: Nucleotide sequences of oligonucleotide primers used in the generation of 5'-distal deletion construct of MYH_{M743-2}

Primer name	Nucleotide sequence (5'----------3')	Length (bp)
P2075F	GG ATC ATA GCA GTA GGT TTA	20
P1482F	CG TGA ATC CAA AAT GGC ACT AC	22
P1006F	CG GAT GAT TCC AAA TAC CCA G	21
P819F	GC CTC ATG AAA TCA AAA CTC	20
P600F	CA AGA ACA ACT GGT ATG AGG TG	22
P500F	CA CTA CAT ATA ACT TGG CTG TGT C	24
P468F	CC TGT GAA TAT AAG GAC CCC TTT G	24
P448F	TT TGG AGA CAT CAG TGT GGA ACA	23
P425F	AG GAG ACC CGT GAG TTG GTG GTA A	24
P340F	CC AAA TTC AAC AAA TCA AAA TGT TCA C	27
Reverse	AT GGT GGC GGC TTA TGT CTG	20

2.2.8 Generation of *MYH_{M743-2}* : *EGFP* transgenic line

For this purpose, RNA encoding functional Tol2 transposase enzyme was transcribed *in vitro* from pCS-TP vector (Kawakami et al., 2004). At first, pCS-TP plasmid was linearized by digestion with *Not*I, and mRNA was synthesized *in vitro* by using mMESSAGE mMACHINE SP6 Kit (Ambion Inc., Texas, USA) and purified by using Ambion MEGAclear™ Kit. To generate the stable transgenic line, the Tol2-based construct (P2075) was co-injected with transposase mRNA in a final concentration of 50 ng/µl and 25 ng/µl of the P2075 construct and transposase mRNA, respectively, at 1-2 cell stage embryos.

The transient embryonic excision assay (TEEA) is important to examine *cis* and *trans* activities of the transposons system. Therefore, about 8-10 h after the microinjection, embryos was subjected to Tol2 excision assay. For this purpose, the injected embryos were transferred one by one to 0.2-ml strip tubes (eight tubes per strip) and lysed in 50 µl of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 200 µg/ml proteinase K at 50°C for 2 h. Proteinase K was inactivated at 95°C for 5 min. One microliter of the lysed DNA sample was used for PCR (35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 3 min). The excision products were amplified by using the primers T2AexL (5'- CGC AAT TAA CCC TCA CTA AAG G-3') and T2AexR (5'-ACC CAA CTG ATC TTC AGC ATC T-3'). The PCR products were analyzed on 2% agarose gel electrophoresis. The excision product was detected from all embryos injected the transposase mRNA and a transposon-donor plasmid containing T2AL200R150G. These result indicated that excision occurred properly in the injected embryos.

As the TEEA showed successful results, EGFP expression in embryos was observed with a MVX10 macro-zoom microscope (Olympus, Tokyo, Japan). The EGFP-positive embryos were identified at 2dpf and then transferred into the fish rearing unit of the Department of Aquatic Bioscience until sexual maturity. Individual flounder fish were outcrossed with wild-type fish for

examination of EGFP-positive expression in the offspring. EGFP-positive offspring of selected founder lines were raised to establish the F1 generation. The F2 generation was then established by incrossing F1 fish.

2.2.9 Generation of deletions and mutations constructs of various *cis*-elements

Inverse PCR-based site-directed mutagenesis technique was used to generate (1) the deletions and mutations of the *cis*-elements such as SRF, MEF2 and MyoD binding sites, and (2) the deletion construct in the conserved region (-664 to -364 bp) between torafugu $MYH_{M743\text{-}2}$ and green spotted pufferfish *MYH13*. Specific forward and reverse primers were designed for the deletion of *cis*-elements (Table 2-3). Mutant constructs of SRF-like binding site (P468M1, P468M2, P468M3 and P468M4) were generated by using mutant reverse primers, each containing 2-bp mutant bases (Table 2-4, bold face). Then the primers were phosphorylated at 37 °C for 1 h and the enzyme was inactivated by heating at 97 °C for 5 min using a GeneAmp PCR system 9700 (Applied Biosystems). A single reaction of 20 µL containing 7 µL each of forward and reverse primers (50 µM), 2 µL of 10× protruding end kinase buffer, 1µL of rATP and 2 µL of T4 kinase. Inverse PCR was performed using KOD FX DNA polymerase (TOYOBO, Osaka, Japan) and the plasmid DNA of a specific construct as template with phosphorylated primers. Then the amplified inverse PCR products were digested by *Dpn*I at 37 °C for 1 h. The digested inverse PCR products were ligated with the DNA Ligation Kit (Mighty Mix-Takara, Tokyo, Japan) and then transformed into JM109 competent cells. Positive colonies were selected by colony PCR and their sequences were confirmed by ABI3100 genetic analyzer (Applied Biosystems). The plasmid DNA for microinjection was isolated from the positive colony of each of deletions and mutations constructs using GenElute™ plasmid Mini-prep Kit (Sigma-Aldrich).

Table 2-3. Nucleotide sequences of oligonucleotide primers used in the generation of deletion constructs of various *cis*-elements from P2075 constructs of MYH_{M743-2}

Expt.	Primer name	Nucleotide sequence (5'----------3')	Length (bp)
SRF deletion constructs	P2075ΔSRF1F	TTCGTAAACATATGGAGATAATTAG	25
	P2075ΔSRF1R	TTTAGAGACATTATTACGAGGGT	23
	P2075ΔSRF1-2F	ACACTACATATAACTTGGCTGTGT	24
	P2075ΔSRF1-2R	GCTGACAACAAAAAAGGAGACAC	23
	P2075ΔSRF1-3F	TTTGGAGACATCAGTGTGGAACA	23
	P2075ΔSRF1-3R	GGGCTTTGCTGACACAGCCAA	21
MyoD deletion constructs	P2075ΔMyoD1F	GGGCAAAAACACTTAACCAAAGAA	24
	P2075ΔMyoD1R	ATGTTTAAAAAGAATCACATGGC	23
	P2075ΔMyoD1-2F	AACATTTACCATGAAAAAGGTGT	23
	P2075ΔMyoD1-2R	GGATGGTTGTTGGGATTTTTTTG	23
	P2075ΔMyoD1-3F	CATTTTACAATTTTAAGCCTCATG	24
	P2075ΔMyoD1-3R	TTCATAAAAATTACTGGGAGTTG	23
MEF2 deletion constructs	P2075ΔMEF2_1F	AAACATATGGAGATAATTAGACAAGATGG	29
	P2075ΔMEF2_1R	GCAGTTTAGAGACATTATTTCGAGG	25
	P2075ΔMEF2_1-2F	CAAACTAATATGTTACAAATAGATATCAAAC	31
	P2075ΔMEF2_1-2R	AATCACTTTTGCTCATATTTTGGC	24
	P2075ΔMEF2_1-3F	AACAAAGCTCATCGTAGAAAAGT	23
	P2075ΔMEF2_1-3R	TTAGTTTGAGATCTATTTTCAGTGC	25
	P2075ΔMEF2_1-5F	TCCAGGTGGCAAAAAGGTCGG	21
	P2075ΔMEF2_1-5R	TGAAAACAACCACATCAATGTTT	23
	P2075ΔMEF2_1-6F	CCAGCCCAAAAAAATCCCAACAA	23
	P2075ΔMEF2_1-6R	TGTGGTTGTCTTGCATATTGTGT	23
	P2075ΔMEF2_1-7F	ATCATTGATTATTGATCTGTTTAGG	25
	P2075ΔMEF2_1-7R	CACTCAACTTGATAATGTCCTAA	23
	P2075ΔMEF2_1-8F	CACAAATTAGTCGAGTCATTATC	23
	P2075ΔMEF2_1-8R	ATCCAGCTTAATACTGTAATTTA	23
Conserved deletion	P2075Δ664-364F	CCACTTAAATATCTTTCCAAATTCAAC	27
	P2075Δ664-364R	GATAATGTCCTAAAAGGAATGTTTGAT	27

Table 2-4. Nucleotide sequences of oligonucleotide primers used in the generation of mutation constructs of SRF binding site in P468 constructs of MYH_{M743-2}

Experiment	Primer name	Nucleotide sequence (5'----------3')	Length (bp)
SRF mutation constructs	P468M1-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M1-R	CAAAGGGGTCCTT**CC**ATTCACAG	23
	P468M2-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M2-R	CAAAGGGGTCCTTATA**CC**CACAG	23
	P468M3-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M3-R	CAAAGGGGT**GG**TTATATTCACAG	23
	P468M4-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M4-R	C**GG**AGGGGTCCTTATATTCACAG	23

Bold two bases indicate mutation sites

2.2.10 Microinjection and EGFP expression analysis

To perform microinjection, fertilized eggs of zebrafish were collected at 15-30 min after fertilization from the zebrafish rearing facility in the Department of Aquatic Bioscience. In microinjection, each EGFP reporter construct was diluted at 50 ng/mL with sterile distilled water containing 0.025% phenol red and introduced into fertilized eggs at the one to two-cell stage. Embryos were reared at 28 °C, and subjected to observation of EGFP expression patterns during development at the interval of every 24 h. The fluorescence derived from transgenes in embryos and larvae during development was observed with a MVX10 macro-zoom microscope (Olympus) and a FV1000 confocal laser scanning microscope (Olympus). Older embryos were anesthetized with 0.6 μM tricaine methyl sulfonate (Sigma-Aldrich) to inhibit movement during observation.

2.2.11 Immunohistochemical analysis

Immunohistochemical staining was performed in whole mount and on the cryosections made from transgenic zebrafish and stable transgenic line embryos after fixation with 4% paraformaldehyde (PFA) (Westerfield, 1993). F310 (Developmental Studies Hybridoma Bank), F59 (Developmental Studies Hybridoma Bank) and anti-EGFP (Clontech) monoclonal antibodies, which are specific to fast-type myosin light chain, slow-type myosin heavy chain and EGFP, respectively, were used as the primary antibodies. For immunohistochemistry, embryos were fixed with 4% PFA in Tris-buffered saline (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% Tween 20 (TBSTw) overnight at 4°C. Fixed embryos were washed with TBSTw, and blocking was performed using a1.5% blocking reagent (Roche , Mannheim, Germany) in TBSTw. Transverse sections were prepared at a thickness of 16 µm with a cryostat Tissue-Tek Cryo3 (Sakura Finetech, Japan) at −20 °C before the first immunoreactions. Living colors EGFP antibody (Clontech) was used at a dilution of 1:1,000 in blocking solution, and MF20 and F59 at 1:20. Immunoreaction with the first antibody was performed overnight at 4 °C. After incubation, embryos were washed with TBSTw and labeled with the second antibodies, anti-mouse IgG Alexa Fluor 555 and anti-rabbit IgG Alexa Fluor 488 (Invitrogen) at a dilution of 1:250 for overnight at 4 °C. The localization of cell nuclei was observed after staining for 10 min with 40, 6-diamidine-20-phenylindole dihydrochloride (DAPI, Roche) diluted at 1 µg/ml in TBSTw. The signals in the whole mount and cryosection samples were visualized using an Olympus Fluo View1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.2.12 Real time PCR analysis

For relative quantification of EGFP expression, total RNAs from 10-12 *EGFP*-expressed embryos of each of injected constructs were isolated by standard procedures with an isogen RNA extraction kit (Nippon Gene) following the instruction of the manufacture. To avoid any possible

contamination with genomic DNAs, the RNA preparation was treated with Turbo DNA-free™ (Applied Biosystems) at 37 °C for 1 h. cDNA templates for use in real-time PCR were synthesized using SuperScript III reverse transcriptase (Invitrogen) in accordance with the manufacturer's recommendation. Real-time PCR primers (Table 2-5) were designed for *EGFP* and the control gene by using primer express 2.0 (Applied Biosystems). A housekeeping gene encoding β-actin (NM_131031) was selected as references for the calculation of relative expression levels of target genes. The specificity of primers was ensured with NCBI primer blast webpage server (www.ncbi.nlm.nih.gov/tools/primer-blast/).

Real-time quantitative PCR was performed in 96-well optical plates with an ABI Prism 7300 Sequence Detection System (Applied Biosystems). After several pilot experiments, the optimal PCR condition was established: PCR reaction mixture contained 10 μL 2×SYBR premix ExTaqII kit (Takara), 10 μM each of forward and reverse primers (Table 2-5), 1 μL diluted template cDNA (about 10 ng) and 0.4 μL ROX reference dye. PCR consisted of pre-incubation at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The absence of nonspecific products was confirmed by dissociation curve analysis (60 to 95 °C). Fluorescence was monitored at the end of each cycle to obtain the amount of PCR products. The point at which the SYBR fluorescent signal was statistically significant above background was defined as the cycle threshold (Ct), the optimal value of which was chosen automatically. PCR efficiency of each primer pair was determined by running standard curves for 10-fold serial dilutions of cDNA templates prepared from EGFP- expressed zebrafish larvae. The transcript quantities were represented as the relative expression levels of target genes to those of reference genes. To exclude the variation caused by different PCR efficiency, the relative expression levels were calculated by using the comparative Ct difference method (Schmittgen and Livak, 2008).

Table 2-5. Nucleotide sequences of oligonucleotide primers used in real-time PCR analysis

Primer name	Nucleotide sequence (5'----------3')	Length (bp)
EGFP -Forward	AGC AAA GAC CCC AAC GAG AA	20
EGFP-Reverse	GCG GCG GTC ACG AAC TC	17
β-actin-Forward	TGC TGT TTT CCC CTC CAT TG	20
β-actin-Reverse	TCT GTC CCA TGC CAA CCA T	19

2.2.13 Statistical analysis

To compare the percentages of embryos with EGFP expression in skeletal, craniofacial and pectoral fin muscles in various constructs and relative quantification of target gene, statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Tukey's test in Statistical Package for Social Science (SPSS) version 11.5 (SPSS Inc., IL, USA). Data were represented as the mean ± SD and the differences were considered significant at $P<0.05$.

2.3 Results

2.3.1 Expression of torafugu *MYH_{M743-2}*

In order to know the information regarding the expression of torafugu fast-type *MYH_{M743-2}*, RT-PCR was performed by using specific forward and reverse primers synthesized based on the sequence in 3' UTR. RT-PCR revealed that the expression of *MYH_{M743-2}* was dependent on the developmental stage. The transcripts of *MYH_{M743-2}* appeared in embryos at 3 dpf and continued to be expressed through successive embryonic and larval development (Fig. 2-1). These results were almost consistent with those reported by Ikeda et al. (2007), who studied the expression of *MYH_{M743-2}* by *in situ* hybridization and RT-PCR analysis.

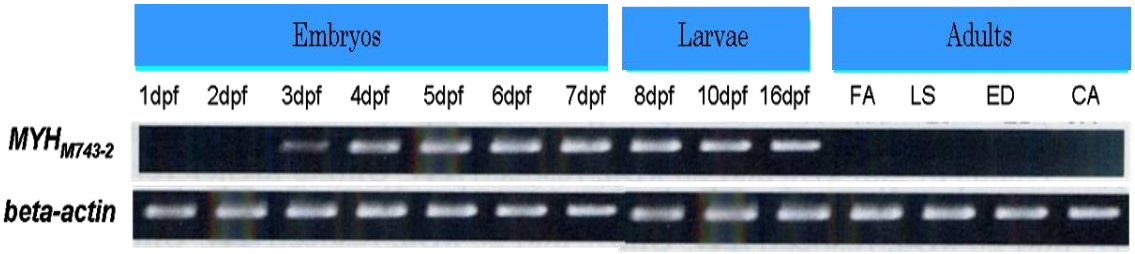

Fig. 2-1: Expression analysis of torafugu *MYH_{M743-2}* from embryos, larvae and various tissues of adult fish. The transcripts of β-actin (U38849) were used as the internal control. dpf, days post fertilization; FA, fast; LS, lateralis superficialis; ED, erectors and depressors; CA, cardiac.

2.3.2 Characterization of 2,075 bp 5'-flanking sequences of torafugu *MYH_{M743-2}*

To determine the regulatory region for the muscle-specific expression, we first characterized the 2,075 bp 5'-flanking region of *MYH_{M743-2}*. The 2,075 bp DNA fragment included an 1,610-bp 5'-upstream region, three exons, and two introns. The transcription start site was determined by 5'-RACE at 465 bp upstream of the ATG start codon in exon 3 (Fig. 2-2). Using the Genomatix Matinspector and TFsearch programs, we found that the 2,075-bp 5'-flanking region contained transcription factor-binding sites for SRF, MEF2 and MyoD, all of which have been implicated in the expressional regulation of muscle-specific genes during development (Fig. 2-2).

Fig. 2-2: The DNA sequence of the 2,075 bp 5'-flanking region of *MYH_{M743-2}*. The transcription initiation site determined by 5'-RACE is indicated by an arrow. Based on the transcription-factor binding site analysis by Genomatix and TFsearch program, MyoD, MEF2, and SRF-like binding motifs are indicated by boxes. Three exons are marked bold. The translation start codon located on the third exon is underlined.

To analyze the transcriptional activity, the 2,075 bp 5'-flanking region of *MYH_{M743-2}* was fused to *EGFP* in pT2AL200R150G vector. The resultant gene construct (P2075) was microinjected into fertilized eggs of zebrafish for transient expression analysis. EGFP expression in injected embryos was monitored by direct observation under a fluorescent microscope. About 90% (n = 105) of the injected embryos showed a strong EGFP expression along skeletal muscle fibers. In zebrafish, the expression was detected at 1 dpf in the somite formation stage (Fig. 2-3A–B). After

hatching, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 2-3C). In addition to the skeletal muscle expression, EGFP expression was also detected in pectoral fin and craniofacial muscles at 4 dpf (Fig. 2-3D-F). These data confirmed that the 2,075 bp 5'-flanking region of torafugu *MYH_{M743-2}* contained the essential regulatory sequences for muscle-specific expression.

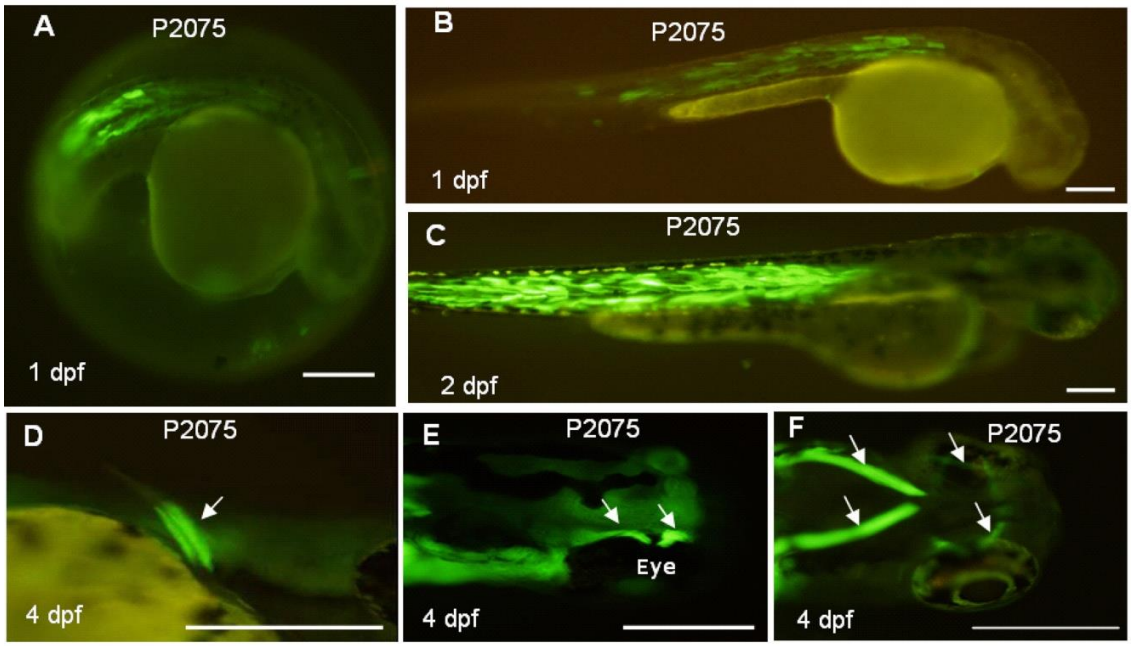

Fig. 2-3: The expression patterns of P2075 construct in embryos and larvae of zebrafish. **A-C**: EGFP expression patterns in whole myotomal region of zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C) injected with the reporter construct of P2075. D-F: EGFP expression patterns in pectoral fin (D) and craniofacial muscles (E, F) in zebrafish larvae at 4 dpf. Arrows indicate muscle fibers expressing EGFP. Scale bars: 100 μm.

2.3.3 Generation and expression pattern of *MYH_{M743-2}: EGFP* transgenic zebrafish

To confirm the expression patterns of P2075 during early muscle development more clearly, we attempted to establish stable transgenic lines. For this purpose, we used the Tol2 transposon vector system, which was originally identified in medaka (Koga et al., 1996) and later adapted as a vehicle to efficiently integrate ectopic DNA into the zebrafish genome (Kawakami et al., 2000, 2004). Transient injection of Tol2 based plasmids was previously shown to be a valuable method

to analyze tissue-specific promoters in zebrafish (Fisher et al., 2006; Korzh, 2007). In this method, P2075 was co-injected with transposase mRNA, which was transcribed from pCS-TP vector. The injected embryos were subjected to the TEEA at 8-10 h after microinjection to confirm whether the excision occurred properly. The excision product was detected from all embryos injected with the transposase mRNA and a transposon-donor plasmid containing T2AL200R150G (Fig. 2-4). Muscle-specific EGFP expressing embryos were selected and raised to adulthood. After 3 months, the surviving founders were mated with wild-type zebrafish to find germline-transmitted transgenic zebrafish.

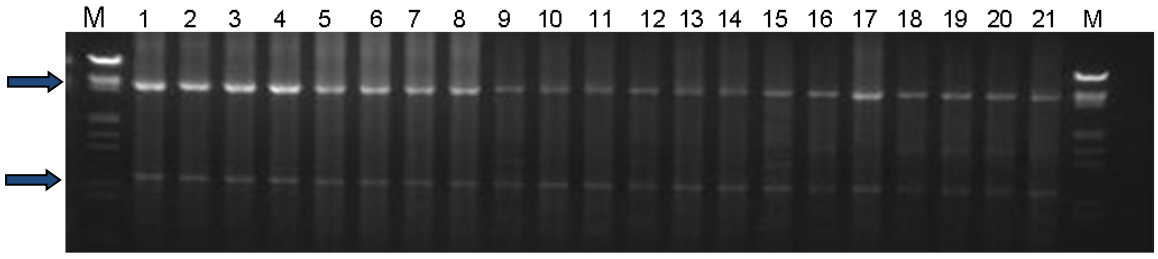

Fig. 2-4: The transient embryonic excision assay. DNA samples prepared from embryos injected with the transposes mRNA and Tol2 based P2075 construct were analyzed by PCR. Top bands represent PCR products from unexcised transposon constructs and bottom bands represent excision products. Lanes 1-21: PCR products amplified from DNA samples prepared from different injected embryo. M, marker.

The 20% (4 out of 20 fish tested) of F0 injected zebrafish were found to be founders, which produced F1 embryos expressing EGFP in the myotomal compartments. About 50% of F2 offspring embryos carried muscle-specific EGFP expression by outcrossing F1 fish with wild-type fish, suggesting that the germline transmission rate of transgene complied with the Mendelian inheritance law. All of the F1 transgenic line fish were mated and produced normal offspring. The temporal and spatial EGFP expression was analyzed in these transgenic lines. Similarly with the transient assay, the embryos of stable zebrafish line also showed a strong expression of the reporter gene along muscle fibers. In the stable transgenic line, the expression

was also detected at 1 dpf (Fig. 2-5A-B). After hatching, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 2-5C). The observation with a fluorescent microscope on 24 h intervals confirmed that zebrafish expressed EGFP in pectoral fin at 3 dpf and craniofacial muscles at 4 dpf (Fig. 2-5D-F). The observed EGFP expression in the stable transgenic line was consistent with the expression pattern of P2075. Taken together, these data suggest that the 2,075-bp 5'-flanking region contained the necessary regulatory elements for MYH_{M743-2} expression in skeletal, craniofacial and pectoral fin muscles.

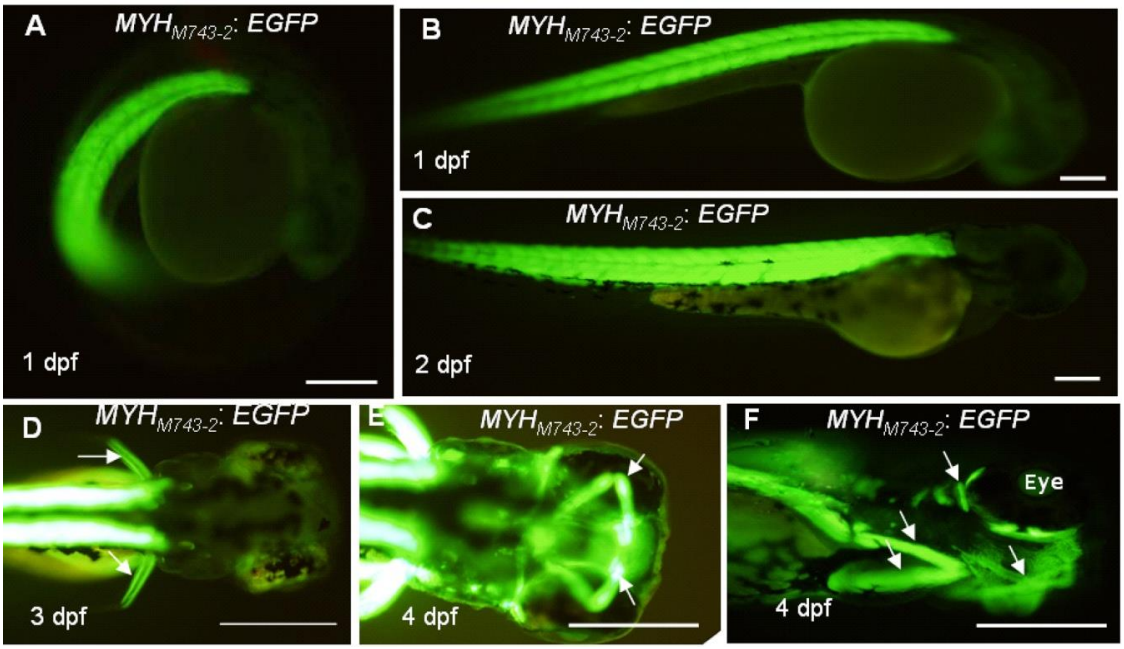

Fig. 2-5: The expression patterns of MYH_{M743-2}:*EGFP* in stable transgenic line embryos and larvae of zebrafish. **A-C**: EGFP expression patterns of MYH_{M743-2}:*EGFP* in the whole myotomal region of stable transgenic line zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C). D-F: EGFP expression patterns in pectoral fin (D) at 3 dpf and craniofacial muscles (E, F) at 4 dpf in stable transgenic line zebrafish larvae. Arrows indicate muscle fibers expressing EGFP. Scale bars: 100 μm.

2.3.4 Immunohistochemical observations

Immunohistochemical observations were conducted to clarify types of muscle fibers that express EGFP. EGFP-expressing embryos were stained with F310 (Crow and Stockdale, 1986) and F59 (Crow and Stockdale, 1986; Devoto et al., 1996) monoclonal antibodies specific to fast-type myosin light chain and slow-type MYHs, respectively. EGFP-expressing muscle fibers of P2075-injected larvae consisted of both fast and slow types reacted with F310 (Fig. 2-6A-B) and F59 antibodies (Fig. 2-6C–D), respectively.

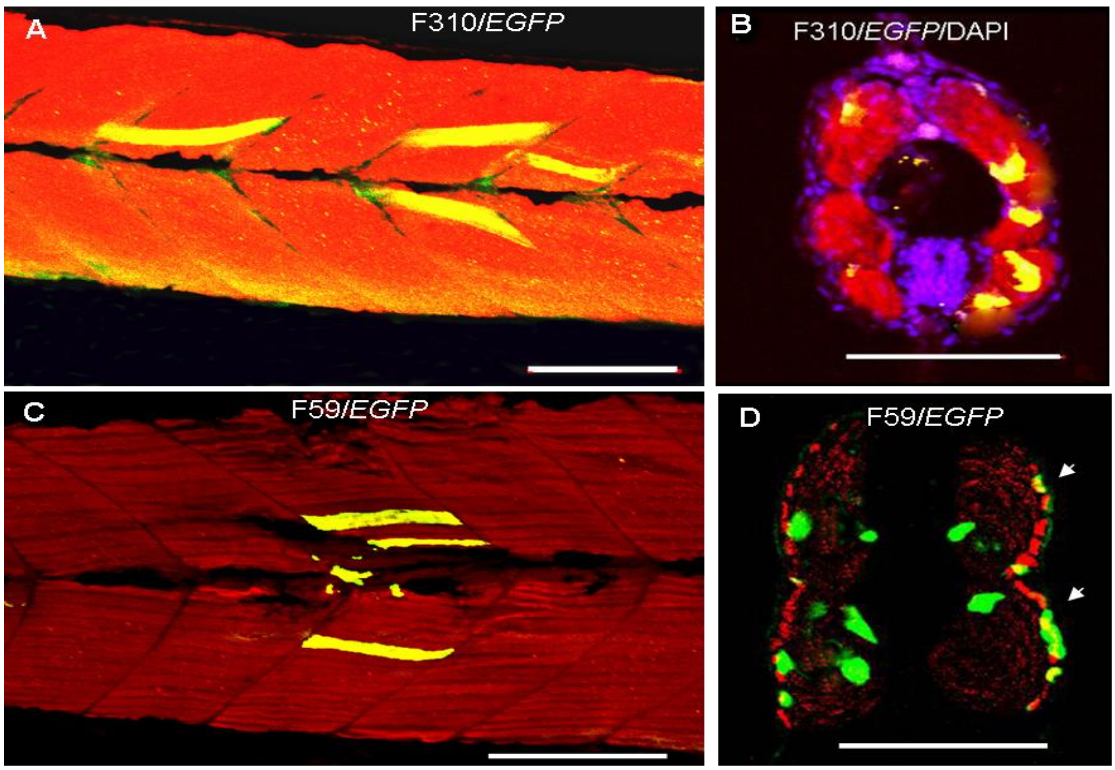

Fig. 2-6: Localization of EGFP expression to both fast and slow muscle fibers in P2075-injected larvae. **A-B**: Fast muscle fibers expressing EGFP as reacted with F310 antibody in a P2075-injected larva at 3 dpf (A, lateral view; B, transverse section). **C-D**, Slow muscle fibers also expressing EGFP as reacted with F59 antibody (C, lateral view; D, transverse section) in a P2075-injected larva at 3 dpf. Some EGFP expressing muscle fibers were stained with F59 antibody (arrows in D). Scale bars: 50 μm.

In situ hybridization demonstrated that the transcripts of $MYH_{M743\text{-}2}$ were restricted to fast muscle fibers in torafugu embryos (Ikeda et al., 2007). Transient transgenic analysis sometimes showed ectopic reporter gene expression. Therefore, immunohistochemical staining was also performed on the cryosection made from the $MYH_{M743\text{-}2}$:*EGFP* stable transgenic line zebrafish larvae at 3 dpf. Interestingly, EGFP-expressing muscle fibers in stable transgenic line larvae consisted of only fast types as reacted with F310 (Fig. 2-7A-C) but not in the slow types as no EGFP muscle fibers reacted with F59 (Fig. 2-7D-F). This study confirmed that $MYH_{M743\text{-}2}$ expression is fast-muscle specific in agreement with the previous findings by Ikeda el al. (2007).

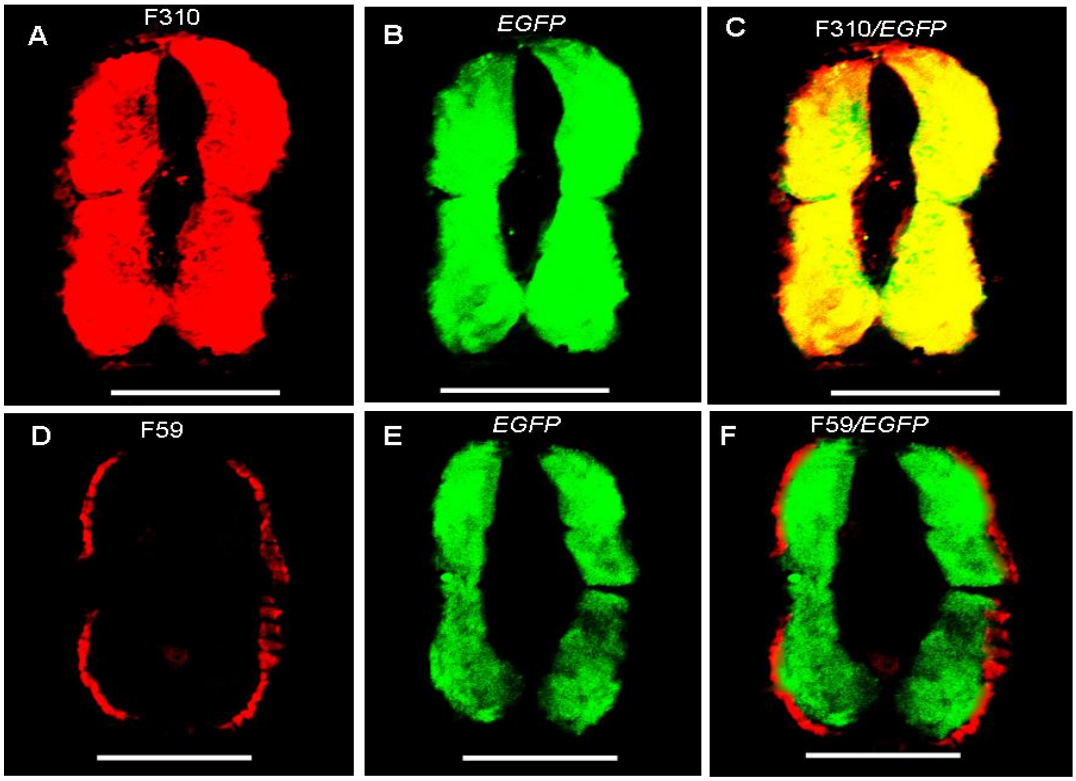

Fig. 2-7: Localization of EGFP expression only to fast muscle fibers in stable transgenic line ($MYH_{M743\text{-}2}$:*EGFP*) larvae. **A-F**: $MYH_{M743\text{-}2}$:EGFP transgene in a stable transgenic line larva showed EGFP expression in fast muscle fibers as reacted with F310 antibody (A, B, C; transverse sections) but not in slow muscle fibers as no EGFP expressing muscle fiber reacted with F59 antibody (D, E, F; transverse sections). Scale bars: 50 µm.

2.3.5 Deletion analysis of torafugu *MYH_{M743-2}* promoter in zebrafish embryos

To identify the minimal promoter that could drive *MYH_{M743-2}* expression in the myotomal compartment, a series of constructs containing progressive deletions from the 5'-end of the 2,075-bp fragment were generated. The resulting six deletions constructs (P1482, P1006, P819, P600, P500 and P340) contained fragments of 1,482, 1,006, 819, 600, 500 and 340 bp, respectively, which were fused to *EGFP* and SV40 polyA signal (Fig. 2-8A). P2075 was generated as a non-deletion control.

When P2075 was microinjected into one to two-cell embryos, approximately 90% of the injected embryos had EGFP expression in the myotomal compartments (Fig. 2-8B). Embryos microinjected with P1482, P1006, P819, P600 and P500 displayed a gradual reduction in percentages of EGFP expression in myotomal compartments (Fig. 2-8B). Notably, EGFP expression was not detected in any embryos microinjected P340. Fluorescent optics also showed that EGFP expression was reduced in embryos microinjected with these deletion constructs and such EGFP fluorescent reduction typically correlated with a smaller *MYH_{M743-2}* promoter region (Fig 2-8C-G). To precisely map the minimal active promoter, three additional deletion constructs, P468, P448 and P425, were generated and microinjected into one to two-cell embryos. Only embryos microinjected with P468 had EGFP expression in the myotomal compartments, and embryos injected with P448 and P425 did not show any EGFP expression in myotomal compartments (Fig. 2-8B). Thus, the 468-bp regulatory sequence contained the minimum basal promoter that could drive EGFP expression in the myotomal compartment. These results also suggest that 20 nucleotides spanning the region from −468 to − 449 bp contain positive *cis*-elements required for *MYH_{M743-2}* expression (Fig. 2-8H).

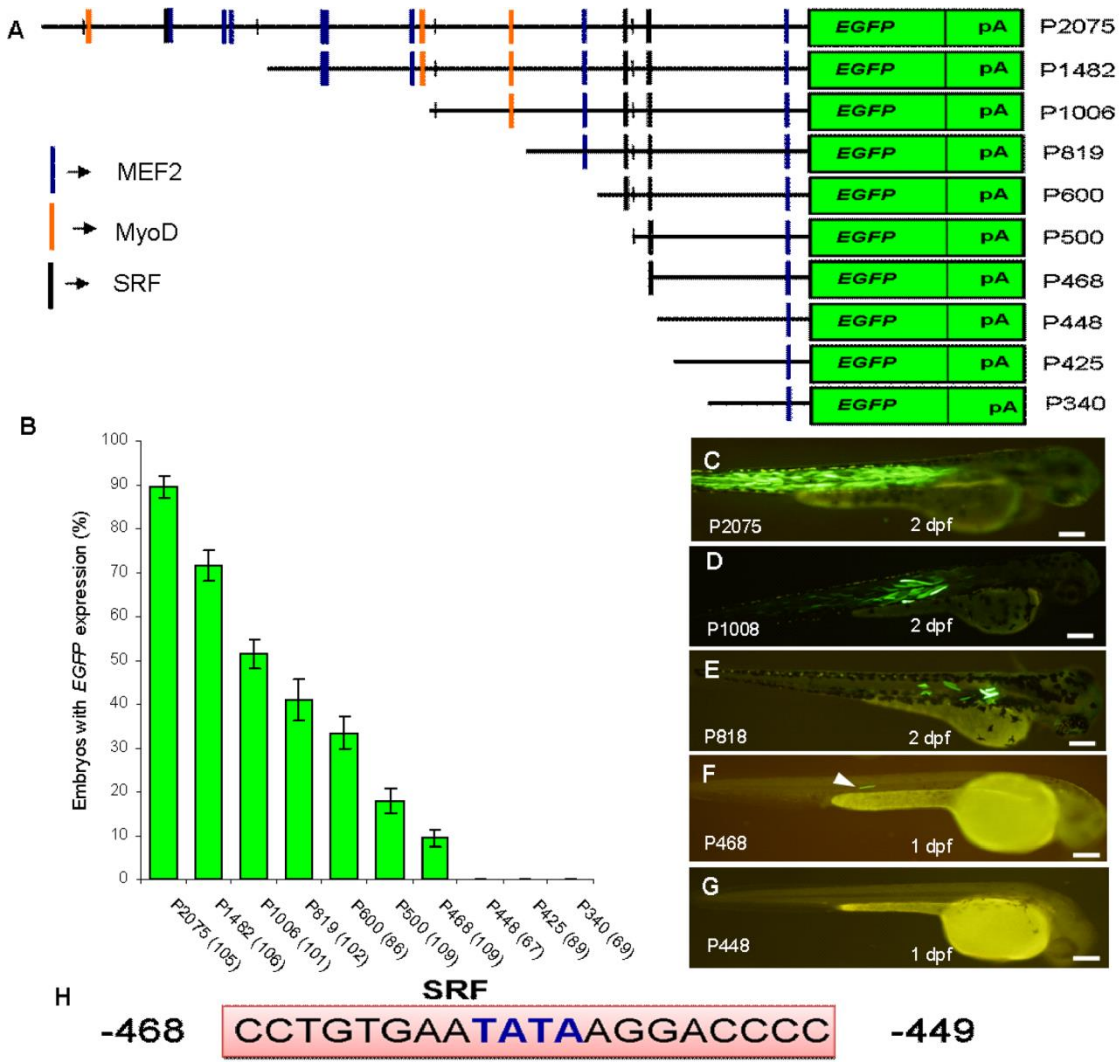

Fig. 2-8: Effects of MYH_{M743-2} promoter deletions on EGFP expression in the myotomal compartments of zebrafish embryos and larvae. **A:** Schematic representations of consensus-binding sites within 2,075-bp upstream region of torafugu $MYH_{M743-2,}$ as well as a deletion series in the torafugu MYH_{M743-2} promoter region. Vertical lines represent consensus-binding sites for transcriptional factors of MEF2, MyoD and SRF. All promoter deletions (black lines) are linked to *EGFP* and SV40-polyA sequence in pT2AL200R150G vector. **B:** Bar graph showing percentages of embryos that express EGFP in the myotomal compartments in microinjection of each deletion construct. The total number of embryos injected with each construct is shown in parentheses. **C-G**: Embryos injected with a series of distal promoter deletion constructs at one- or two-cell stages were examined for transient EGFP expression at 1-2 dpf using fluorescent microscopy. Reduced EGFP fluorescence typically correlates with a smaller MYH_{M743-2} promoter (C-F) and no EGFP expression was observed in P448 (G). An arrowhead indicates a single fiber expressing EGFP in P468 (F). Scale bars: 100 µm. **H:** 20 nucleotides spanning the region from -468 to -449 contain positive *cis*-elements required for MYH_{M743-2} expression. Blue color with bold face in the SRF sequence indicates a central core sequence.

2.3.6 SRF-like binding elements are necessary for directing MYH_{M743-2} expression

Deletion analysis suggested that the 20 nucleotides spanning the region from −468 to −449 bp (CCTGTGAATATAAGGACCCC) may contain positive *cis*-elements required for MYH_{M743-2} expression. Genomatix Matinspector program predicted a transcription factor known as SRF-like binding site within this 20 bp sequence of MYH_{M743-2}. SRF is a member of the MADS box superfamily of transcription factors (Shore and Sharrocks, 1995) and regulates skeletal as well as cardiac and smooth muscle genes by binding to a DNA sequence known as the CArG box (Camoretti-Mercado et al., 2003; Miano, 2003). A growing number of genes have been shown to contain functional CarG boxes, particulary those encoding proteins involved with a cell's cyto-architecture (Minty and Kedes, 1986; Philippar et al., 2004; Zhang et al., 2005; Sun et al., 2006). Further, conditional knockout studies in mice have demonstrated a critical role of SRF in the normal organization of the cyto-contractile apparatus in cardiac, smooth and skeletal muscle cells (Miano et al., 2004; Li et al., 2005). Zebrafish has two SRFs (*srf1* and *srf2*), with partially overlapping expression patterns in larave at 3 and 7 dpf (Davis et al., 2008). In order to know the information regarding the expression of zebrafish *srf1* and *sfr2* more precisely, RT-PCR was performed by using specific forward and reverse primers. RT-PCR revealed that the transcripts of both *srf1* and *sfr2* appeared in embryos as early as 2 hpf and continued to be expressed through successive embryonic and larval development (Fig. 2-9).

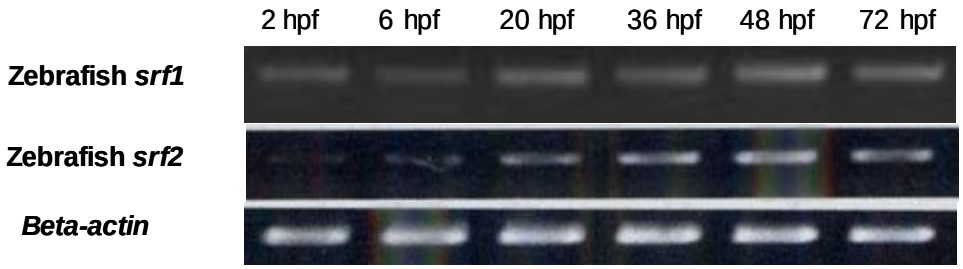

Fig. 2-9: Expression analysis of s*rf1* and *sfr2* in embryos and larvae of zebrafish. The transcripts of β-actin (NM_131031) were used as the internal control.

Therefore, we hypothesized that this SRF-like binding site may be necessary to mediate MYH_{M743-2} expression in the myotomal compartments. To examine this, four primers, each having two altered bases within and outside of SRF-like binding site, were used to generate mutant forms of P468 (Table 2-6). Embryos microinjected with P468M1, containing two base substitutions in the central core sequence, showed the complete disappearance of EGFP expression in the myotomal compartments (Table 2-6). Mutation of two bases adjacent to the core sequence (P468M2 and P468M3) caused a marked reduction of EGFP expression in the myotomal compartments (Table 2-6). In contrast, dinucleotide replacement (P468M4), which was apart from the SRF-like binding site, resulted in relatively higher levels of EGFP expression compared with P468M2 and P468M3 (Table 2-6).

Table 2-6. Mutations in the SRF-like binding site in the P468 construct and their effects on EGFP expression in the myotomal compartments

Constructs	Sequence and mutation	EGFP expression (%)
P468	5'- CCTGTGAA**TATA**AGGACCCCTTTGGAGA	9.17 (n = 109)
P468M1	5'- CCTGTGAA**TGGA**AGGACCCCTTTGGAGA	0 (n = 89)
P468M2	5'- CCTGTGGG**TATA**AGGACCCCTTTGGAGA	2.67 (n = 75)
P468M3	5'- CCTGTGAA**TATA**ACCACCCCTTTGGAGA	3.84 (n = 78)
P468M4	5'- CCTGTGAA**TATA**AGGACCCCTCCGGAGA	5.81 (n =8 6)

Underlined, SRF-like binding site; bold, central core elements of the SRF-like binding site. n, total number of microinjections for each construct. Blue font color indicates mutation within and outside the SRF-like binding site

Genomatix MatInspector program identified three SRF-like binding sites within the 2,075-bp regulatory region of MYH_{M743-2}. To examine functional redundancy of these SRF-like binding sites, we deleted these sites in P2075 and established the P2075ΔSRF1, P2075ΔSRF1-2 and P2075ΔSRF1-3 with deletion of one, two, and three SRF-like binding sites, respectively (Fig. 2-10A). Embryos microinjected with P2075ΔSRF1 did not significantly reduce EGFP expression percentages in myotomal compartments compared to P2075-injected embryos (Fig. 2-10A). P2075ΔSRF1-2 injection exhibited a marked reduction in EGFP expression, and embryos microinjected with P2075ΔSRF1-3 displayed the largest reduction in EGFP expression percentage (Fig. 2-10A). Fluorescent optics showed that EGFP expression pattern in myotomal compartments was variable from embryo to embryo. Therefore, EGFP expressing embryos were grouped into three categories (high, medium and low), according to the intensity of fluorescence by visual observation under a fluorescent microscope (Fig. 2-10B-D). The purpose of this grouping was to investigate whether there was any correlation between the levels of transient expression and the deletion of SRF-like binding sites. Deleting all three SRF-like binding sites resulted in a marked reduction of high-EGFP expressing embryos and an increase of medium- to low-EGFP expressing embryos (Fig 2-10A). In order to confirm this, we validated the reduction of EGFP expression in P2075ΔSRF1-2 and P2075ΔSRF1-3 using real-time PCR analysis (Fig. 2-10E). The deletion of all SRF-like binding sites reduced EGFP expression in P2075, although it failed to eliminate MYH_{M743-2} expression completely. Collectively, these data suggest that SRF-like binding sites, together with other *cis*-elements, are necessary for directing MYH_{M743-2} expression.

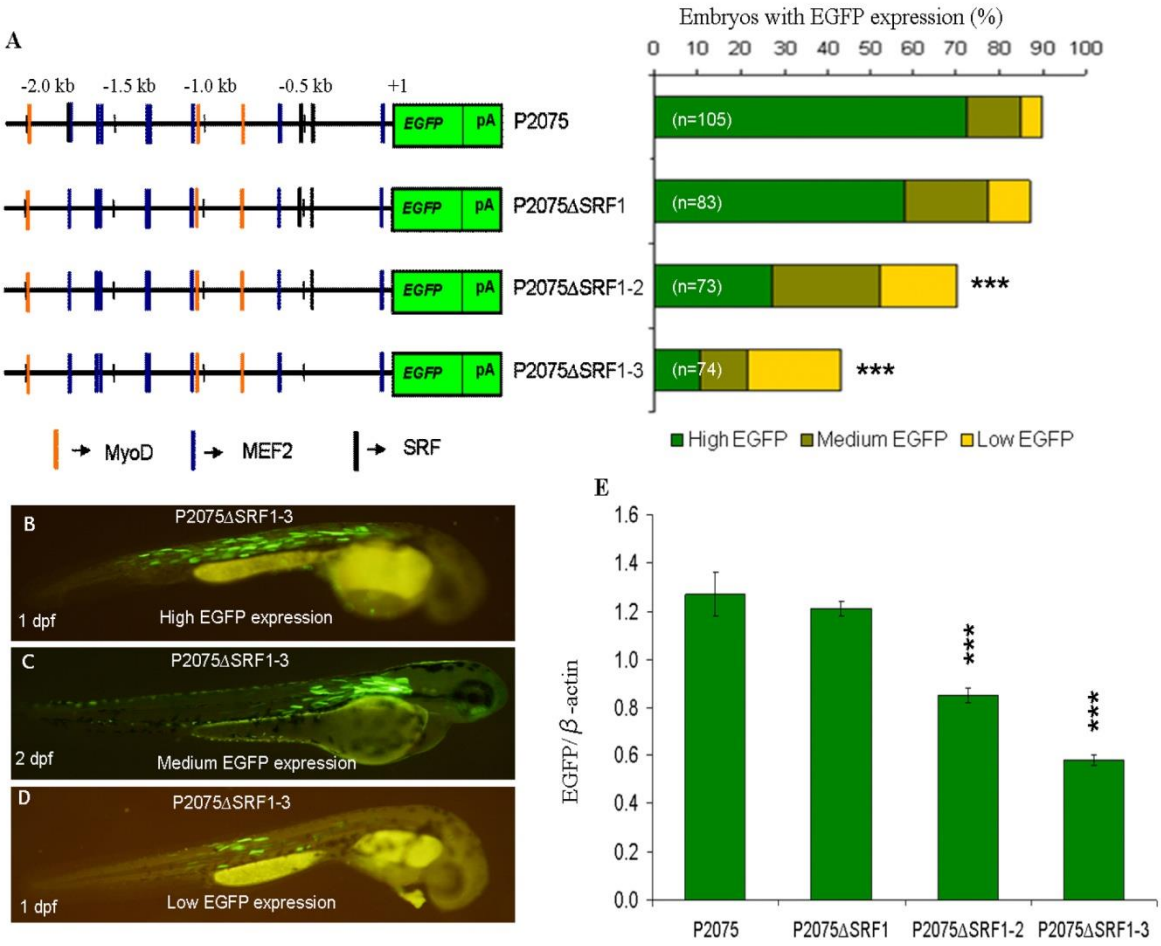

Fig. 2-10: Effects of SRF-like binding sites deletions on EGFP expression in the myotomal compartments. **A**: Schematic representation showing SRF-like binding sites deleted one by one and the percentages of EGFP-expressing embryos (high, medium and low) in each SRF-deletion constructs. The total number of embryos injected with each construct is shown in parentheses. **B-D:** Lateral view showing high (B), medium (C) and low (D) EGFP expression in myotomal compartments in embryos injected with the P2075ΔSRF1-3 construct. **E:** Bar graph showing reduced relative EGFP expression in the P2075ΔSRF1-2 and P2075ΔSRF1-3 constructs using real-time PCR analysis. Difference are significant in ANOVA followed by Tukey test at ***<0.001.

2.3.7 MyoD and MEF2 binding sites are involved in the transcriptional control of *MYH_{M743-2}* expression in skeletal muscle

Deletion analysis confirmed that EGFP expression in myotomal compartments typically correlated with a smaller *MYH_{M743-2}* promoter region. These results indicate that the key *cis*-acting element(s) that regulates the transcription of *MYH_{M743-2}* is located within the deleted promoter region. Analysis of the 2,075 bp 5'-flanking region by Genomatix MatInspector and TFsearch programs identified three putative MyoD and eight putative MEF2 binding sites (Fig. 2-2). These binding sites have been reported to be involved in the transcriptional regulation of many muscle-specific genes during development in fish (Du et al., 2006; Kobiyama et al., 2006; Liang et al., 2008). To determine the role of these MyoD and MEF2 binding sites, we deleted these sites individually or in various combinations (Fig. 2-11A). All deletion mutant constructs were microinjected into zebrafish embryos for transient expression analysis. Deleting any one of these three MyoD or eight MEF2 binding sites alone had little or no effect on the *MYH_{M743-2}* promoter activity and EGFP expression from these single MyoD or MEF2 binding site deletion constructs was almost comparable with the wild-type construct (P2075) (data not shown). Therefore, we decided to generate the multiple MyoD and/or MEF2 binding site-deleted mutant constructs by deleting these sites one by one (Fig. 2-11A). Deleting multiple MyoD and/or MEF2 binding sites significantly reduced EGFP expression percentage compared with the non-deletion construct (P2075) (Fig. 2-11A). However, EGFP expression levels in myotomal compartments were variable from embryo to embryo. Therefore, similarly with the SRF-like binding site deletion analysis, EGFP-expressing embryos were also grouped into high, medium and low categories. Deleting all three MyoD or eight MEF2 binding sites resulted in a significant reduction of high-EGFP expressing embryos (Fig 2-11A). The reduction of such EGFP expression in MyoD and MEF2 binding sites deletion constructs were also validated by using real-time PCR analysis (Fig. 2-11B). These findings suggest that the deletion of MyoD and

MEF2 binding sites reduced EGFP expression in myotomal compartments. However, it should be noted that EGFP expression was not completely inhibited in skeletal muscles in all MyoD and/or all MEF2 binding sites deletion constructs. Collectively, these data suggest that multiple transcriptional factors including MyoD and MEF2 participate in the transcriptional regulation of MYH_{M743-2} expression.

2.3.8 MyoD binding sites participates in the transcriptional control of MYH_{M743-2} expression in craniofacial and pectoral fin muscles

At larval stage (10 dpf) of torafugu, the indigenous transcripts of fast-type MYH_{M743-2} were localized to epaxial and hypaxial domains of myotome, and eye and pectoral fin muscles (Asaduzzaman et al., 2013). Similarly with the indigenous expression, EGFP expression was also observed in craniofacial and pectoral fin muscles in both P2075-injected and stable transgenic line larvae. Because MYH_{M743-2} was expressed in several types of muscle, it would be interesting to determine if the same or distinct regulatory elements are responsible for its transcriptional regulation in skeletal, craniofacial and pectoral fin muscles using transient expression and transgenic approach. Previous studies reported that MRFs such as MyoD and Myf5 had a significant role in driving pectoral fin and head myogenesis (Lin et al., 2006; Hinits et al., 2009). We therefore selected MyoD and MEF2 (in our interest) binding sites to investigate their roles in the transcriptional control of MYH_{M743-2} expression in craniofacial and pectoral fin muscles. During expression analyses, we observed that all skeletal muscle-specific EGFP-expressing embryos did not show EGFP expression necessarily in the above two muscles and that there were no embryos which exhibited EGFP expression only in these two muscles. Therefore, we calculated EGFP expression in these two muscles in terms of percentage in myotomal compartments-specific EGFP-expressing embryos. In non-deletion construct (P2075), about 53.2% and 44.2% of the muscle-specific EGFP-expressing embryos showed EGFP expression in craniofacial and pectoral fin muscles, respectively, at 4 dpf (Fig. 2-11C). In the case of

craniofacial muscle, only embryos microinjected the construct deleting all MyoD binding sites could reduce EGFP expression (Fig 2-11C). For pectoral fin muscle, deleting MyoD binding sites gradually reduced EGFP expression (Fig. 2-11C). However, the deletion of all MEF2 binding site did not significantly reduce EGFP expression irrespective of pectoral fin and craniofacial muscles. These data suggest that the MyoD binding sites, but not the MEF2 binding sites, are crucial for MYH_{M743-2} promoter activity in craniofacial and pectoral fin muscles. It should be noted that the deletion of all MyoD binding elements could not completely abolish EGFP expression in these two muscles. Therefore, additional regulatory sequences other than MyoD binding sites, but not MEF2 binding site, might also be involved in the transcriptional control of MYH_{M743-2} expression in these muscles.

2.3.9 The −664 to −364 bp is a key regulatory region in the MYH_{M743-2} promoter

Initially, we aligned -2,075 bp 5'-flanking region of torafugu MYH_{M743-2} with the corresponding region of orthologues from zebrafish. The sequence analysis showed that there was no homology in the 5'-flanking region of torafugu MYH_{M743-2} with those of zebrafish (data not shown). However, torafugu MYH_{M743-2} showed homology over 55% in the 5' flanking region from −664 to −364 bp with the corresponding region of green spotted pufferfish orthologous gene, *MYH13* (Fig. 2-12A), suggesting a similar regulatory element function for these two genes. Therefore, to examine the functional roles of this conserved region, we deleted the −664 to −364 bp region from P2075 by an inverse PCR technique and established the P2075Δ664-364 construct. When P2075 was microinjected into one to two-cell embryos, approximately 90% of them showed EGFP expression in the myotomal compartments (Fig. 2-12B). However, the embryos microinjected with P2075Δ664-364 showed no fluorescence of EGFP in the whole myotomal region (Fig. 2-12C). These results indicate that the key *cis*-acting element(s) that regulates muscle-specific expression of MYH_{M743-2} is located in this region.

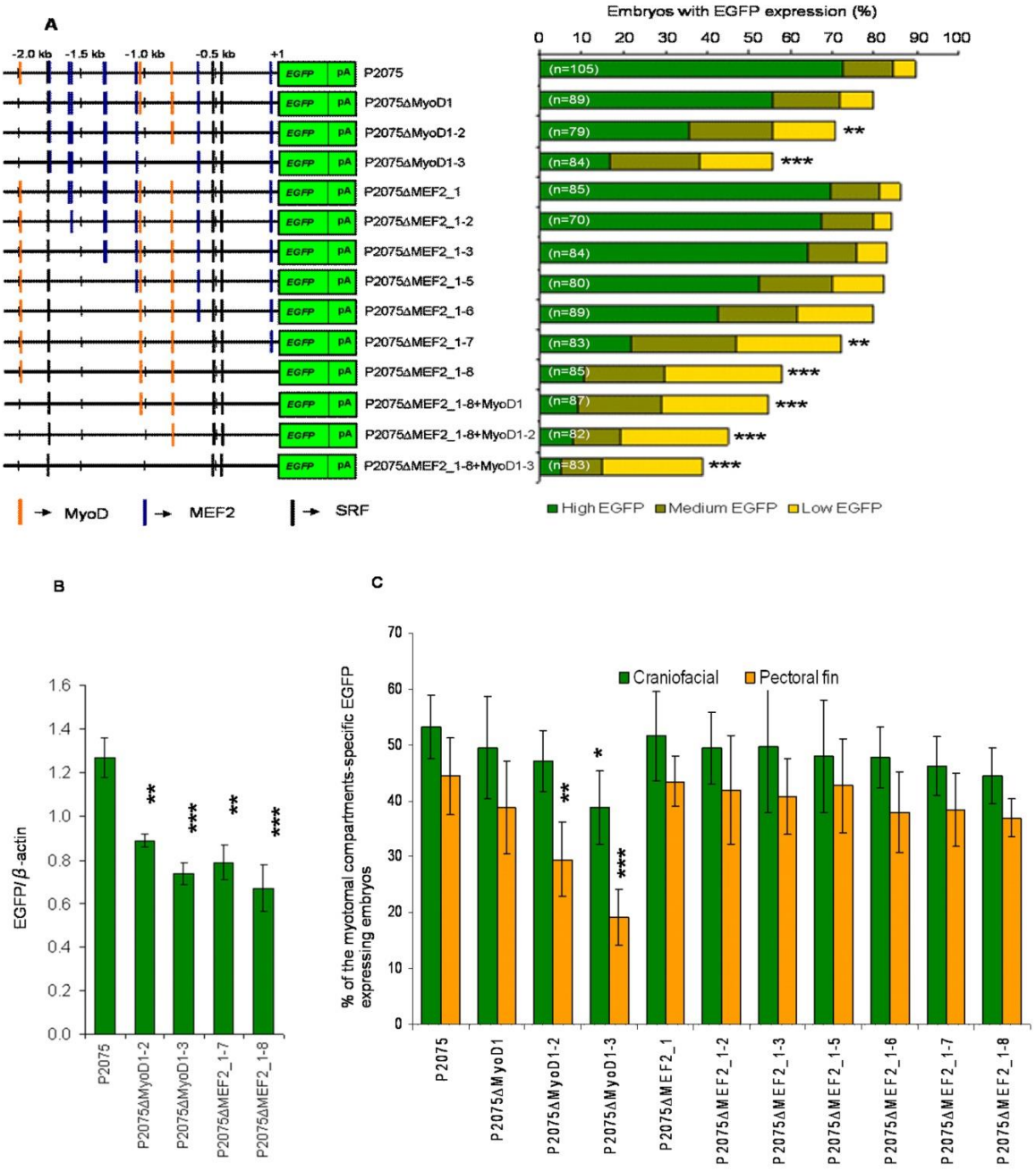

Fig. 2-11: Effects of MyoD and/or MEF2 binding sites deletions on transgene EGFP expression in the myotomal compartments, pectoral fin and craniofacial muscles. **A**: Schematic representation of different constructs in which MyoD and/or MEF2 binding elements were deleted one by one and the percentages of EGFP-expressing embryos (high, medium and low) in each MyoD and/or MEF2 binding site deletion constructs. The total number of embryos injected with each construct is shown in parentheses. **B:** Bar graph showing reduced relative EGFP expression in MyoD and MEF2 binding sites deletion constructs using real-time PCR analysis. **C**: Bar graph showing percentages of embryos showing EGFP expression in craniofacial and pectoral fin muscles to those expressing EGFP in myotomal compartments.

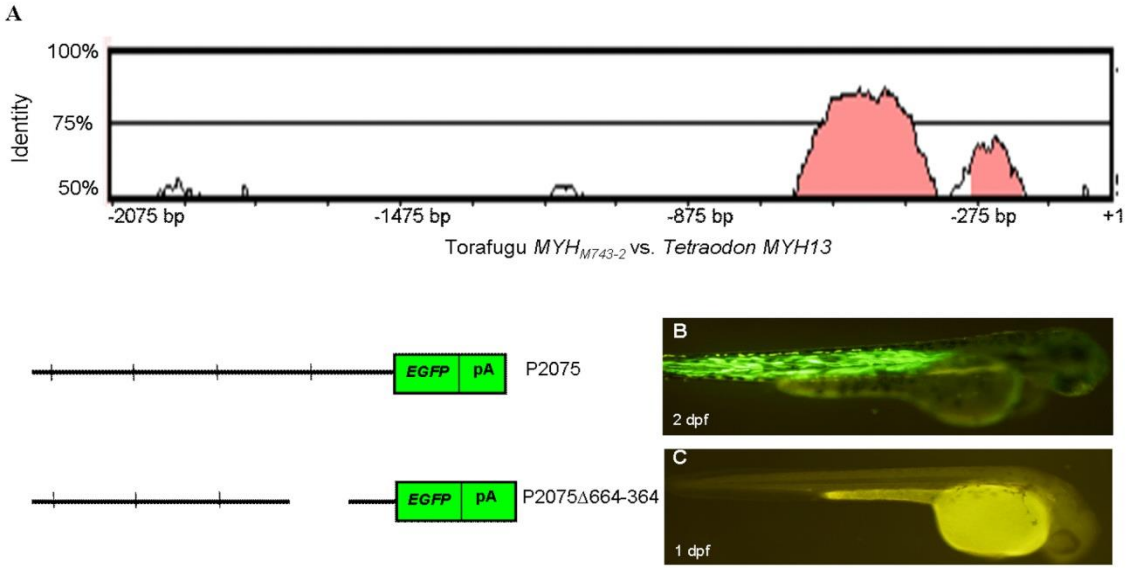

Fig. 2-12: The -664 to -364 bp region in the 5'-flanking region of MYH_{M743-2} crucial for its promoter activity. **A**:. rVISTA plot showing homology (-664 to -364 bp region) in pairwise sequence alignments between −2075 bp of torafugu MYH_{M743-2} and the corresponding region of the *Tetraodon* orthologue. Peaks are shown relative to their positions in MYH_{M743-2} and their percent identities (50%–100%) are indicated on the vertical axis. **B**: EGFP expression was observed in the whole myotomal region in larvae microinjected with the P2075 construct. **C**: No EGFP expression was observed in embryos injected with the P2075Δ664-364 construct.

2.4 Discussion

In embryonic and larval stages of torafugu, multiple sarcomeric *MYH*s are expressed in different muscles in a sequential fashion. A greater understanding of factors regulating the expression of these embryonic- and larval-type *MYH*s in different muscles would provide insights into how these muscles are established and maintained. Although a considerable progress has been made in elucidating the molecular genetics underlying the muscle-specific expression of *MYH*s in carp and medaka mostly in a temperature dependent manner (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008), little is currently known regarding the molecular mechanisms governing gene expression even within the fast subfamily at embryonic and larval stages of torafugu. We recently reported that the 2.1 kb 5'-flanking region of torafugu $MYH_{M743\text{-}2}$ regulated developmental muscle-specific expression (Yasmin et al., 2011). This study identified several regulatory elements involved in the expression of $MYH_{M743\text{-}2}$ in skeletal, craniofacial and pectoral fin muscles by transient and transgenic analyses.

2.4.1 The 2,075 bp 5'-flanking region of $MYH_{M743\text{-}2}$ is sufficient for muscle-specific expression

Combining both transient and stable transgenic techniques, we demonstrated here that a 2,075-bp fragment upstream of torafugu $MYH_{M743\text{-}2}$ could recapitulate muscle-specific gene expression. In transient transgenic assay, the 2,075 bp fragment of torafugu $MYH_{M743\text{-}2}$ was cloned into a Tol2-based vector to reduce mosaicism, and significantly increase sensitivity. The expression patterns of the 2,075 bp construct (P2075) from the transient transgenic assay were confirmed by generating stable transgenic fish lines for P2075. In both transient and stable transgenic analyses, strong EGFP expression was observed along the whole myotomal regions in zebrafish embryos. This observation was in agreement with an earlier report by Ikeda et al. (2007), where the transcripts of $MYH_{M743\text{-}2}$ were localized in the whole myotome of torafugu embryos.

Embryonic *MYHs* have been also reported to be expressed distinctly in craniofacial and pectoral fin muscles in common carp (Ikeda et al., 2010). Similarly, we observed zebrafish larvae expressed *EGFP* in pectoral fin and craniofacial muscles. These results were also in agreement with the expression of embryonic MYH orthologues in zebrafish (Xu et al., 2000) and medaka (Ono et al., 2006, 2010). The endogenous expression of *MYH$_{M743-2}$* by *in situ* hybridization was also found to be expressed in pectoral fin and eye muscles of torafugu by our laboratory member. EGFP expression patterns suggests the functional requirement of *MYH$_{M743-2}$* during larval pectoral fin muscle development which entirely consist of fast fibers (Thorsen and Hale, 2005; Patterson et al., 2008). It also suggests that *MYH$_{M743-2}$* functions in craniofacial muscles development which consists of both fast and slow fibers at larval stage (Hernandez et al., 2005). The whole mount *in situ* hybridization for torafugu embryos and larvae showed the *MYH$_{M743-2}$* expression specific to fast-type muscle (Ikeda et al., 2007). The orthologous genes of *MYH$_{M743-2}$* have been also reported to be expressed only in fast muscle of common carp (Nihei et al., 2006), zebrafish (Xu et al., 2000) and medaka embryos (Ono et al., 2006). While the injected P2075 showed EGFP expression in both fast and slow muscle fibers of larvae as revealed by immunohistochemical analysis, zebrafish larvae from the stable transgenic line expressed EGFP in fast muscle fibers only. Finally, we can say that expressions directed by the 2,075 bp 5'-flanking region of torafugu *MYH$_{M743-2}$* in skeletal, craniofacial and pectoral fin muscles in zebrafish is consistent with its endogenous expression patterns in torafugu. Taken together, these data suggest that this region of torafugu *MYH$_{M743-2}$* is sufficient for muscle-specific expression in zebrafish.

2.4.2 SRF-like binding sites as a critical element of *MYH$_{M743-2}$* promoter activity

SRF is a transcription factor, which binds to a serum response element associated with a variety of genes including muscle-specific genes such as those encoding actin and MYH. By regulating the expression of these muscle-specific genes, SRF controls muscle development and function

(Camoretti-Mercado et al., 2003; Miano, 2003). It binds to the 10 bp sequence CArG box [CC(A/T)$_6$GG], a regulatory element identified in many muscle-specific genes (Miano, 2003). In this study, we revealed that the minimum 468-bp basal promoter drove EGFP expression in the myotomal compartment and suggested that the 20 nucleotides spanning from 468 to 449 bp and containing a SRF-like binding site may be required for MYH_{M743-2} expression. The predicted SRF-like binding site within this 20 bp basal promoter contained a CArG-like box that differed from the consensus sequence by two nucleotides (CCTG**TGAATATAAGG**ACCCC, with bold face for CArG-like box and differing nucleotides underlined). Mutations in the central core sequence (TATA→TGGA) of this SRF-like binding site completely eliminated MYH_{M743-2} expression in the myotomal compartment, suggesting that this SRF-like binding site was necessary for mediating the minimum basal expression of MYH_{M743-2}. In general, no insertions or deletions are allowed in the central AT core as this would disrupt the planar topology of the G base and alter the inherent bending of the CArG element that appears critical for SRF binding (Pellegrini et al., 1995). Substitutions with C or G are best tolerated at the first and last positions of the AT core whereas similar substitution at the middle severely impaired SRF binding (Leung and Miyamoto, 1989; Hautmann et al., 1998; Miano et al., 2000). A very recent study reported that a single nucleotide difference (at 8th base pair position) in CArG motif of human *MyHC-IIb* sequence greatly reduced or abolished SRF binding (Harrison et al., 2011). Therefore, further studies are needed to confirm whether the difference at 1st and 2nd base pair position (TG in stead of CC) in the CArG motif of 486-bp promoter alters the SRF binding.

A considerable progress has been made in elucidating the role of SRF underlying the transcriptional regulation of muscle-specific expression of *MYH* in mammals. A previous study showed that a CArG-like box (SRF binding site) residing in the -120 to -80 bp promoter region act as an enhancers of *MyHC-IIb* transcriptional activity predominantly in fast muscle of adult mice (Allen et al., 2005). *In silico* analysis combined with deletion and site-directed mutagenic analyses revealed that the low transcriptional activity of human *MyHC-IIb* is largely the result of

reduced SRF binding within the proximal promoter region (Harrison et al., 2011). Deletion studies using transgenic mice demonstrated that multiple SRF binding sites were involved in the expressional regulation of the smooth muscle-α actin gene (Mack and Owens, 1999). Functional analyses also indicated a major role of SRF in skeletal muscle fiber growth and maturation in mice (Arsenian et al., 1998; Li et al., 2005). Consistent with all of these studies, we revealed that deleting all three SRF-like binding sites together significantly reduced EGFP expression in myotomal compartments. This data argue that SRF-like binding sites participate in the transcriptional activity of MYH_{M743-2} expression. Although the role of SRF in transcriptional control of *MYH* expression has been reported in mammals (Allen et al., 2005; Harrison et al., 2011), the combined deletion and mutation analyses in this study provided the new insight that SRF binding sites within the upstream promoter regulate the transcriptional activity of *MYH* in fish too.

2.4.3 MyoD and MEF2 binding sites participate in MYH_{M743-2} transcriptional activity in skeletal muscle

Members of MRF (MyoD, myogenin, myf5, and MRF4) and MEF2 have all been shown to regulate the transcription of numerous muscle-specific genes. The transcriptional regulation of these genes is often correlated with the presence of DNA consensus sequences in the regulatory region like E box (CANNTG) and AT rich sequence [(C/T)TA(T/A)$_4$TA(A/G)]. In this study, deleting multiple MyoD and/or MEF2 binding sites together significantly reduced EGFP expression in myotomal compartments of fish. These findings argue that E box to bind MyoD family and AT rich sequences to bind MEF2 may regulate MYH_{M743-2} expression in skeletal muscles. The 5'-flanking region in three adult fast skeletal *MYH*s of mouse was found to have *cis*-elements such as MEF2 binding site, E-box and NFAT binding site (Lakich et al., 1998; Swoap, 1998). Members of the MRF and MEF2 families of myogenic transcription factors appear to be necessary for high level, muscle-specific expression of *MYH-IIb*, whereas NFAT preferentially activates the *MYH-IIa* promoter. It was also shown that the expression of common

carp fast skeletal *MYH*, named *FG2*, required the sequence in the 5'-flanking region between -901 to -824 bp, which contained both E box and MEF2 binding site (Gauvry et al., 1996). Previous studies also reported that MEF2 binding sites are crucial for a temperature-dependent expression of *MYH*s in medaka and common carp (Kobiyama et al., 2006; Liang et al., 2008). Functional analysis also indicated a major role of MyoD and Myf5 for fast muscle myogenesis in individual somites in zebrafish (Hinits et al., 2009). All of these studies indicate that MyoD and MEF2 binding sites are involved in the transcriptional regulation of *MYH*s expression in fish and mammals. It should be noted that deleting all MyoD and/or MEF2 binding sites in the torafugu MYH_{M743-2} regulatory region did not completely abolish its activity in directing EGFP expression in skeletal muscles, suggesting that other regulatory element(s) might be involved in its expression. Regulation of muscle specific *MYH*s expression by multiple regulatory sequences appears to be a common mechanism for the control of muscle-specific gene expression. Supporting this idea, because the deletion of −664 to −364 bp completely abolished the MYH_{M743-2} expression, it remains to be determined which *cis*-elements within this region regulate its expression in fast skeletal, craniofacial and pectoral fin muscles.

2.4.4 MyoD also participates in the transcriptional regulation of MYH_{M743-2} expression in craniofacial and pectoral fin muscles

MyoD has an important role in abaxial pectoral fin and craniofacial muscle formation in zebrafish. Inactivation of Myf5 and/or MyoD leading to malformation of different populations of craniofacial muscles indicates distinct functions of Myf5 and MyoD during craniofacial myogenesis (Lin et al., 2006). Unlike most craniofacial muscles, pectoral fin muscle is somite derived and composed exclusively of fast fibers (Patterson et al., 2008). Hinits et al. (2009) showed that MyoD knockdown prevented myosin accumulation in pectoral fin buds. Therefore, MyoD is also the major MRF required for fast pectoral myogenesis. Because MYH_{M743-2} was also predominantly expressed in both craniofacial and pectoral fin muscles, we hypothesize that MyoD binding site may regulate MYH_{M743-2} expression in these muscles. In our observation,

deleting multiple binding sites of MyoD reduced EGFP expression in craniofacial and pectoral fin muscles. This data suggest that E-box binding to MyoD participate in MYH_{M743-2} expression in the two muscles. However, the deletion of all MyoD binding sites did not completely inhibit EGFP expression in these muscles, suggesting that other regulatory elements might be involved in the transcription regulation of MYH_{M743-2} expression, as in the case of skeletal muscle. Lin et al. (2006) showed that MyoD knockdown completely abolished red fluorescent protein (RFP) signal in some of the extraocular (superior rectus, medial rectus, inferior rectus, lateral rectus) and ventral pharyngeal muscles in the Tg (α-actin:RFP) fish, but other extraocular and dorsal pharyngeal muscles retained RFP with slightly reduced signals. Because of a certain level of mosaic problem with the transient expression assay, we did not characterize which craniofacial muscles are actually affected EGFP expressions by the deletion of MyoD binding sites. Further studies of MyoD knockdown by using MYH_{M743-2}:*EGFP* transgenic fish are required for this purpose. This ambiguity is the next target of our research.

CHAPTER 3

Stimulatory and inhibitory mechanisms of slow muscle-specific myosin heavy chain gene expression in fish: transient and transgenic analysis of torafugu *$MYH_{M86\text{-}2}$* promoter in zebrafish embryos

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Abstract

The myosin heavy chain gene, $MYH_{M86\text{-}2,}$ exhibited restricted expression in slow muscle fibers of torafugu embryos and larvae, suggesting its functional roles for embryonic and larval muscle development. However, the transcriptional mechanisms involved in its expression are still ambiguous. The present study is the first extensive analysis of slow muscle-specific $MYH_{M86\text{-}2}$ promoter in fish for identifying the *cis*-elements that are crucial for its expression. Combining both transient transfection and transgenic approaches, we demonstrated that the 2,614 bp 5'-flanking sequences of $MYH_{M86\text{-}2}$ contains a sufficient promoter activity to drive gene expression specific to superficial slow muscle fibers. By cyclopamine treatment, we also demonstrated that the differentiation of such superficial slow muscle fibers depends on hedgehog signaling activity. The deletion analyses defined an upstream fragment necessary for repressing ectopic $MYH_{M86\text{-}2}$ expression in the fast muscle fibers. The transcriptional mechanism that prevents $MYH_{M86\text{-}2}$ expression in the fast muscle fibers is mediated through Sox6 binding elements. We also demonstrated that Sox6 may function as a transcriptional repressor of $MYH_{M86\text{-}2}$ expression. We further discovered that nuclear factor of activated T cells (NFAT) binding elements play a key role and myocyte enhancer factor-2 (MEF2) binding elements participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression.

3.1 Introduction

Vertebrate skeletal muscle is heterogeneous tissue consisting of various types of muscle fibers and the fiber-type specification is crucial for muscle development. Several unique features of fish skeletal muscle make them an excellent model organism to study such fiber-type specification mechanisms. The most important feature is the anatomical separation of fiber types in contrast with higher vertebrates including mammals in which different types of muscle fibers form a mosaic within the same muscle. Fast-twitch fibers occupy a bulk of fish trunk myotomal muscle and are located deeply in the myotome, whereas slow-twitch fibers lie under the lateral line on the surface of myotome (Bone, 1978). In zebrafish fast and slow muscle cells are originated from different cell lineages, and the latter is differentiated at early developmental stages from adaxial cells, namely paraxial mesodermal cells which exist in direct contact with notochord (Devoto et al., 1996; Dagget et al., 2007). Adaxial cells migrate laterally from either sides of the notochord to the superficial part in the trunk and form slow muscle in a superficial region beneath the skin or remain as muscle pioneers in the horizontal myoseptum (Devoto et al., 1996; Felsenfeld et al., 1991). These slow muscle fibers are mononuclear and span the entire length of each somite. Following the slow muscle formation, an undifferentiated somitic mesoderm differentiates into fast muscle. In contrast to slow muscle fibers, fast muscle fibers undergo fusion to generate multinucleated fibers.

Terminal differentiation and specification of various types of muscle cells require expression of specific myosin heavy chain (MYH) isoforms. Recent development of genome databases has revealed that MYH genes (*MYH*s) consist of multigene family in the vertebrate genome (Weiss and Leinward, 1996). Among *MYH*s family members in fish, specific *MYH*s are expressed during embryonic and larval development. These lines of information indicate that the expression of *MYH*s contributes to the formation of specific muscle-fiber types during development of fish.

Therefore, expressional regulation of *MYH*s is critical for terminal differentiation of various types of muscle fibers, molecular basis of the complex expression of *MYH*s is still ambiguous.

The torafugu *Takifugu rubripes* genome has been proposed as a model for rapid characterization of vertebrate genes due to its smallest size among vertebrates (Brenner et al., 1993). Our laboratory members have investigated the genomic organization of sarcomeric and non-sarcomeric *MYH*s of torafugu by *in silico* analysis of the total genome database, revealing that it contains 20 sarcomeric *MYH*s which are organized in different clusters (Watabe and Ikeda, 2006; Ikeda et al., 2007). Detailed expression analysis by our laboratory members showed the strictly regulated spatial and temporal expression patterns of *MYH*s in torafugu musculature (Akolkar et al., 2010). Comprehensive *in silico* analysis of torafugu genome revealed that there are two paralogous isoforms of MYH_{M86}, $MYH_{M86\text{-}1}$ and $MYH_{M86\text{-}2}$ (Ikeda et al., 2007). Recently, it was demonstrated that $MYH_{M86\text{-}1}$ was predominantly expressed in fast muscle fibers with different diameters in adult torafugu (Akolkar et al., 2010). In contrast, $MYH_{M86\text{-}2}$ exhibited restricted expression in slow muscle fibers of torafugu embryos and larvae only (Asaduzzaman et al., 2013). However, transcriptional regulatory mechanisms involved in the spatio-temporal expression of $MYH_{M86\text{-}2}$ have remained unknown.

Skeletal muscle cell differentiation is coordinated by endocrine, paracrine, and autocrine inductive factors that activate discrete intracellular signal transduction pathways, resulting in the modulation of transcription factor activity and the reprogramming of gene expression. The myogenic regulatory factors (MRF) family of transcriptional activators (Bergstrom et al., 2002; Berkes and Tapscott, 2005) and the myocyte enhancer factor-2 (MEF2) family of transcription factors (Berkes and Tapscott, 2005; Jordan et al., 2004) are believed to underlie the expression of most of muscle specific genes. Another pathway postulated as having a regulatory role in fiber type-specific gene expression is the calcineurin-NFAT (nuclear factor of activated T cells) pathways (Chin et al., 1998). MEF2 is also activated by calcineurin as well as by

calcium-calmodulin kinase and may activate transcription of some slow fiber-specific genes (Wu et al., 2000, 2001). Sox6, a member of the Sox family of transcription factors, is a multi-faceted transcription factor involved in the terminal differentiation of fetal skeletal muscle during myogenesis (Higawara et al., 2007). The transcriptional activities of *MYH*s have been extensively investigated on fast skeletal *MYH*s in fish (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008; Asaduzzaman et al., 2011). However, it is still far from comprehensive understanding of the transcription factors functioning in the regulation of slow-type *MYH*s expression in fish. To the best of our knowledge, this is the first report on the transcriptional regulation of exclusively slow muscle-specific *MYH* expression in fish by transient transfection and transgenic approaches.

In the present study, we examined transcriptional regulatory mechanism of a torafugu slow muscle-specific *MYH,* named $MYH_{M86\text{-}2}$. Combined analysis of torafugu genome data and transgenic approach in zebrafish successfully retrieved promoter-containing sequences which were sufficient for slow muscle-specific gene expressions. We established a stable transgenic zebrafish line that expresses EGFP in slow muscle fibers under the control of a $MYH_{M86\text{-}2}$ promoter. The deletion analyses defined an upstream fragment necessary for repressing ectopic $MYH_{M86\text{-}2}$ expression in the fast muscle fibers. We demonstrated that Sox6 may function as a transcription suppressor of $MYH_{M86\text{-}2}$ expression, and described an important Sox6 mediated-fast muscle-specific repression mechanism for governing slow muscle-specific expression. We further discovered that NFAT binding elements play a key role for directing $MYH_{M86\text{-}2}$ expression in slow muscle fibers and demonstrated that MEF2-binding elements participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression.

3.2 Material and Methods

3.2.1 Experimental fish

Embryos and larvae of torafugu at different developmental stages were reared and different tissues of adult fish were collected as described in section 2.2.1 of Chapter 2. Zebrafish embryos and adult fish were also reared and maintained as described in section 2.2.1 of Chapter 2.

3.2.2 Reverse transcription, amplification and sequence determination

Total RNAs were prepared from whole embryos from 1 to 8 dpf and larvae at 10 and 16 dpf, and various tissues of adult torafugu by using ISOGEN (Nippon Gene, Tokyo, Japan). 5 µg of total RNAs were reverse-transcribed by using oligo-dT primer (Akolkar et al., 2010) and superscript reverse transcriptase III™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 3'-rapid amplification of cDNA ends (RACE)-PCR was performed to determine the nucleotide sequence at the 3'-untranslated region (UTR) of MYH_{M86-2} by using a specific forward primer 5'-GGA AGC TGA CCT CGT CCA GGT T-3' and an adapter primer 5'-CCA GTG AGC AGA GTG ACG-3'. The amplified DNA fragments were sub-cloned and sequenced by the method described in section 2.2.2 of Chapter 2.

RT-PCR was performed to examine the expression of MYH_{M86-2} by using gene specific forward primer 5'-CAT GTC CAG GCT GAG GAA GGT TCA -3' and reverse primer 5'-GCA GTA AAA CGG TTT ATT GAG AT-3' based on the nucleotide sequences in 3' UTR. The transcript of torafugu β-actin (U38849) was used as the internal control for RT-PCR.

3.2.3 *In situ* hybridization

The cDNA fragment of about 250 to 300 bp which contained 3'UTR nucleotide sequences and those encoding a C-terminal part of MYH_{M86-2} was amplified and subcloned into pGEM-T vector

(Promega). Digoxigenin (DIG)-labelled antisense riboprobes were synthesized using T7 and SP6 RNA polymerases (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization was performed in embryos at 4 dpf and on transverse sections (16 μm thickness) for larvae at 16 dpf and juveniles (4.5 g bodyweight) of torafugu by the method of Darby et al. (2006). Samples were rehydrated with 75% methanol/phosphate buffered-saline with 0.1% tween 20 (PBSTw), 50% methanol/PBSTw and 25% methanol/PBSTw consecutively at room temperature for 5 min each. Subsequently, the samples were washed for 5 min twice in PBSTw. Then the samples were permeabilized with proteinase K (Takara) at room temperature for a time period depending on the developmental stages. After several rinses in PBSTw and re-fixation in 4% PFA, the samples were completely dehydrated before hybridization with riboprobes. Hybridization was performed in a humidified incubator at 58°C for at least 16 h followed by two high-stringency washes for 30 min in 2 x saline-sodium citrate (SSC) and two rinses in 1xSSC at the same temperature, respectively. Unhybridized probes were removed after incubation with RNase A in solution containing 0.01 M Tris-HCl (pH 8.0) and 0.5 M NaCl at 58°C for 30 min, followed by two washes in 0.1xSSC for 30 min each at room temperature. Then the samples were blocked with 2% blocking solution for 60 min at room temperature. Blocking buffer was replaced with a solution containing alkaline phosphatase-conjugated anti-DIG antibody (1: 2000 in blocking solution) (Roche Applied Science) for 1 h at room temperature. Unbound DIG antibodies were removed after six washes in PBSTw for 15 min each followed by incubation with a staining buffer containing 0.1 M NaCl, 1.1 M Tris-HCl (pH 9.5) and 0.05 M $MgCl_2$ three times for 5 min each. Finally the samples were stained with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) (Roche Applied Science) for detection of riboprobes. The serial tissue sections were examined with a stereomicroscope MVX10 (Olympus, Tokyo, Japan).

3.2.4 Immunohistochemical analysis

For immunohistochemistry, larvae were fixed with 4% PFA in Tris-buffered saline (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% Tween 20 (TBSTw) overnight at 4°C. Transverse sections were prepared at a thickness of 16 µm with a cryostat Tissue-Tek Cryo3 (Sakura Finetech) at −20°C before the first immunoreactions. Immunohistochemical analysis was carried out by the method described in section 2.2.11 of Chapter 2.

3.2.5 Bioinformatics

The nucleotide sequence of *$MYH_{M86\text{-}2}$* determined by 3'RACE was used to identify the genomic sequence of *$MYH_{M86\text{-}2}$* using ensemble genome browser (http://www. ensembl.org). MyoD and MEF2 binding elements in the 2614 bp 5'-flanking region of *$MYH_{M86\text{-}2}$* were predicted by MatInspector, V7.1 (http://www.genomatix.de). Putative Sox6 binding sites based on sequence (A/T)(A/T)CAA(A/T)G (Cantu et al., 2011) and NFAT binding site based on core sequence GGAAA or TTTCC (Latinis et al., 1997) were manually screened in the 2614 bp 5′-flanking region of torafugu *$MYH_{M86\text{-}2}$*.

3.2.6 Construction of reporter vectors

The 5'-flanking sequences of different length (5500 bp, 5000 bp, 3750 bp, 2614 bp, 2000 bp and 1500 bp) from the start codon in *$MYH_{M86\text{-}2}$* were amplified with specific primers and the genomic DNA as a template. A total of 6 forward primers and a common reverse primer were designed to amplify these fragments (Table 3-1). The constructs were prepared and plasmid DNA for microinjection was isolated by the method as described in section 2.2.7 of Chapter 2. Each of these constructs contained a specific *$MYH_{M86\text{-}2}$* promoter region, *EGFP*, and SV40 polyA signal.

Table 3-1: Nucleotide sequences of oligonucleotide primers used in the generation of 5'-distal deletion construct of MYH_{M86-2}

Experiment	Primer name	Nucleotide sequence (5'----------3')	Length (bp)
Generation of deletion constructs	P5500F	CAGCAGCAAGAATAGAAAGA GTCG	24
	P5000F	CCAAGAAGAAAGAACTGGAGGAG	23
	P3750F	TTCACGGATGACAAAGGGGCTTTG	24
	P2614F	CAACGTGTGTTTGGATGAGTTAATG	25
	P2000F	GACACATACACACGTGGGTCACACA	25
	P1500F	CAGTTGACAGATCCTTCACTTAT	23
	Reverse	GATGGCACCTTAGGATACTGAAAG	24

3.2.7 Generation of *MYH_{M86-2}: EGFP* transgenic zebrafish line

To generate the stable transgenic line, the Tol2-based construct (P2614) of MYH_{M86-2} was co-injected with transposase mRNA into one- to two-cell staged embryos. The stable transgenic line of *MYH_{M86-2}: EGFP* was generated by the method described in section 2.2.8 of Chapter 2.

3.2.8 Generation of deletion constructs of various *cis*-elements

Inverse PCR-based site-directed mutagenesis technique was used to generate the deletion constructs of Sox6, NFAT, MyoD and MEF2 binding sites. Specific forward and reverse primers were designed for the individual deletion each of Sox6, NFAT, MyoD and MEF2 binding sites (Table 3-2). The individual and multiple deletion constructs of various *cis*-elements from 5'-flanking region of MYH_{M86-2} were generated by the method described in section 2.2.9 of Chapter 2.

Table 3-2: Nucleotide sequences of oligonucleotide primers used in the generation of deletion constructs of various *cis*-elements from P2614 constructs of MYH_{M86-2}

Expt.	Primer name	Nucleotide sequence (5'----------3')	Length
MyoD binding site deletion	P2614ΔMyoD-1F	GTTTACTGTCATGCATTATTGTCAAG	26
	P2614ΔMyoD-1R	TCCCTGTCCCTGGTGACAATAG	22
	P2614ΔMyoD-2F	GCTATAAATTTGGACACAAGGAG	23
	P2614ΔMyoD-2R	ACATTATAGTTTTAATATACACAGTTG	27
MEF2 binding site deletion	P2614ΔMEF2-1F	ATTTTTTGTATTACACAATTAAACACCAATTAT	33
	P2614ΔMEF2-1R	TAAATTTTGTTCAGTTTAAATTAACTTTATTTATTTA	37
	P2614ΔMEF2-2F	AGGGGTACTTTGACCTTCCCTGA	23
	P2614ΔMEF2-2R	TTGTGAATACTTGAAATGTCTGAATATAATTG	23
	P2614ΔMEF2-3F	TTATATACATGTATAATTTATTTTACATGTATATACA	37
	P2614ΔMyoD-3R	TAAATAGAGGACAAATATAAAAGTGCC	27
NFAT binding site deletion	P2614ΔNFAT-1F	GAACTGCCTAAATATGTACTGTGC	24
	P2614ΔNFAT-1R	ATTTATTACAGTAAGAGGCAGATTTC	26
	P2614ΔNFAT-2F	TTTATTGAAGAACAAAGGCCAAGC	24
	P2614ΔNFAT-2R	TGACCTTGTTGAATGTCACCTGT	23
	P2614ΔNFAT-3F	AAAACCATATTCTGTTGCCTCTCG	24
	P2614ΔNFAT-3R	AAGCACATAAATGTCAGGGGTTTA	24
	P2614ΔNFAT-4F	TTTTTCAATCCAACAAGGTGTGGA	24
	P2614ΔNFAT-4R	ACACATCACACGGTACAAATGAAT	24
Sox6 binding site deletion	P2614ΔSox6-1F	GCCAAGCAATAATATGTGGTAAATTTAG	28
	P2614ΔSox6-1R	CTTCAATAAAGGAAATGACCTTGTTG	26
	P2614ΔSox6-2F	TTGTATTTATTTATTGAAACAAGCAGTTG	29
	P2614ΔSox6-2R	AATGATCTTCAGAGTTATTAATTCTAAAC	29
	P2614ΔSox6-3F	TAATCATGAAACAATGAGACAACATG	26
	P2614ΔSox6-3R	GAGGTAGGTTTTGAATTAATGTAAATG	27
	P2614ΔSox6-4F	AGACAACATGAGATGCATGTAGTG	24
	P2614ΔSox6-4R	TCATGATTACATTGTAGAGGTAGGTT	26
	P2614ΔSox6-5F	AAAATCCCTTCTTTCAGTATCCTAA	25
	P2614ΔSox6-5R	AAAAATGTCTCTGCTATTTATTTCATTAT	29

3.2.9 Microinjection

To perform microinjection, fertilized eggs of zebrafish were collected at 15-30 min after fertilization from the zebrafish rearing facility in the Department of Aquatic Bioscience. In microinjection, each EGFP reporter construct was diluted at 50 ng/mL with sterile distilled water containing 0.025% phenol red and introduced into fertilized eggs at the one to two-cell stage. Embryos were reared at 28 °C, and subjected to observation of EGFP expression patterns during development at the interval of every 24 h. The fluorescence derived from transgenes in embryos and larvae during development was observed with a MVX10 macro-zoom microscope (Olympus) and a FV1000 confocal laser scanning microscope (Olympus). Older embryos were anesthetized with 0.6 μM tricaine methyl sulfonate (Sigma-Aldrich) to inhibit movement during observation.

3.2.10 Cyclopamine treatment

Fertilized eggs of *$MYH_{M86\text{-}2}$: EGFP* stable transgenic zebrafish line were obtained by crossing matured heterozygous F1 transgenic fish. Transgenic embryos at 2-4 cell stages were transferred into 2.5, 5 and 10 μg ml^{-1} cyclopamine solution (Wako, Osaka, Japan) containing 0.25, 0.5 and 1% ethanol and incubated at 28.5 °C. Control embryos for 2.5, 5 and 10 μg ml^{-1} cyclopamine treatment were developed in 0.25, 0.5 and 1% ethanol, respectively, containing water without cyclopamine. EGFP expressions in cyclopamine treated and untreated control embryos were observed from 1 dpf to 5dpf.

3.2.11 Real-time PCR analysis

For relative quantification, the reaction was performed in a total volume of 20 μl, containing 10 μl 2×SYBR premix ExTaqII kit (Takara), 10 μm each of primers, 1 μl diluted template cDNA (about 10 ng) and 0.4 μl ROX reference dye. Real-time quantitative PCR was performed with an ABI Prism 7300 Sequence Detection System (Applied Biosystems). PCR consisted of

pre-incubation at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The samples were analyzed in triplicates. A housekeeping gene encoding β-actin (NM_131031) was selected as reference for the relative expression levels of target genes, which were calculated using the comparative C_T difference method (Schmittgen and Livak, 2008). Primers used in real-time PCR for *EGFP* are listed in Table 3-3.

Table 3-3. Nucleotide sequences of oligonucleotide primers used in real-time PCR analysis

Primer name	Nucleotide sequence (5'----------3')	Length (bp)
EGFP -Forward	AGC AAA GAC CCC AAC GAG AA	20
EGFP-Reverse	GCG GCG GTC ACG AAC TC	17
β-actin-Forward	TGC TGT TTT CCC CTC CAT TG	20
β-actin-Reverse	TCT GTC CCA TGC CAA CCA T	19

3.2.12 Statistical analysis

To compare the percentages of embryos with EGFP expression in superficial slow muscles for various constructs and relative quantification of target gene, statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Tukey's test in Statistical Package for Social Science (SPSS) version 14 (SPSS Inc., IL, USA). Data were represented as the mean ± SE and the differences were considered significant at $P<0.05$.

3.3 Results

3.3.1 *MYH_{M86-2}* expression is restricted to slow muscle fibers in torafugu embryos and larvae

In silico analysis of torafugu genome showed that *MYH_{M86}* encodes two paralogous muscle-specific isoforms, *MYH_{M86-1}* and *MYH_{M86-2}* (Ikeda et al., 2007). Previously we demonstrated that *MYH_{M86-1}* was predominantly expressed in fast muscle fibers with different diameters in adult torafugu (Akolkar et al., 2010). RT-PCR using highly specific primers synthesized on the basis of the 3'UTR sequences of *MYH_{M86-2}* revealed its expression in developmental stage-dependent and tissue-specific manners (Fig. 3-1A-E). The transcripts of *MYH_{M86-2}* appeared in embryos at 4 dpf and continued to be expressed in later embryonic and larval development stages (Fig. 3-1A). However, no transcripts of *MYH_{M86-2}* were detected in adult muscles, suggesting their important role in embryonic and larval muscle development of torafugu. The *in situ* hybridization for torafugu embryos at 4dpf (Fig. 3-1B-C) and larvae at 16 and 10 dpf (Fig. 3-1D-E) confirmed that the expression of *MYH_{M86-2}* was restricted to slow and pectoral fin muscles. Thus, an expression pattern of *MYH_{M86-2}* was in a marked contrast to its previously reported adult fast-type paralogous gene, *MYH_{M86-1}* (Akolkar et al., 2010).

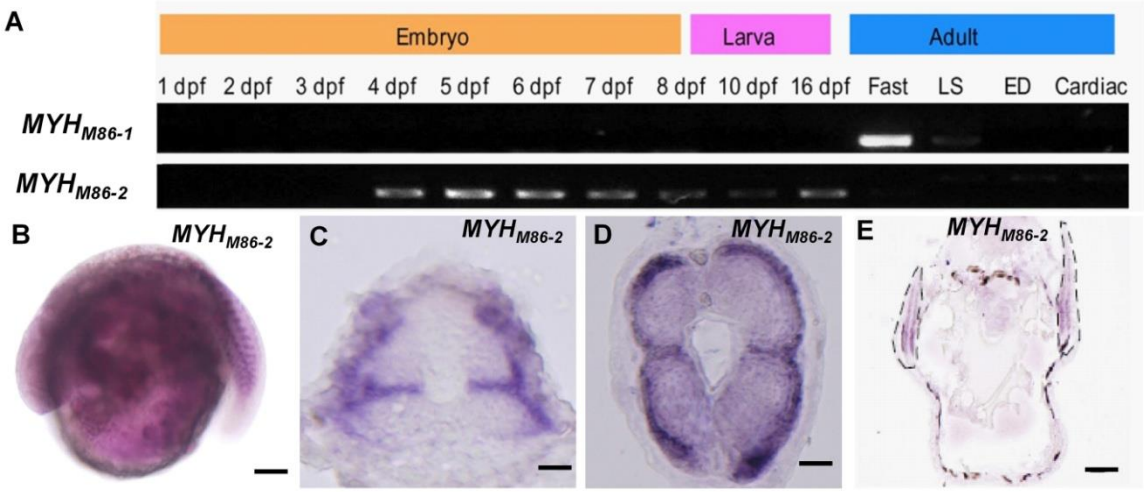

Fig. 3-1: Endogenous expression patterns of *MYH_{M86-2}* in torafugu. A) In contrast to *MYH_{M86-1}*, *MYH_{M86-2}* expression was restricted to embryonic and larval stages. B-E) *In situ* hybridization showing the transcripts of *MYH_{M86-2}* were localized at superficial slow muscle in torafugu embryos at 4dpf (B,C) and larvae at 16 dpf (D) and pectoral fin muscles in larvae at 10 dpf (E)

3.3.2 Slow muscle specificity of the torafugu $MYH_{M86\text{-}2}$ promoter

In situ hybridization study showed that $MYH_{M86\text{-}2}$ is highly expressed in slow muscle fibers and pectoral fin muscles of torafugu embryos and larvae (Fig. 3-1B-E). To examine whether or not the 5'-flanking sequence of torafugu $MYH_{M86\text{-}2}$ would function similarly in zebrafish, we constructed a reporter vector containing the 2,614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ fused to the open reading frame (ORF) of *EGFP* in pT2AL200R150G vector. The resultant gene construct, P2614, was microinjected into one- to two-cell zebrafish embryos for transient EGFP expression analysis. The EGFP expression in injected embryos was monitored by direct observation under fluorescence microscope. Over 93% (n=113) of the injected embryos exhibited EGFP expression at 1 dpf in the somite formation stage (Fig. 3-2A). After hatching at 2 dpf, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 3-2B-C). In addition to the myotomal muscle expression, EGFP expression was also detected in pectoral fin muscles at 4 dpf (Fig. 3-2D). However, a very few embryos showed ectopic EGFP expression in cardiac muscles (data not shown). Immunohistological observations were conducted to clarify types of muscle fibers that express EGFP. EGFP-expressing embryos were stained with F310 (Crow et al., 1986) and F59 (Crow et al., 1986; Devoto et al., 1996) monoclonal antibodies specific to fast-type myosin light chain and slow-type MYHs, respectively. EGFP-expressing muscle fibers of P2614-injected larvae were contained in only slow types as reacted with F59 antibody (Fig. 3-2E-F) but not in the fast types as EGFP expressing muscle fibers did not react with F310 antibody (Fig. 3-2G). Therefore, the observed EGFP expression of transient analysis in zebrafish embryos was consistent with the endogenous $MYH_{M86\text{-}2}$ expression in torafugu embryos and larvae. These data confirmed that the 2,614 bp 5'-flanking region of torafugu $MYH_{M86\text{-}2}$ contained the essential regulatory sequences for slow muscle-specific expression.

3.3.3 Characterization of $MYH_{M86\text{-}2}$ expression using $MYH_{M86\text{-}2}$: *EGFP* transgenic zebrafish

To confirm the slow muscle-specificity of the 2,614 bp 5'-flanking sequence of $MYH_{M86\text{-}2}$, we next attempted to establish a stable transgenic zebrafish line carrying the $MYH_{M86\text{-}2}$: *EGFP* transgene. For this purpose, we used the Tol2 transposon vector system (Kawakami et al., 2000, 2004). In this method, the P2614 construct was co-injected with transposase mRNA, which was transcribed from pCS-TP vector. Superficial slow muscle-specific EGFP-expressing embryos were selected and raised to adulthood. Stable muscle-specific EGFP-expressing zebrafish line (F1) was established from 24 founders tested. In F2 offspring, 48-50% embryos carried muscle-specific EGFP expression by outcrossing F1 fish with wild-type fish. All of the F1 transgenic line fish were mated, producing normal offspring. The temporal and spatial EGFP expression was analyzed in this transgenic line. Similarly with the transient transgenic assay, stable transgenic line embryos showed EGFP expression at 1 dpf (Fig. 3-2H). After hatching at 2 dpf, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 3-2J-L). The observation with a fluorescent microscope also confirmed that zebrafish expressed EGFP in pectoral fin muscles at 3 dpf (Fig. 3-2I). To further confirm that the EGFP-expressing muscle fibers are indeed slow types, we performed the immunohistochemical analysis as reported previously. The EGFP-expressing muscle fibers of stable transgenic line larvae were found only in the slow type (Fig. 3-2M-N) but not in the fast type (Fig. 3-2O-P). The observed EGFP expression in the stable transgenic line zebrafish embryos and larvae was consistent with the expression pattern of P2614-injected zebrafish embryos and larvae, and endogenous expression of $MYH_{M86\text{-}2}$ in torafugu embryos and larvae. Taken together, these data suggest that the 2,614-bp 5'-flanking region contained the necessary regulatory elements for $MYH_{M86\text{-}2}$ expression in slow and pectoral fin muscles.

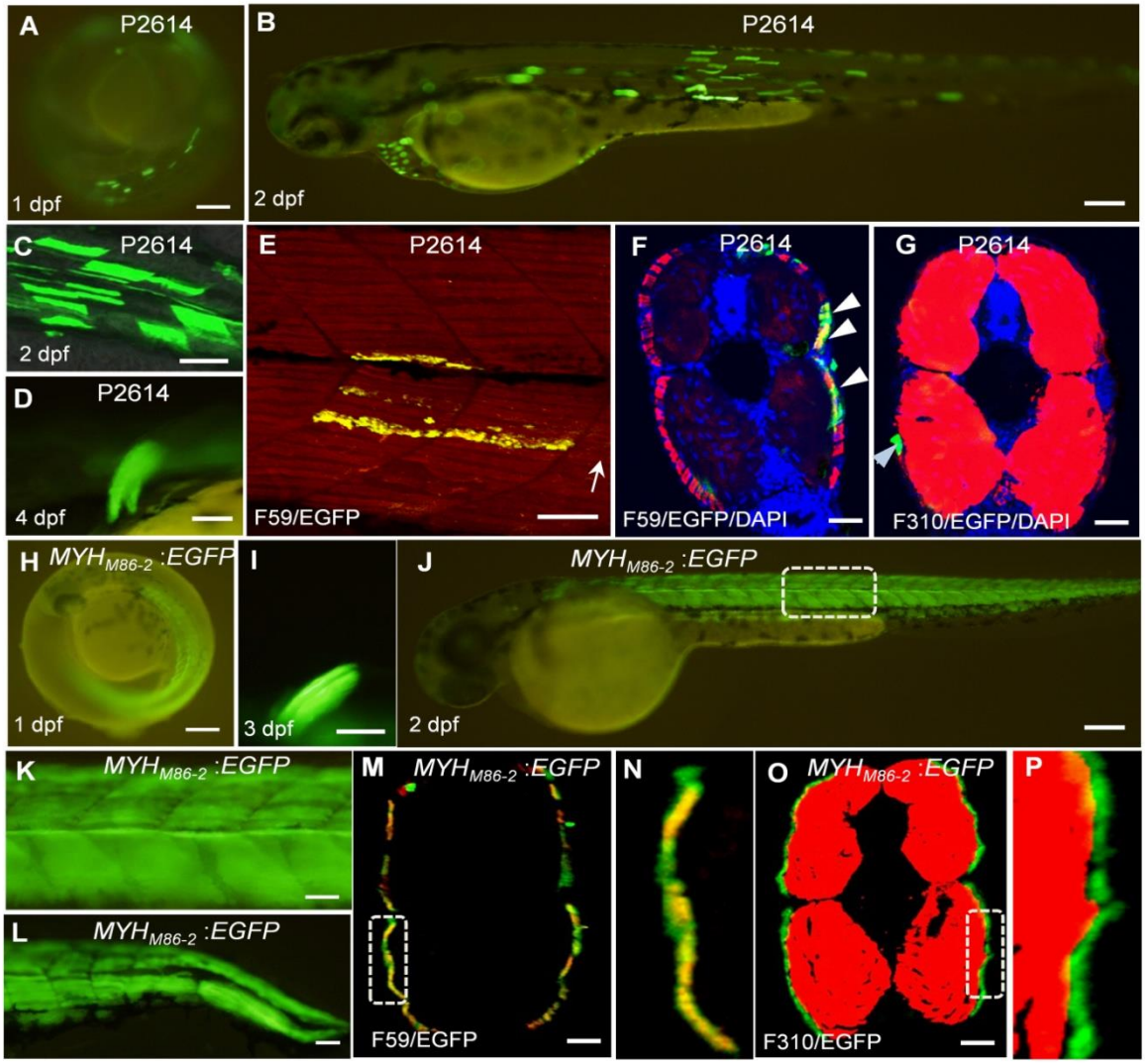

Fig. 3-2: The consistent expression patterns of P2614 with *$MYH_{M86\text{-}2}$:EGFP* transgene in stable transgenic line embryos and larvae of zebrafish. A-D) EGFP expression patterns of P2614 in whole myotomal region of embryo (A) and larva (B,C) and in pectoral fin (D). C) Indicates a higher magnification view of the expressed transgene. E-G) EGFP-expressing muscle fibers in P2614-injected larvae consisting of only superficial slow types as reacted with F59 antibodies (E, lateral view; F, transverse section) but not fast types (G, transverse section). H-L) EGFP expression patterns of *$MYH_{M86\text{-}2}$:EGFP* in whole myotomal region of stable transgenic line embryos (H) and larva (J,K,L) and in pectoral fin muscles (I). (K) and (L), indicates a higher magnification view of the expressed transgene in trunk and posterior regions, respectively. M-P) EGFP expressing muscle fibers in *$MYH_{M86\text{-}2}$:EGFP* stable transgenic larvae consisting of only superficial slow types (M,N) but not fast types (O,P). (N) and (P) are the magnified view of the boxed area of (M) and (O), respectively. Arrowheads indicate EGFP expression in superficial slow muscle fibers. Green colors indicate EGFP expression, red colors indicate slow (for F59) or fast (for F310) muscle fibers and merge (yellow) indicate EGFP expression in superficial slow muscle fibers. Scale bars: 100 μm in A,B,H,J and 20 μm in others.

3.3.4 *$MYH_{M86\text{-}2}$* promoter activity depends on the hedgehog signaling

Hedgehog signaling is important for slow muscle development in vertebrates. In zebrafish, adaxial cells differentiate into slow muscle cells by receiving hedgehog signaling. To further characterize EGFP expression in slow muscle fibers of *$MYH_{M86\text{-}2}$*: *EGFP* transgenic fish, we examined the involvement of hedgehog signaling pathway, a positive regulator of adaxial cell differentiation, in *$MYH_{M86\text{-}2}$* expression using cyclopamine, a well-known hedgehog signaling inhibitor (Incardona et al., 1998; Chen et al., 2002). The stable transgenic line embryos were treated with three different concentrations (2.5, 5 and 10 µg/ml) of cyclopamine where no change was observed in the survival rate of embryos. By treatment with cyclopamine, zebrafish embryos showed fused-eye, a representative phenotype by hedgehog signaling inhibition (Fig. 3-3A-B). Depending on the dose, cyclopamine treated embryos showed reduced or complete elimination of EGFP expression in slow muscle fibers (Fig. 3-3C-G). The rate of embryos at 2dpf expressing *$MYH_{M86\text{-}2}$*: *EGFP* transgene in slow muscle fibers was significantly decreased after cyclopamine treatment and found to be only 4.0% (2.5 µg/ml) and 2.5% (5 µg/ml) compared to 92% in those without treatment (Fig. 3-3H). However, the stable transgenic line zebrafish embryos treated with 10 µg/ml cyclopamine showed complete inhibition of *$MYH_{M86\text{-}2}$*: *EGFP* transgene expression (Fig. 3-3H), indicating that hedgehog signaling pathways control differentiation of slow muscle fibers where the *$MYH_{M86\text{-}2}$* promoter works.

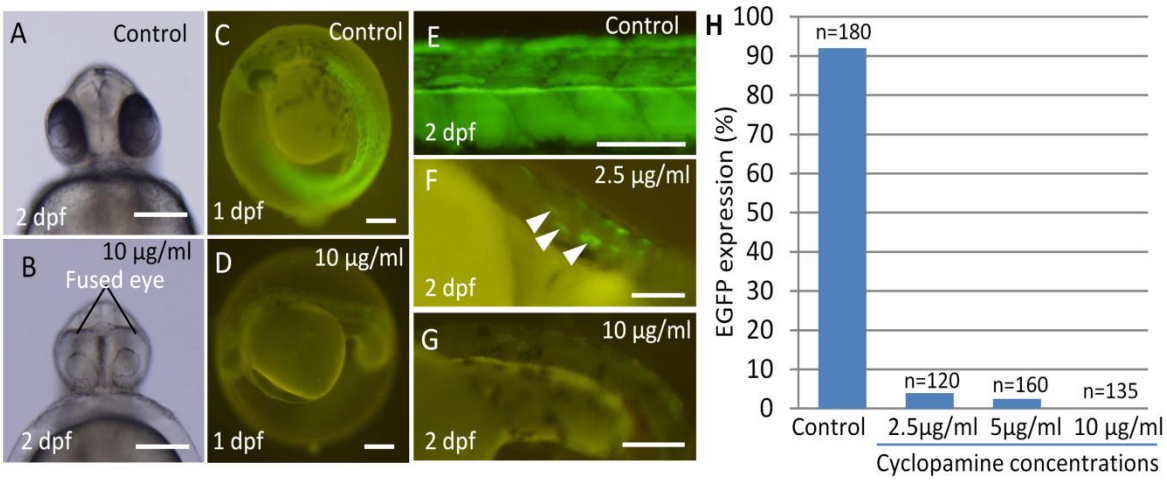

Fig. 3-3: Hedgehog signaling and *MYH$_{M86-2}$* promoter activities. A-G) Inhibition of the hedgehog signaling by cyclopamine treatment on the transgene EGFP expression. Stable transgenic line (*MYH$_{M86-2}$:EGFP*) zebrafish embryos at 2-4 cell stages were transferred into 2.5, 5 and 10 µg/ml cyclopamine containing 0.25, 0.5 and 1% ethanol. Control embryos were developed in 0.25, 0.5 and 1% ethanol containing water without cyclopamine. A,B) Ventral views of head of zebrafish embryo. Inhibition of hedgehog signaling caused fused eye phenotype (B). C,E) Lateral views showing control embryos at 1 dpf (C) and larvae at 2 dpf (E) displayed normal EGFP expressions in superficial slow muscle fibers. F) Lateral view showing reduced (only few fibers) EGFP expression in larvae treated with 2.5 µg/ml cyclopamine. D,G) Lateral views showing complete inhibition of EGFP expression in embryos at 1 dpf (D) and larvae at 2 dpf (G) treated with 10 µg/ml cyclopamine. H) The rate of transgenic embryos expressing the *MYH$_{M86-2}$*: *EGFP* transgene in larvae at 2dpf with and without cyclopamine treatment. Scale bars: 100 µm.

3.3.5 Characterization of key upstream fragments in the *MYH$_{M86-2}$* promoter

To identify the key regulatory regions for the slow muscle-specific expression, we performed a deletion analysis within the 5.5-kb 5'-flanking region of *MYH$_{M86-2}$*. A series of constructs containing progressive deletion from the 5'-end of the 5.5-kb fragment were generated and analyzed by transient expression in zebrafish embryos. The resulting six deletion constructs (P5500, P5000, P3750, P2614, P2000 and P1500) contained fragments of 5,500, 5,000, 3,750,

2,614, 2,000 and 1500 bp, respectively, which were fused to *EGFP* and SV40 polyA signal in pT2AL200R150G vector. Deleting the 5'-flanking region up to -2614 bp upstream of the $MYH_{M86\text{-}2}$ promoter had no or marginal effect on the activity and slow muscle-specificity of the promoter (Fig. 3-4A-H, 3-5). EGFP expression remained strong and specific in slow and pectoral fin muscles. However, deleting the 5'-flanking region from -2,614 to -2,000 bp upstream of the $MYH_{M86\text{-}2}$ promoter resulted in significant reduction of EGFP expression in slow muscle fibers (Fig. 3-4I-J, 3-5). This finding indicates that *cis*-acting element(s) in between -2,614 to -2,000 bp 5'-flanking region may participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression. Interestingly, deleting the 5'-flanking region from -2,000 to -1,500 bp upstream of the $MYH_{M86\text{-}2}$ promoter resulted in ectopic EGFP expression in fast muscle fibers (Fig. 3-4K-L). Such ectopic EGFP expression in the fast muscle fiber was further confirmed by immunohistochemical analysis using the F310 monoclonal antibody specific to fast-type myosin light chain (Fig. 3-4M). Therefore, the deletion analysis defined an upstream fragment necessary for repressing ectopic $MYH_{M86\text{-}2}$ expression in the fast muscle fibers. It also suggests that the *cis*-element(s) located between -2,000 to -1,500 bp upstream of the $MYH_{M86\text{-}2}$ promoter mediate the transcriptional mechanism that prevents its expression in fast muscle fibers.

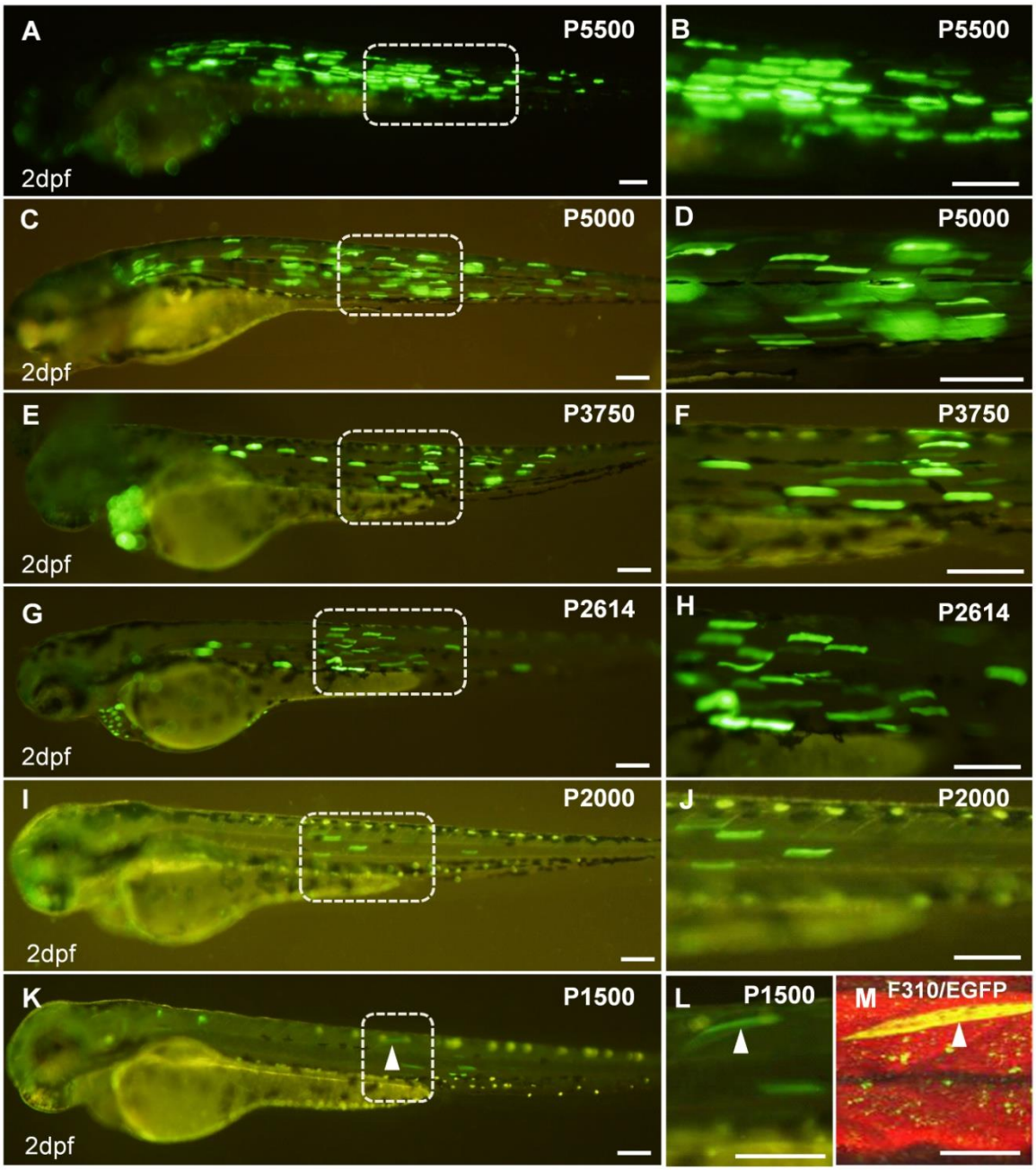

Fig. 3-4: Effects of torafugu *MYH_{M86-2}* promoter deletions on transgene EGFP expression in the superficial slow muscle fibers of zebrafish larvae at 2dpf. A-J) EGFP expression in superficial slow muscle fibers of zebrafish embryos injected with a series of distal promoter deletion constructs that contain the 5500 bp (A,B), 5000 bp (C,D), 3750 bp (E,F), 2614 bp (G,H) and 2000 bp (I,J) 5'-flanking sequences. All panels in the right side are the magnified view of boxed areas of the left side panels. K-M) Deleting the 500 bp (-2000/-1501) of 5'-flanking region of the *MYH_{M86-2}* resulted in ectopic EGFP expression in fast muscle fiber. M) Immunohistochemistry confirming the ectopic EGFP expression in the fast muscle fiber as reacted with F310 antibody (an arrowhead). L,M) A higher magnification of the boxed region in (K). Arrowheads indicate EGFP expressing fast muscle fibers. Scale bars: 100 μm.

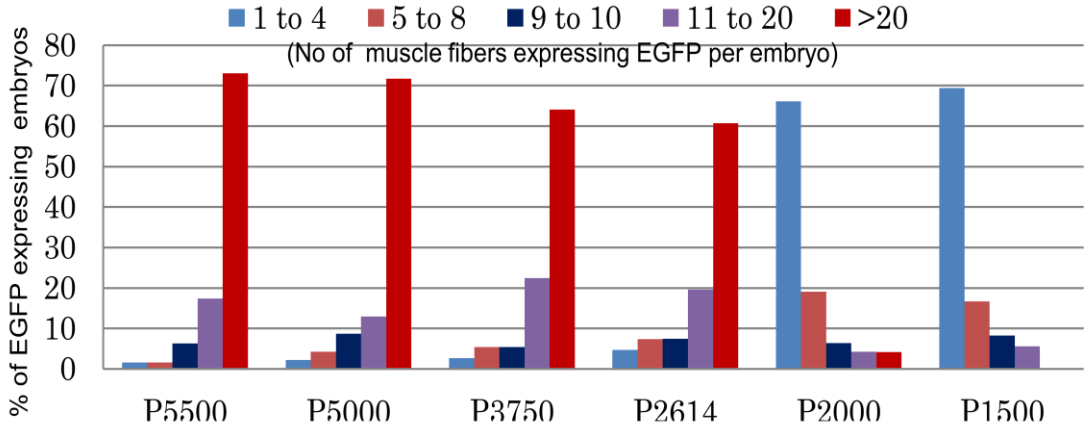

Fig. 3-5: Effects of torafugu *MYH$_{M86-2}$* promoter deletions on the number of muscle fibers expressing transgene EGFP in the superficial slow muscle fibers of zebrafish larvae at 2dpf.

3.3.6 *MYH$_{M86-2}$* promoter harbors fast muscle-specific repression elements to govern slow muscle-specific expression

During deletion analysis, careful examination of zebrafish embryos injected with P1500 constructs revealed that approximately 70-80% embryos had ectopic EGFP expression in fast muscle fibers (Fig. 3-4K-M). On the other hand, nonspecific EGFP expression in fast muscle fibers was typically observed in less than 10% of embryos injected with other deletion constructs (P5500, P5000, P3750, P2614 and P2000), suggesting that the fast muscle-specific ectopic EGFP expression is sequence specific, possibly due to regulatory *cis*-elements in the *MYH$_{M86-2}$* promoter. To identify the transcription factor(s) involved in regulating slow muscle-specific *MYH$_{M86-2}$* transcription, we used an *in silico* approach to predict transcription factor binding sites, focusing on those previously reported to be involved in muscle specificity. *In silico* analysis revealed five putative Sox6 binding sites based on sequence (A/T)(A/T)CAA(A/T)G, which spanned the regions at -1953/-1947 (Sox6_1), -1525/-1519 (Sox6_2), -808/-802 (Sox6_3), -792/-786 (Sox6_4) and -41/-35 (Sox6_5) in the 2,614 bp 5'-flanking region of *MYH$_{M86-2}$* (Fig. 3-6).

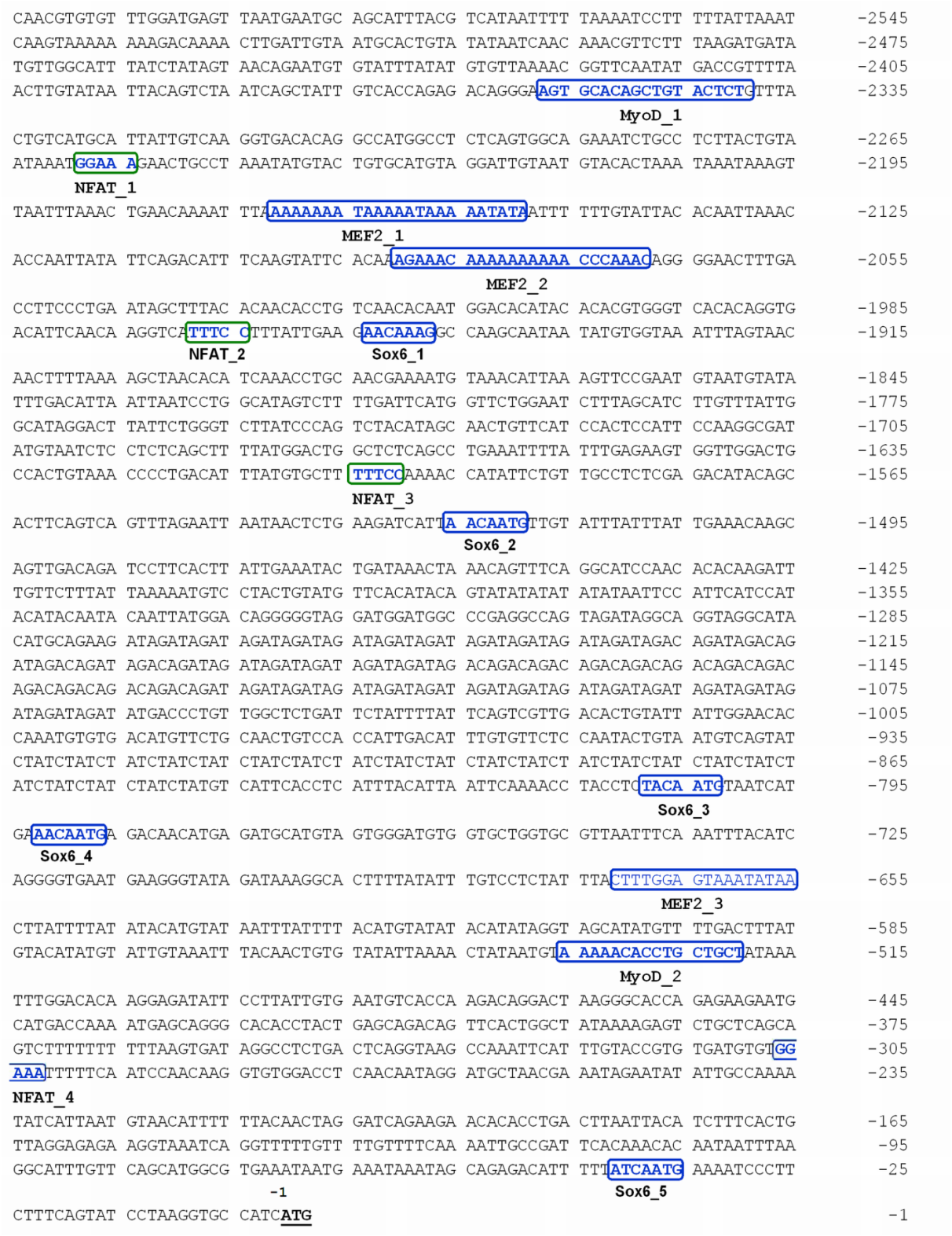

Fig. 3-6: The DNA sequence of the 2,614 bp 5'-flanking region of *MYH_{M86-2}*. The translation start codon is underlined. MyoD and MEF2 binding elements were predicted by MatInspector program. Putative Sox6 and NFAT binding elements were manually screened based on sequences (A/T)(A/T)CAA(A/T)G and GGAAA or TTTCC, respectively.

Interestingly, first two Sox6 binding sites from 5'-site (Sox6_1 and Sox6_2) were located within the -2000/-1500 bp region of the *$MYH_{M86\text{-}2}$* promoter, the deletion of which caused ectopic EGFP expression in fast muscle fibers. It has been reported that Sox6 plays a critical role in vertebrate skeletal muscle fiber type specification (Hagiwara et al., 2007; Hagiwara, 2011). We thus deleted one site or multiple sites of Sox6 in combination from the P2614 construct to determine the role of these Sox6 binding sites. Deleting Sox6_1, Sox6_2, Sox6_3 and Sox6_4 binding sites individually or in various combinations caused ectopic EGFP expression in fast muscle fibers but the deletion of Sox6_5 binding site did not cause any ectopic EGFP expression in fast muscle fibers (Fig. 3-7, 3-8), which was comparable to the slow muscle-specific expression pattern of the P2614 injection (Fig. 3-7A-B).

In addition to slow muscle-specific expression, such ectopic EGFP expression in fast muscle fibers larvae injected with Sox6_1, Sox6_2, Sox6_3 and Sox6_4 binding sites deletion constructs was further confirmed by immunohistochemical analysis (Fig. 3-9A-N). Immunohistochemical analysis also confirmed that the deletion of Sox6_5 did not show any ectopic EGFP expression in fast muscle fibers (Fig. 3-9O-P). These data suggest that Sox6 binding sites spanning the region -1953/-1947 (Sox6_1), -1525/-1519 (Sox6_2), -808/-802 (Sox6_3) and -792/-786 (Sox6_4) are functional for repressing the *$MYH_{M86\text{-}2}$* expression in fast muscle fibers.

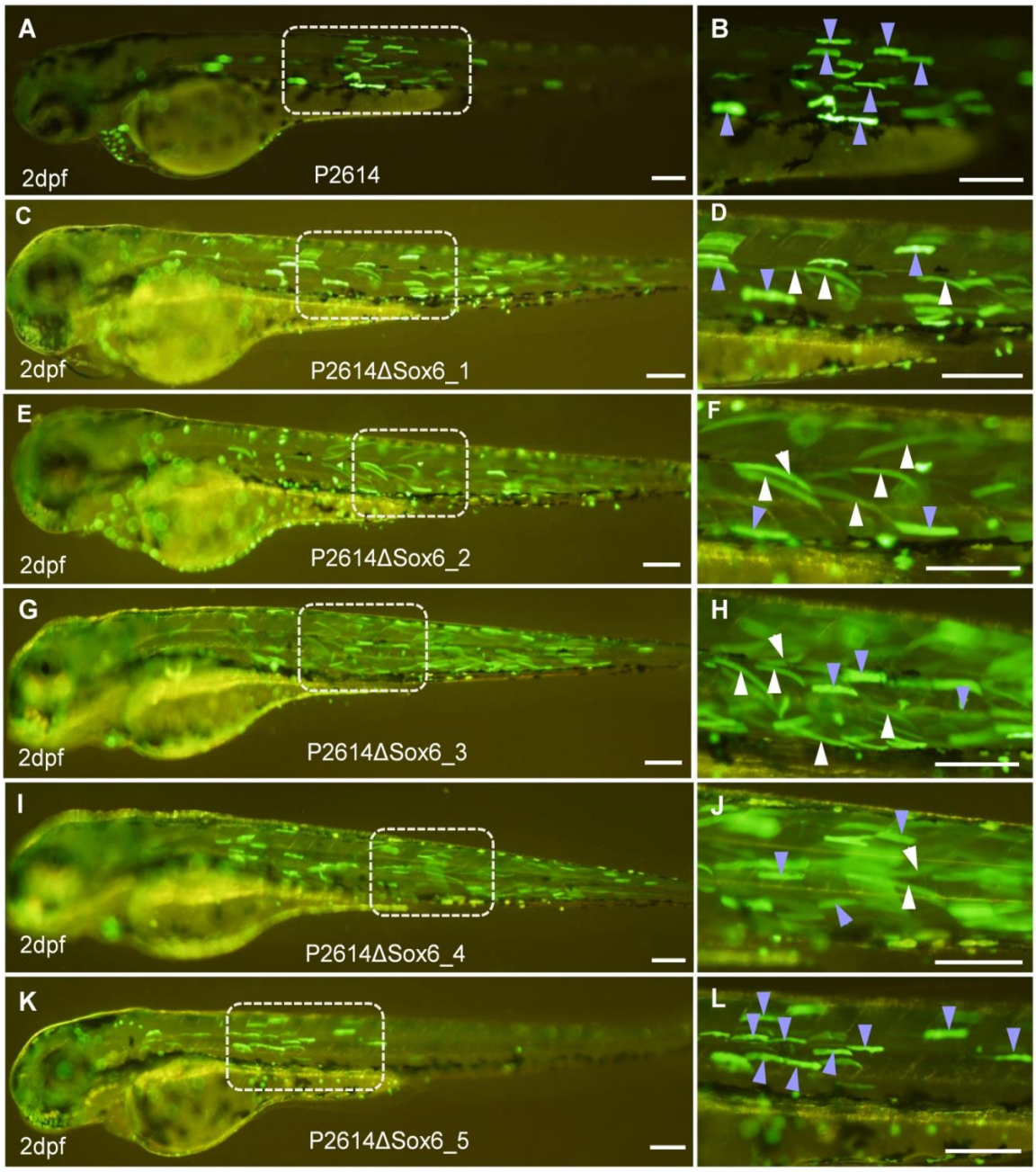

Fig. 3-7: Individual deletion of Sox6 binding elements causes ectopic EGFP expression in fast muscle fibers. A-H) Fluorescent optics showing larvae microinjected with the individual Sox6 binding sites deletion constructs of P2614ΔSox6_1 (C,D), P2614ΔSox6_2 (E,F), P2614ΔSox6_3 (G,H) and P2614ΔSox6_4 (I,J) revealed ectopic EGFP expression in fast muscle fibers. K-L) Embryos microinjected with the deletion constructs P2614ΔSox6_5 did not show any ectopic EGFP expression in fast muscle fibers. All panels in the right side are the magnified view of boxed areas of the left side panels. White arrowheads indicate EGFP expressing fast muscle fibers and purple arrowheads indicate EGFP expressing slow muscle fibers. Scale bars: 100 μm.

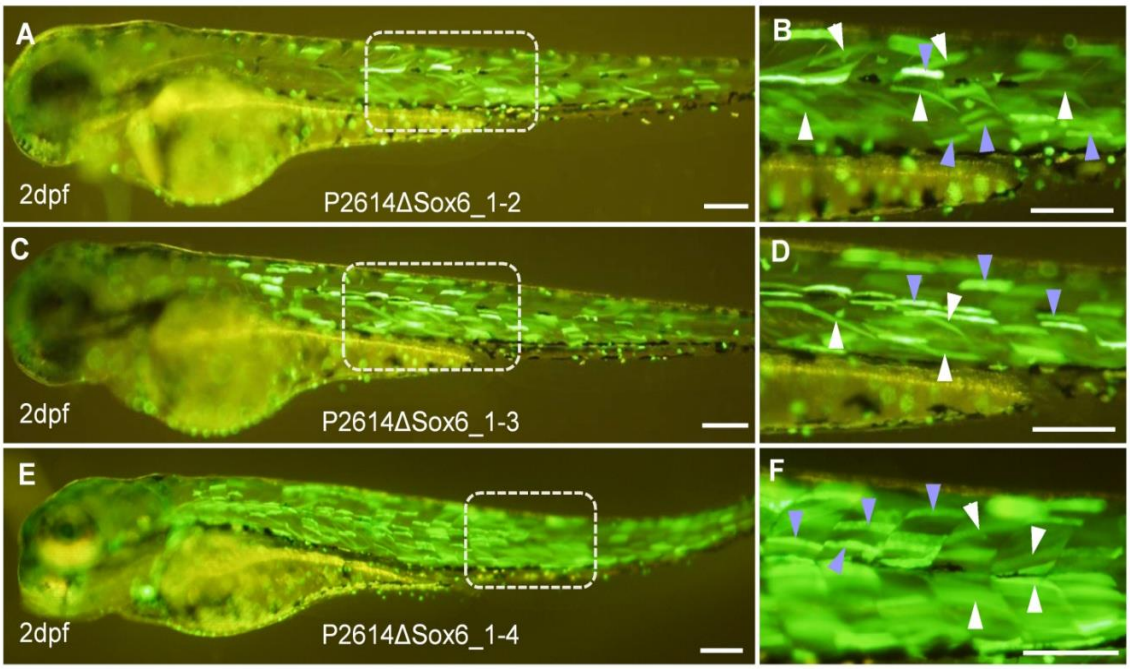

Fig. 3-8: Multiple deletions of Sox6 binding elements cause ectopic EGFP expression in fast muscle fibers. A-F) In consistent with individual Sox6 binding site deletion constructs, larvae microinjected with multiple Sox6 binding sites deletion constructs of P2614ΔSox6_1-2 (A,B), P2614ΔSox6_1-3 (C,D) and P2614ΔSox6_1-4 (E,F) displayed similar but enhanced ectopic EGFP expression in fast muscle fibers. All panels in the right side are the magnified view of boxed areas of the left side panels. White arrowheads indicate EGFP expressing fast muscle fibers and purple arrowheads indicate EGFP expressing slow muscle fibers. Scale bars: 100 μm.

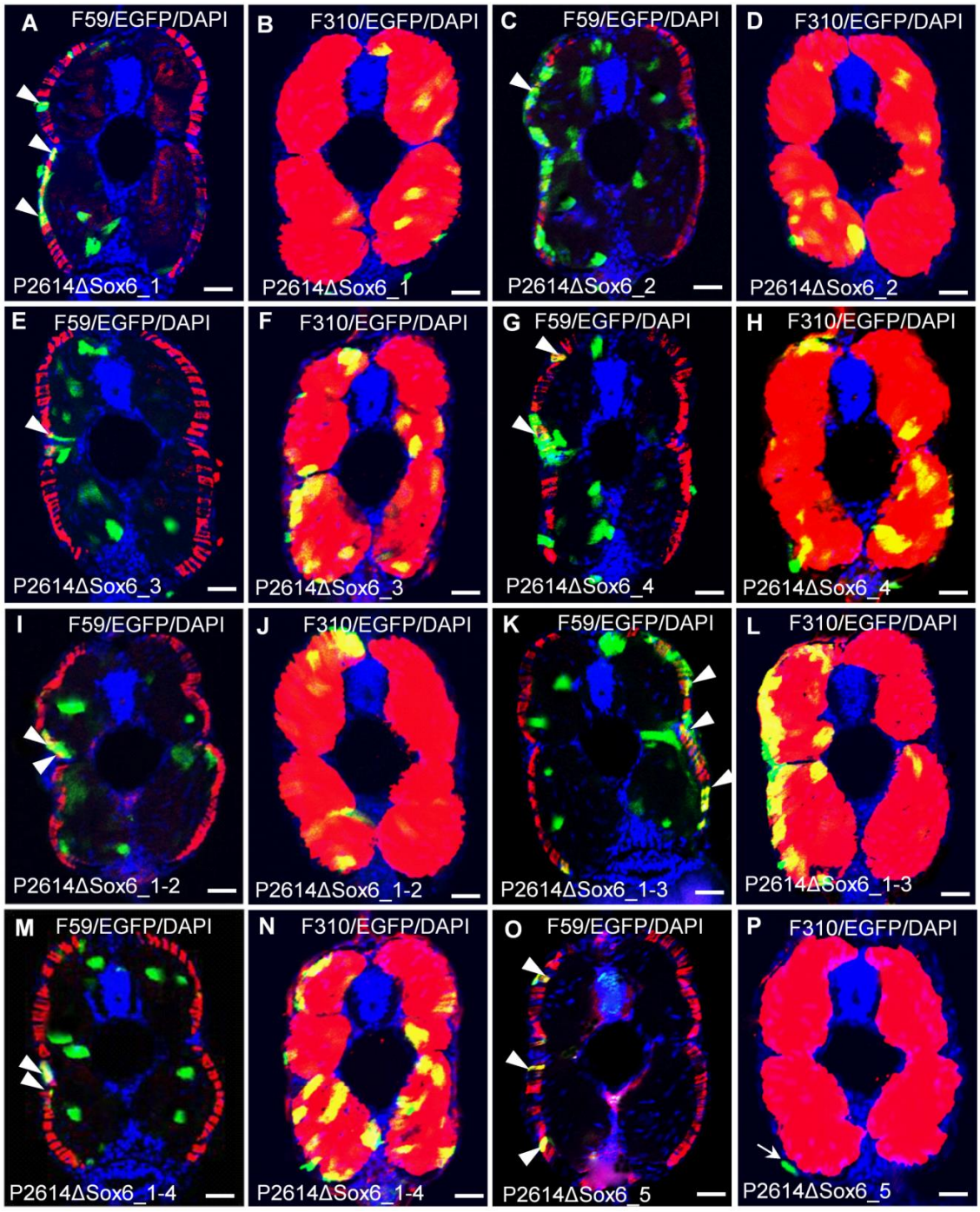

Fig. 3-9: Immunohistochemistry localizing ectopic EGFP expression in fast muscle fibers due to the deletion of Sox6 binding elements. A-H) EGFP expression was localized in both slow and fast muscle as reacted with F59 and F310 antibodies in larvae microinjected with the individual Sox6 binding sites deletion constructs. I-N) Enhanced EGFP expression was also localized in both slow and fast muscle fibers in larvae microinjected with multiple Sox6 binding sites deletion constructs. O-P) Embryos microinjected with the deletion construct of P2614ΔSox6_5 did not show any ectopic EGFP expression in fast muscle fibers. Arrowheads indicate EGFP expressing slow muscle fibers. Arrows indicate EGFP expression not in the fast muscle fiber. Scale bars: 20 μm.

3.3.7 Sox6 function as a transcriptional repressor of $MYH_{M86\text{-}2}$ promoter activity

The loss of Sox6 activity led to the up-regulation of numerous slow fiber–specific genes in mice and zebrafish, suggesting that Sox6 functions as a transcriptional suppressor of slow fiber-specific genes (Hagiwara et al., 2007; von Hofsten et al., 2008). Embryos microinjected with Sox6_1 (-1953/-1947), Sox6_2 (-1525/-1519), Sox6_3 (-808/-802) and Sox6_4 (-792/-786) binding sites deletion constructs showed a marked increase of EGFP expression in skeletal muscle (Fig. 3-7C-J) compared to the P2614 injected embryos (Fig. 3-7A-B). Such effects were more pronounced when these binding sites were deleted in various combinations rather than individually (Fig. 3-8A-F). This observation originally led us to hypothesize that the deletion of Sox6 binding sites may cause over-expression of EGFP in the skeletal muscle and increase the $MYH_{M86\text{-}2}$ promoter activity. Such enhanced expression of EGFP in Sox6 binding sites deletion constructs was validated by real-time PCR analysis (Fig. 3-10). In consistence with the data by microscopic observation of EGFP expressing embryos, real-time PCR data demonstrated that EGFP mRNA expression was significantly higher in embryos microinjected with the Sox6_1, Sox6_2, Sox6_3 and Sox6_4 binding sites deletion constructs compared to the P2614 injected embryos (Fig. 3-10). Deleting multiple Sox6 binding sites resulted in further increase of EGFP mRNA expression in skeletal muscle (Fig. 3-10). These data suggested that Sox6 function as a transcriptional suppressor of $MYH_{M86\text{-}2}$ promoter activity. In contrast, EGFP mRNA expression from the embryos microinjected with Sox6_5 deletion constructs was comparable with the embryos microinjected with wild type P2614 constructs (Fig. 3-10).

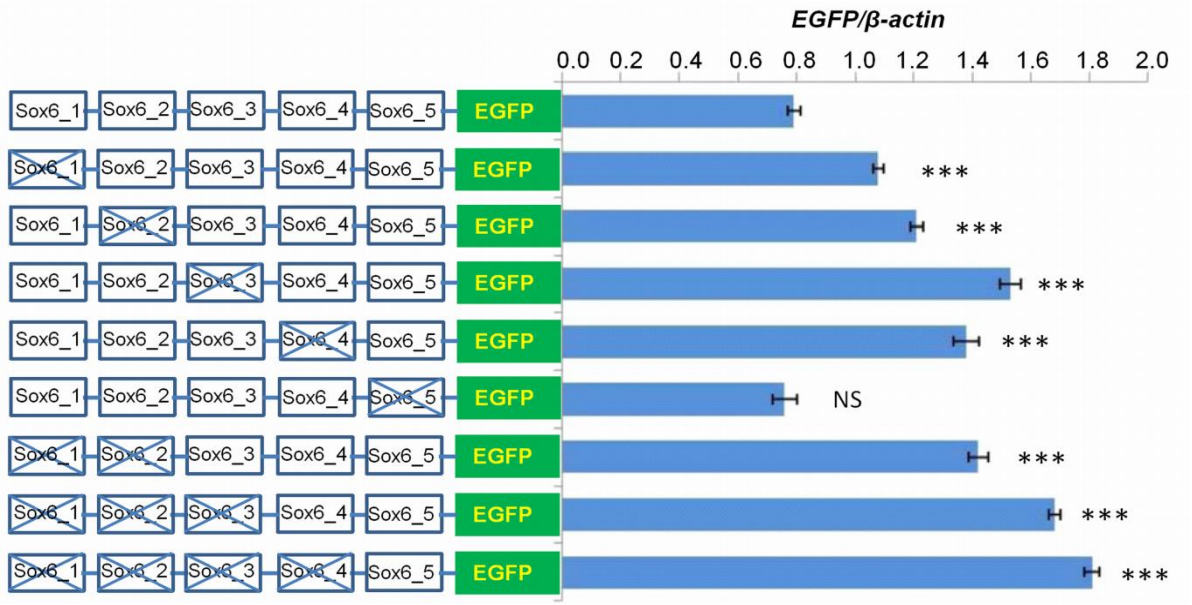

Fig. 3-10: Sox6 functioning as a transcriptional repressor of *MYH$_{M86-2}$* expression as revealed by real-time PCR analysis. Bar graphs show relative EGFP mRNA expression folds in larvae at 2dpf microinjected with different Sox6 binding elements deletion constructs. NS, not significant; *** P<0.001.

3.3.8 NFAT elements play a key role in the regulation of *MYH$_{M86-2}$* expression

During deletion analysis, we observed that the deletion of 614-bp (-2614/-2001) 5'-flanking region of *MYH$_{M86-2}$* significantly reduced the number of EGFP expressing muscle fibers per embryos (Fig. 3-5). It suggested that the 614 nucleotides in the region spanning from 2614 to 2001 may contain positive *cis*-elements required for *MYH$_{M86-2}$* expression. We used an *in silico* approach to predict transcription factor binding sites within this 614-bp promoter region of *MYH$_{M86-2}$*, focusing on those previously reported to be involved in the expressional regulation of slow-muscle specific genes. *In silico* analysis predicted a putative site for NFAT at -2258/-2254 (NFAT_1) based on the core motif: GGAAA or TTTCC within the -2,614 to -2000 bp promoter sequence of *MYH$_{M86-2}$* (Fig. 3-6). The calcineurin/NFAT pathways appear to be involved in the slow muscle-specific gene expression (Calvo et al., 1999; Jiang et al., 2004; Naya et al., 2010). In slow twitch soleus muscle of mice, NFAT has been identified as a key transcription factor for the activity-dependent reprogramming of *MYH* expression (McCullagh et al., 2004). Therefore, we

hypothesized that this NFAT binding site might have a significant role in the expressional regulation of slow muscle-specific $MYH_{M86\text{-}2}$ gene. Upon getting positive results for the deletion of this NFAT binding site (Fig. 3-11A), we became interested about the presence of any other NFAT binding elements outside of this region. Based on the same approach, *in silico* analysis also revealed another three putative NFAT binding sites at -1968/-1964 (NFAT_2), -1604/-1600 (NFAT_3) and -306/-302 (NFAT_4) bp upstream regions from the start codon of $MYH_{M86\text{-}2}$ (Fig. 3-6). In order to determine which NFAT binding sites are functional in the transcriptional regulation of $MYH_{M86\text{-}2}$, 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 the P2614 construct. Embryos injected with the deletion constructs of either NFAT_1, NFAT_3 or NFAT_4 site significantly reduced EGFP expression in slow muscle fibers compared to the embryos injected with the P2614, and elimination of multiple and all the sites of NFAT elements showed more pronounced effects (Fig. 3-11A). This suggests the relative importance of sites NFAT_1, NFAT_3 and NFAT_4, with a comparatively minimal role of NFAT_2 binding site in NFAT induced transcriptional activation of $MYH_{M86\text{-}2}$. The deletion of NFAT binding sites also reduced the number of EGFP expressing muscle fibers (Fig. 3-11B-J). In control (non-deletion of any NFAT binding sites), most embryos had EGFP expression in more than 20 muscle fibers (Fig. 3-11B,J). However, the deletion of NFAT binding sites significantly reduced the number of muscle fibers expressing EGFP per embryos (Fig. 3-11C-J). Such effects were more pronounced when all NFAT binding sites were deleted and under such conditions most embryos showed EGFP expression in only 1-2 muscle fibers (Fig. 3-11I-J). These results clearly demonstrated that NFAT binding sites play a key role in the transcription regulation of $MYH_{M86\text{-}2}$ expression. However, the deletion of all NFAT binding sites could not completely abolish the EGFP expression, suggesting that NFAT binding sites require other *cis*-elements for directing the $MYH_{M86\text{-}2}$ expression.

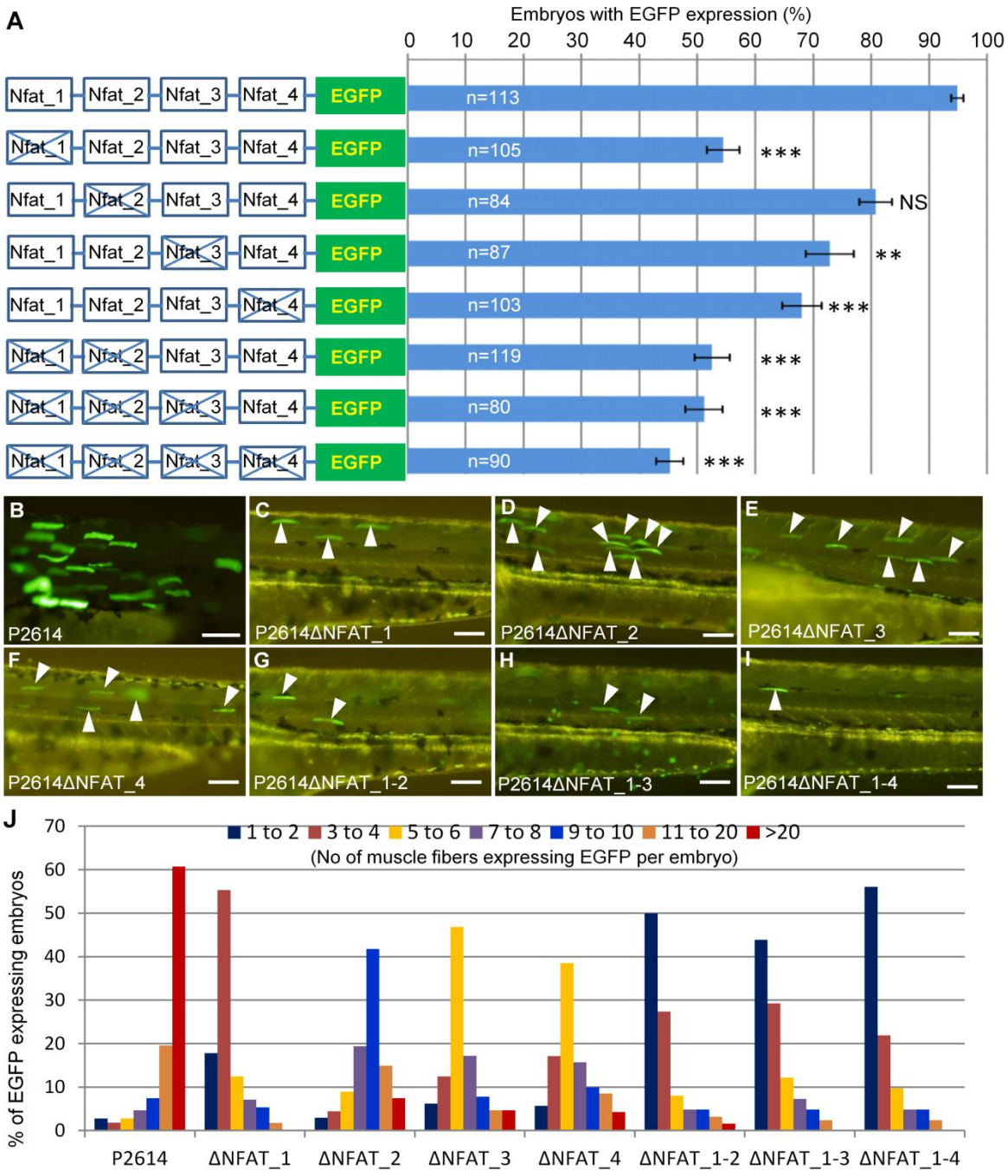

Fig. 3-11: NFAT binding *cis*-elements playing a key role in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression. A) Bar graph showing percentages of embryos that expressed EGFP in superficial slow muscle fibers in microinjection with different NFAT binding elements deletion constructs. B-I) All panels are side views showing EGFP expression in superficial slow muscles fibers of zebrafish larvae at 2 dpf in different NFAT binding sites deletion constructs. Arrowheads indicate EGFP expressing slow muscle fibers. J) Bar graph showing the number of EGFP expressing superficial slow muscle fibers per embryo in different NFAT binding sites deletion constructs. Scale bars 50 µm. NS, not significant; ** P<0.01; *** P<0.001.

3.3.9 MEF2 but not MyoD elements participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression

Of interest, analysis of the 2,614 bp 5′-flanking region of $MYH_{M86\text{-}2}$ by the Genomatix Matinspector program identified three putative MEF2 binding sites in the region spanning -2171/-2149 (MEF2_1), 2090-/-2068 (MEF2_2) and -671/-655 (MEF2_3) and two MyoD binding sites spanning at -2357/-2339 (MyoD_1) and -535/-520 (MyoD_2) (Fig. 3-6). These binding sites have been reported to be involved in the transcriptional regulation of many muscle-specific genes during development in fish (Kobiyama et al., 2006; Liang et al., 2008; Asaduzzaman et al., 2011). Therefore, we deleted these MEF2 and MyoD binding sites individually or in various combinations from the P2614 construct (Fig. 3-12A-B). Deleting any one of these three MEF2 or two MyoD binding sites alone had little or no effect on the $MYH_{M86\text{-}2}$ promoter activity. EGFP expression from this single MEF2 or MyoD binding site deletion was comparable with the wild-type P2614 construct (Fig. 3-12A-B). Therefore, we decided to generate the multiple MEF2 or MyoD binding site-deleted mutant constructs by deleting these sites successively (Fig. 3-12A-B). Embryos microinjected with both two MyoD binding sites deletion construct did not significantly reduced EGFP expression percentage in slow muscle fibers compared to wild type P2614-injected embryos (Fig. 3-12A,D). These data suggest that MyoD binding sites do not participate in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression. However, deleting multiple MEF2 binding sites significantly reduced EGFP expression percentages in slow muscle fibers (Fig. 3-12B). Fluorescent optics showed that deleting all three MEF2 binding sites significantly reduced the number of muscle fibers expressing EGFP per embryos (Fig 3-12E-F). It should be noted that EGFP expression was not completely inhibited in slow muscle fibers. These data indicate that these MEF2 binding sites in the $MYH_{M86\text{-}2}$ promoter were involved in the slow muscle-specific expression, however, additional regulatory sequences might also be involved in regulating $MYH_{M86\text{-}2}$ gene expression in skeletal muscles.

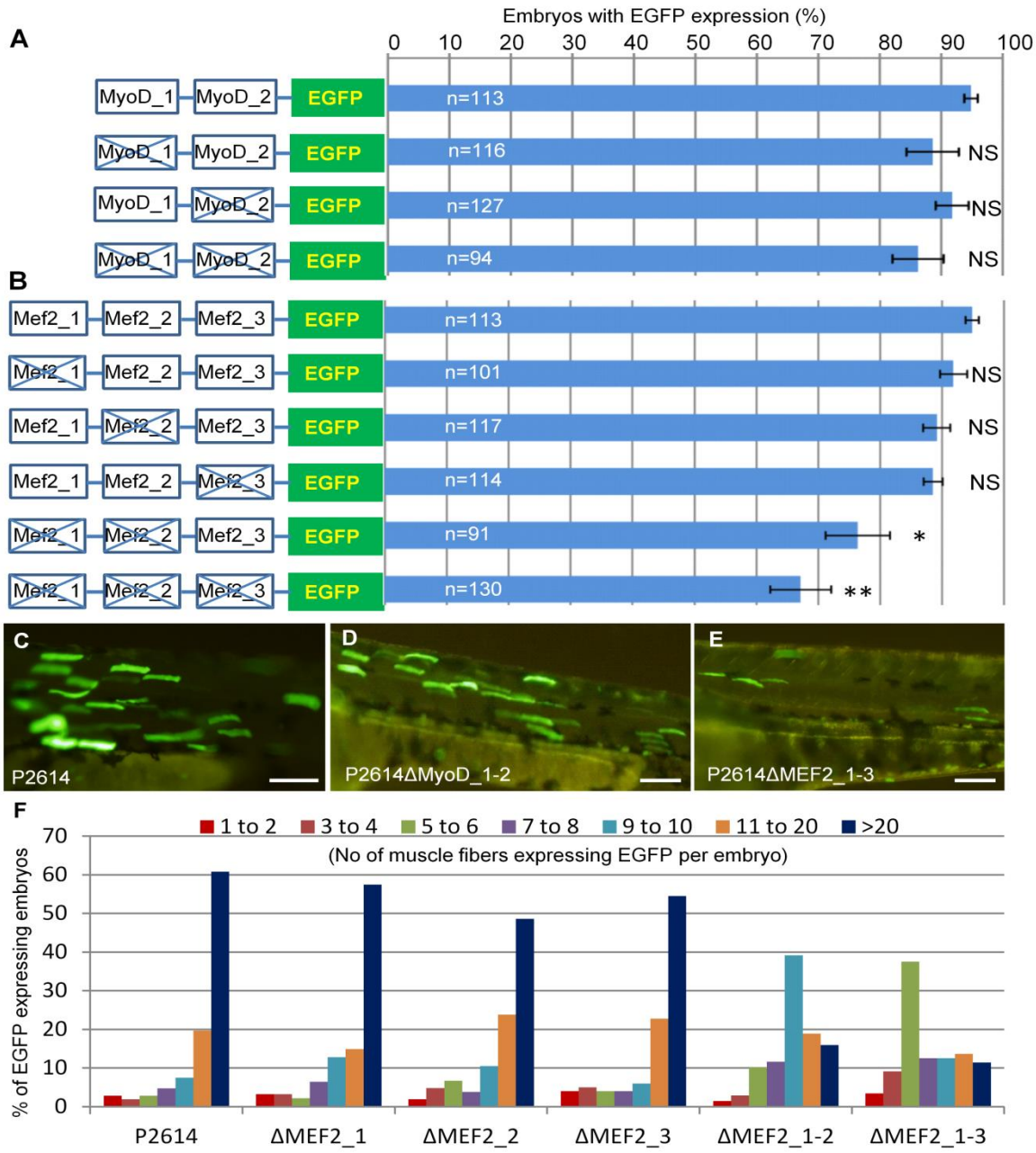

Fig. 3-12: Effects of MyoD or MEF2 binding sites deletions on transgene EGFP expression in slow muscle fibers. A-B) Bar graph shows percentages of embryos that expressed EGFP in the superficial slow muscle fibers in microinjection with different MyoD (A) and MEF2 (B) binding elements deletion constructs. C-E) Fluorescent optics show similar EGFP expression in larva injected with P2614 (C) and P2614ΔMyoD_1-2 (D) constructs and reduced EGFP expression in P2614ΔMEF2_1-3 construct (E). All panels are side views showing EGFP expression in superficial slow skeletal muscles fibers of zebrafish larvae at 2 dpf. F) Bar graph showing the number of EGFP expressing superficial slow muscle fibers per embryo in different MEF2 binding sites deletion constructs. Scale bars 50 µm. NS, not significant; * P<0.05; ** P<0.01.

3.4 Discussion

Multiple sarcomeric *MYH*s are expressed in a sequential fashion in different muscles at embryonic and larval stages of fish. A greater understanding of factors regulating the expression of these embryonic- and larval-type *MYH*s would provide insights into how these muscles are established and maintained. The transcriptional regulation of these *MYH*s consists of a complex process that is often regulated by a cohort of transcriptional factors. The regulatory region of these *MYH*s requires a specific combination and co-operation of various transcription factors unique to each muscle fiber lineage at different stages of differentiation to achieve gene expression in an efficient and specific manner. $MYH_{M86\text{-}2}$ is highly expressed in slow muscle fibers of torafugu embryos and larvae (see Fig. 1A-E), suggesting its functional roles for embryonic and larval muscle development. However, very little is known about the mechanisms by which its expression is regulated. Therefore, the present study was attempted to identify the *cis*-elements that are crucial for the expressional regulation of torafugu $MYH_{M86\text{-}2}$ by transient transfection and transgenic approaches in zebrafish embryos.

In teleost myogenesis, adaxial cell-derived slow muscle formation is crucial. This is the first step of specific muscle fiber formation (Devoto et al., 1996). Our combined analysis of torafugu genome data and transgenic approach using zebrafish successfully retrieved 2614 bp upstream region of $MYH_{M86\text{-}2}$ containing sufficient promoter which acts in superficial slow muscle fibers (see Fig. 3-2). The superficial slow muscle-specific expression patterns with the P2614 construct by the transient transgenic assay were confirmed by generating stable transgenic zebrafish lines. Apart from the slow muscle-specific expression, both the endogenous $MYH_{M86\text{-}2}$ and the reporter gene were also found to be expressed in pectoral fin muscles (see Fig. 3-1 and 3-2). Embryonic *MYH*s have been also reported to be expressed distinctly in pectoral fin muscles in common carp (Ikeda et al., 2010), zebrafish (Xu et al., 2010) and medaka (Ono et al., 2006, 2010). This is consistent with the functional requirement of larval pectoral fin muscles which mainly consist of

fast fibers initially and change to slow type during later stages for the initiation of maneuvering and resting in a horizontal position relative to the body during slow swimming (Thorsen and Hale, 2005; Patterson et al., 2008). However, we did not examine the promoter activity in pectoral fin muscles in the present study. Our findings demonstrated that reporter gene expression patterns in transient and stable transgenic zebrafish well recapitulated intact $MYH_{M86\text{-}2}$ expressions in torafugu (see Figs. 3-1 and 3-2). Torafugu and zebrafish are phylogenically far from each other in the teleost lineage. Therefore, the activity of our promoter is supposed to be conserved among teleost.

During development, zebrafish embryo develop series of anatomically distinct slow twitch muscle fibers that characteristically express distinct members of a tandem array of slow *MYH*s (Elworthy et al., 2008). Based on the expression of slow *MYH*s repertoire and hedgehog signal dependencies, the subsets of slow muscle fibers can be classified into three distinct groups. The first group, called as primary slow muscle fibers, differentiated from adaxial cells. Primary slow muscle fibers express an *MYH* named *smyhc1* and its differentiation depends on the hedgehog signaling (Elworthy et al., 2008). Following primary slow muscle differentiation, some secondary slow fibers (2^{nd} group) also express *smyhc1* but they can differentiate normally in the absence of hedgehog signaling activity, in contrast to their primary counterparts (Elworthy et al., 2008; Barresi et al., 2001). Conversely, other secondary slow fibers (3^{rd} group) express *smyhc2* and their differentiation depends on the hedgehog signaling activity. In our study, cyclopamine treatment completely repressed reporter gene expression by the $MYH_{M86\text{-}2}$ promoter (see Fig. 3-3), suggesting that EGFP expressing cell may correspond to hedgehog signaling dependent slow muscle fibers in zebrafish.

The spatial and temporal control of $MYH_{M86\text{-}2}$ expression lies in its regulatory *cis*-elements within 2614 bp. By promoter dissection, we observed that the deletion of a distal 500 bp region (-2000/-1501) caused the ectopic $MYH_{M86\text{-}2}$ expression in fast muscle fibers (see Fig. 3-4K-M).

This suggests existence of an inhibitory mechanism that mediates the repression of $MYH_{M86\text{-}2}$ expression in fast muscle fibers, which in turn ensures its expression in slow muscle fibers. We identified five Sox6 binding sites in the 2614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ including first two Sox6 binding sites (Sox6_1 and Sox6_2) in the 500 bp distal region (see Fig. 3-6). Notably, the deletion of these Sox6 binding sites (except Sox6_5) in the context of -2614 bp upstream region relieved the fast muscle-specific repression (see Figs. 3-7, 3-8 and 3-9). In zebrafish, transcription of *Sox6* was clearly detectable at the 10-somite stage in the lateral region of each somite and the levels of its transcript reached their peak at 1 dpf and gradually diminished thereafter, though persisting at least until 6 dpf (Wang et al., 2011). Recent investigations using several model organisms have proposed an important function for Sox6 to play a conserved role in vertebrate skeletal muscle fiber-type specification. In mice, analysis of the *MYH-β* promoter revealed a Sox consensus sequence that likely functions as a negative cis-regulatory element (Hagiwara et al., 2007), implicating that Sox6 plays a critical role in the fiber type differentiation of fetal skeletal muscle. Our findings thus describe an important Sox6 binding element which mediates fast muscle-specific repression mechanism to govern the slow muscle-specific expression, a novel extension of the roles of Sox6 binding elements in regulating muscle fiber type-specific differentiation in fish. It has also been suggested that in mice as well as in zebrafish Sox6 directly represses the transcription of slow muscle fiber-enriched genes by binding to conserved cis-regulatory elements (Hagiwara et al., 2007; von Hofsten et al., 2008). In addition, in both zebrafish and mice overexpression of Sox6 resulted in a decreased expression of slow fiber-specific genes (von Hofsten et al., 2008; van Rooij et al., 2009). Very recent study on mice has also demonstrated that Sox6 binds to conversed cis-regulatory elements in slow twitch-fiber genes to represses their transcription (Quiat et al., 2011). These results identify Sox6 as a robust regulator of muscle contractile phenotype and metabolism, and elucidate a mechanism by which functionally related muscle fibers type-specific genes are collectively controlled. We also demonstrated that deleting Sox6 binding elements significantly increased the reporter gene

mRNA expression (see Fig. 3-10). Thus, in addition to fast muscle-specific repressive function, Sox6 may also function as a transcriptional suppressor of MYH_{M86-2} promoter activity.

We demonstrated that the key regulatory sequence appeared to be located within the 2614 bp 5'-flanking sequence of MYH_{M86-2}, because the deletion of the 5'-flanking sequence up to 2614 bp region had marginal effect on the slow muscle specificity and the activity of the promoter in skeletal muscle (see Fig. 3-4). Further promoter dissection suggested that 614 bp spanning the region from -2614 to -2001 bp may contain positive *cis*-elements required for slow muscle-specific MYH_{M86-2} expression. *In silico* analysis identified four putative NFAT binding sites in 2614 bp 5'-flanking region of MYH_{M86-2} including first one (NFAT_1) in the 614 bp region (see Fig. 3-6). Several studies on various model systems have reported that calcineurin/NFAT pathways appear to be involved in the transcriptional regulation of slow muscle-specific gene expression including slow *MYH*s (Naya et al., 2010; Calvo et al., 1999; Jiang et al., 2004). Therefore, it was of interest to assess the role of the NFAT binding sites in the regulation of slow muscle-specific MYH_{M86-2} promoter. Our study demonstrated that NFAT binding sites play a crucial role in the transcriptional regulation of MYH_{M86-2} expression (see Fig. 3-11). The role of NFAT binding sites in the transcriptional regulation of slow *MYH*s promoters has been well documented in avian and mammals muscles cells. So far, reports on the relevance of NFAT binding sites in slow *MYH* promoters are contradictory. During the innervations -induced expression of an avian *MYH-slow* promoter two proximal NFAT binding sites play a prominent role (Jiang et al., 2004) while mutation of NFAT binding sites in a distal enhancer of a rat *MYH-β* promoter had no effect on its activity (Giger et al., 2004). Transient transfection assays demonstrated that the calcineurin/NFATc1 signaling pathway is essential for slow *MYH-β* promoter activation during transformation of mouse C2C12 myotubes (Meissner et al., 2007). Relatively recent study (Calabria et al., 2009) provided evidence that the transcription of a slow muscle-specific gene, *MYH-slow*, is controlled in a cooperative way by all 4 NFAT family

members *in vivo*. It was also evident that the transcription of slow and fast *MYH*s uses different combinations of NFAT family members, ranging from *MYH-slow*, which uses all 4 NFAT isoforms, to *MYH-2B*, which only uses NFATc4. A very recent study (Meissner et al., 2011) also showed detailed investigation of the prominent role of an NFATc1 binding site for the slow *MYHI/β* promoter activity and gene expression induced by increased intracellular Ca^{2+}. In consistence with the other studies on avian and mammalian muscles, our study also demonstrated an important role of NFAT binding sites in the transcriptional regulation of slow muscle-specific *MYH* expression in fish. However, it should be noted that deleting all four NFAT binding sites from the 2614 bp regulatory region did not completely abolish its activity in directing EGFP expression in slow muscle fibers, suggesting that other regulatory element(s) might be also involved in its expression.

Members of MRF (MyoD, myogenin, myf5, and MRF4) and MEF2 have all been shown to regulate the transcription of numerous muscle-specific genes. Our previous study (Asaduzzaman et al., 2011) demonstrated that both MyoD and MEF2 binding *cis*-elements participate in the transcriptional regulation of fast muscle-specific torafugu $MYH_{M743\text{-}2}$. Therefore, it was of interest to examine whether or not the MyoD and MEF2 binding sites similarly participate in the regulation of slow muscle-specific torafugu $MYH_{M86\text{-}2}$. Sequence analysis identified two MyoD and three MEF2 binding *cis*-elements in the 2614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ (see Fig. 3-6). We revealed that MEF2, but not MyoD, participates in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression (see Fig. 3-12). As like mammals, three types of zebrafish MEF2 genes (MEF2A, MEF2C and MEF2D) encode factors that function as DNA binding transcriptional activators of muscle specific promoters (Ticho et al., 1996). The consistent expression patterns of MEF2 isoforms in the developing somites suggested their potential roles to bind with $MYH_{M86\text{-}2}$ promoters and may participate in the transcriptional regulation of its expression. Previous studies reported that MEF2 binding sites are crucial for the transcriptional regulation of fast

muscle-specific *MYH*s in fish (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008; Asaduzzaman et al., 2011) and mammals (Allen et al., 2002; Meissner et al., 2007). Our results suggest that MEF2 is also active in the transcriptional regulation of slow muscle-specific *MYH*s in fish. In mammals, MEF2 was found to be active in both type I slow-twitch fibers and type IIa fast-twitch fibers. The involvement of MEF2 in the transcriptional regulation of slow muscle-specific *MYH*s represents a second pathway from calcineurin to DNA (Wu et al., 2000; Mckinsey et al., 2002). MEF2 activity may be regulated by intracellular calcium transients transduced by the calcium-dependent phosphatase, calcineurin, leading to the activation of transcription of some slow fiber-specific genes (Wu et al., 2000). In consistence with our findings, MEF2 was also found to be required for innervations-dependent expression of an avian *MYH-slow* promoter *in vivo* (Jiang et al., 2004). In calcium-ionophore-treated C2C12 myotubes, transient transfection studies revealed that a MEF2-D transcription factor transactivate the *MYH-β* promoter in a calcineurin-dependent manner (Meissner et al., 2007). Thus, MEF2 appears to be participated in the transcriptional regulation of $MYH_{M86\text{-}2}$, however, additional regulatory sequences might also be involved in regulating $MYH_{M86\text{-}2}$ expression. Regulation of *MYH*s expression by multiple regulatory sequences appears to be a common mechanism for the control of muscle-specific gene expression. Therefore, combined deletion of multiple *cis*-elements would provide more clear insight about transcriptional regulation of $MYH_{M86\text{-}2}$. In mammalian skeletal muscle, recent study showed that the increased intracellular Ca^{+2}level of slow muscle fibers can also activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) pathway, which in turn was shown to enhance transcriptional activity of slow *MYHI/β* promoter and gene activation by post-translation modification of NFAT (Meissner et al., 2011). In addition, the above pathways directly phosphorylated MEF2A and MEF2C in their transactivation domains and enhance their transcriptional activity (Ma et al., 2005). Therefore, besides the mentioned combined deletion of multiple *cis*-elements, these

findings should also be taken into account for future experiments to understand the mechanisms of $MYH_{M86\text{-}2}$ expression in fish.

In summary, our study on the torafugu $MYH_{M86\text{-}2}$ promoter suggests that slow muscle-specific expression is achieved through an inhibitory mechanism that suppresses expression in the fast muscle fibers. In addition, we conclude that NFAT binding elements play a key role and MEF2 binding elements participate in the transcriptional regulation of the torafugu $MYH_{M86\text{-}2}$. However, a putative consensus binding sequence may not necessarily bind the predicted transcription factors and deletions of complete putative binding sites may affect the binding of other transcription factors to adjacent sequences which can then subsequently be the cause of the measured effects. Therefore, the next important steps of our research would be to investigate whether these transcription factors interact with other cofactors to activate $MYH_{M86\text{-}2}$, and whether and how these transcription factors bind to the $MYH_{M86\text{-}2}$ promoters.

CHAPTER 4

Expressional regulation of myosin heavy chain genes associated with muscle hyperplasia in fish

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Abstract

The relationship of *MYH*s expression with muscle fiber formation has not been well understood in fish. In torafugu, the transcript of $MYH_{M2528\text{-}1}$ appeared in embryos at 6 dpf and continued to be expressed in later embryonic and larval development successively as well as in adult fast and slow skeletal muscles. Interestingly, the transcripts of $MYH_{M2528\text{-}1}$ were localized specifically in the dorsal and ventral extremes of larval myotome suggesting its involvement in stratified hyperplasia. Apart from the involvement in stratified hyperplasia in torafugu larvae, *in situ* hybridization study showed that $MYH_{M2528\text{-}1}$ is also involved in mosaic hyperplasia in juvenile torafugu where its transcripts were expressed in fast fibers with small diameters as well as inner parts of superficial slow muscle fibers.

4.1 Introduction

Molecular, genetic and cellular features of skeletal muscle growth have been documented in a number of vertebrate species. In mammals, all the muscle fibers required for growth are produced before birth and subsequent growth is mainly by fiber hypertrophy alone (Rowe and Goldspink, 1969). Teleost fish represent a good model for studying vertebrate myogenesis, since slow and fast muscle fibers are arranged in anatomically discrete layers and myotube production is not restricted to early developmental stages, reflecting the large difference between embryonic and final adult body size (Weatherley et al., 1988). Furthermore, fast and slow muscles have a distinct embryological origin (Devoto et al., 1996) and show different patterns of postembryonic growth (Barresi et al., 2001; Johnston et al., 2004).

Embryonic slow muscle fibers are differentiated at early developmental stages from adaxial cells, namely paraxial mesodermal cells which exist in direct contact with notochord. Adaxial cells migrate laterally from either side of the notochord to the superficial part in the trunk and form slow muscle in superficial region beneath the skin or remain as muscle pioneers in the horizontal myoseptum (Devoto et al., 1996). In subsequent stages, slow muscle fibers recruited new fibers mainly through SH based on the identification of germinal growth zones over a partial cross-sectional area of slow muscle and/or range of body sizes (Barresi, 2001; Johnston et al., 2004). However, previous study reported new evidence that SH in slow muscle relies on the three different germinal zones arising sequentially to generate multiple waves of muscle fiber recruitment during ontogeny (Lee, 2010).

Embryonic fast muscle fibers arise from the lateral presomitic mesoderm (Devoto et al., 1996), and additional fibers are produced from discrete germinal zones in the late embryo, larval, and early juvenile stages by stratified hyperplasia (Johnston et al., 1995; Barresi et al., 2001). The final and most important stage of myogenesis involves the formation of new fast muscle fibers

throughout myotomes, called mosaic hyperplasia because of the resulting pattern of fiber diameters (Rowlerson et al., 1995; Johnston et al., 2003). Myotube production in fast muscle only continues until the fish has attained ~40–45% of its maximum body length, with subsequent growth restricted to an expansion in the diameter and length of muscle fibers, and nuclear accretion (Weatherley et al., 1988; Johnston et al., 2003). However, in response to injury and as a component of the repair response, new myotube production can be initiated even after the end of fiber recruitment (Rowlerson et al., 1997). The nuclei for fiber recruitment and hypertrophy are derived from a population of MPCs that are equivalent to the muscle satellite cells found in mammals (Johnston and Hall, 2004).

Morphological, functional and metabolic characteristics of skeletal muscle are mainly related to the cellular expression of different MYH isoforms and muscle fibers are characterized mainly based on MYHs they contain (Cobos et al., 2001). Fish are known to posses highly conserved MYH multigene family, although *MYH*s are much more than their higher vertebrate counterparts (Kikuchi et al., 1999; Watabe and Ikeda, 2006; Ikeda et al., 2007). Although the precise functions of each *MYH* in fish have been not well understood, subsets of fibers in fish skeletal muscles are identified by the transcripts of *MYH*s, suggesting that they play distinct physiological roles. Small diameter fibers that appear by mosaic hyperplasia in adult fast muscle of fish express distinct *MYH*s (Ennion et al., 1995; Akolkar et al., 2010), although their expression in early development remains unclear.

The torafugu genome has been proposed as an ideal model for rapid characterization of vertebrate genes due to its smallest size and compactness, generally with short introns and a low proportion of repetitive elements (Aparicio et al., 2002). The genomic organization of sarcomeric and non-sarcomeric *MYH*s of torafugu has been investigated by *in silico* analysis of the total genome database, revealing that it contains 20 sarcomeric *MYH*s which are organized in different clusters (Watabe and Ikeda, 2006; Ikeda et al., 2007). The relationship of *MYH*s expression with muscle

fiber formation has not been well understood in fish and even in amniotes. In adult torafugu musculature, detailed expression analysis showed the strictly regulated spatial and temporal expression patterns of *MYH*s (Akolkar et al., 2010). Among these *MYH*s in adult torafugu musculature, $MYH_{M2528\text{-}1}$ was expressed in fast fibers with relatively small diameters and slow fibers with relatively large diameters, implying that this gene is possibly associated with mosaic hyperplasia. However, it is still ambiguous to confirm whether or not this gene is involved in SH during development of torafugu.

4.2 Materials and Methods

4.2.1 Experimental fish

Juveniles (4 to 6 g) of torafugu were collected from Oshima Fisheries Hatchery Company, Nagasaki, Japan, and brought to The University of Tokyo, where they were reared in a 500 L tank in seawater at 18°C. Embryos and larvae of torafugu at different developmental stages were reared and different tissues of adult fish were collected as described in section 2.2.1 of Chapter 2.

4.2.2 Reverse transcription, amplification and sequence determination

Total RNAs were prepared from whole embryos from 1 to 8 dpf and larvae at 10 and 16 dpf, and various tissues of adult torafugu. 5 µg of total RNAs were reverse-transcribed by using oligo-dT primer (Akolkar et al., 2010) and superscript reverse transcriptase III™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 3'-RACE-PCR was performed to determine the nucleotide sequence at the 3'-untranslated region (UTR) of *MYH*$_{M2528\text{-}1}$ by using a specific forward primer 5'-AGC TGG AAG GTG ACC TTG TTC AGG-3' and an adapter primer 5'-CCA GTG AGC AGA GTG ACG-3'. The amplified DNA fragments were sub-cloned and sequenced as described in section 2.2.2 of Chapter 2.

RT-PCR was performed to examine the expression of *MYH*$_{M2528\text{-}1}$ to be restricted to embryonic and larval stages in torafugu and any further possible expression in different tissues of adult by using gene specific forward primer 5'-TCG GCA GGG GAA AGG ACG CTG AAG -3' and reverse primer 5'-TTG CGT ATA GAT TTA TTG CAC TT-3' based on the nucleotide sequences in 3' UTR. The transcript of torafugu β-actin (U38849) was used as the internal control for RT-PCR.

4.2.3 *In situ* hybridization

The cDNA fragment of about 250 to 300 bp which contained 3'UTR nucleotide sequences and those encoding a C-terminal part of $MYH_{M2528\text{-}1}$ was amplified by using gene specific forward primer 5'-TGA CCA GGT ACA GGA AGG TG -3' and reverse primer 5'-TTG AAT TGC GTA TAG ATT TAT TGC-3' and subcloned into pGEM-T vector (Promega). Digoxigenin (DIG)-labelled antisense riboprobes were synthesized using T7 and SP6 RNA polymerases (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization was performed on transverse sections (16 μm thickness) for larvae at 16 dpf and juveniles (4.5 g body weight) of torafugu by the method described in section 3.2.3 of Chapter 3.

4.3 Results

4.3.1 Expression of torafugu *MYH*$_{M2528\text{-}1}$

RT-PCR revealed that the expression of torafugu *MYH*$_{M2528\text{-}1}$ was dependent on the developmental stage. The transcripts of *MYH*$_{M2528\text{-}1}$ first appeared in embryos at 6 dpf and continued to be expressed in embryonic and larval development successively as well as in adult fast and slow skeletal muscles (Fig. 4-1A). Interestingly, the transcripts of *MYH*$_{M2528\text{-}1}$ were localized specifically in the craniofacial muscles of head (Fig. 4-1B) and dorsal and ventral extremes of larval myotome (Fig. 4-1C-D). These results were further confirmed by *in situ* hybridization in juvenile torafugu (4.5 g body weight), where the transcripts of *MYH*$_{M2528\text{-}1}$ were found to be expressed in fast fibers with small diameters as well as the inner part of LS and ED slow muscle fibers (Fig. 4-2). The transcripts of *MYH*$_{M2528\text{-}1}$ were also found to be expressed in myocepta (Fig. 4-2).

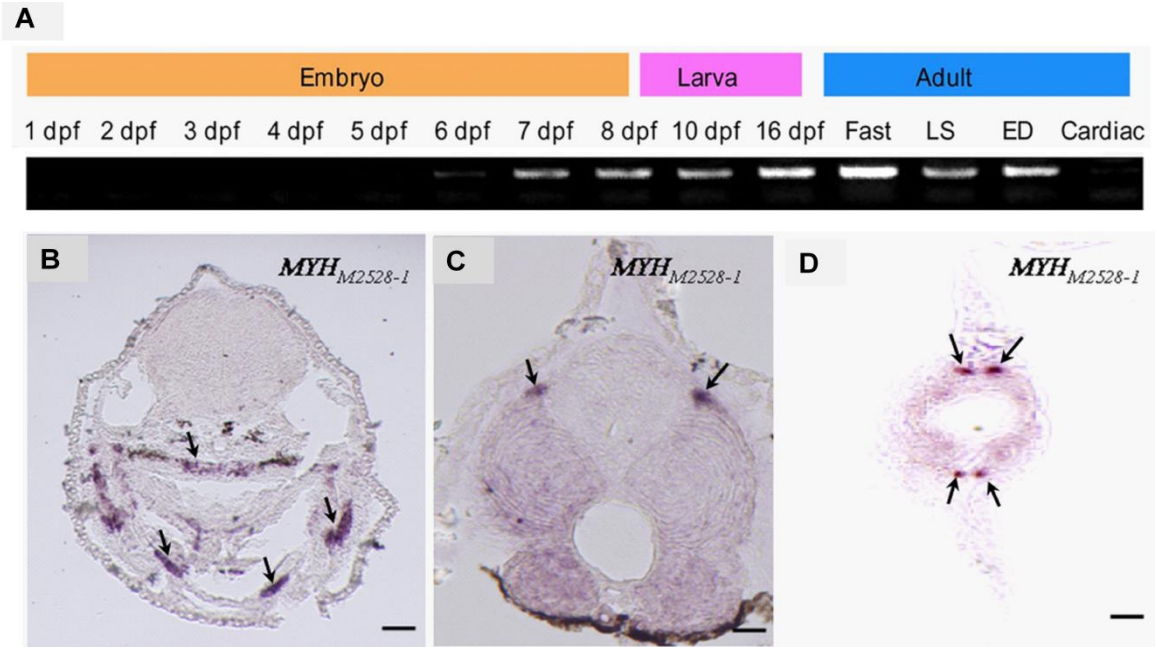

Fig. 4-1: Expression patterns of *MYH*$_{M2528\text{-}1}$ in different developmental stages of torafugu. A: RT-PCR showed the expression of *MYH*$_{M2528\text{-}1}$ during development and adult muscle of torafugu. B-D: *In situ* hybridization showed localization of *MYH*$_{M2528\text{-}1}$ in torafugu larvae at 10 dpf. Scale bar 20 μ m

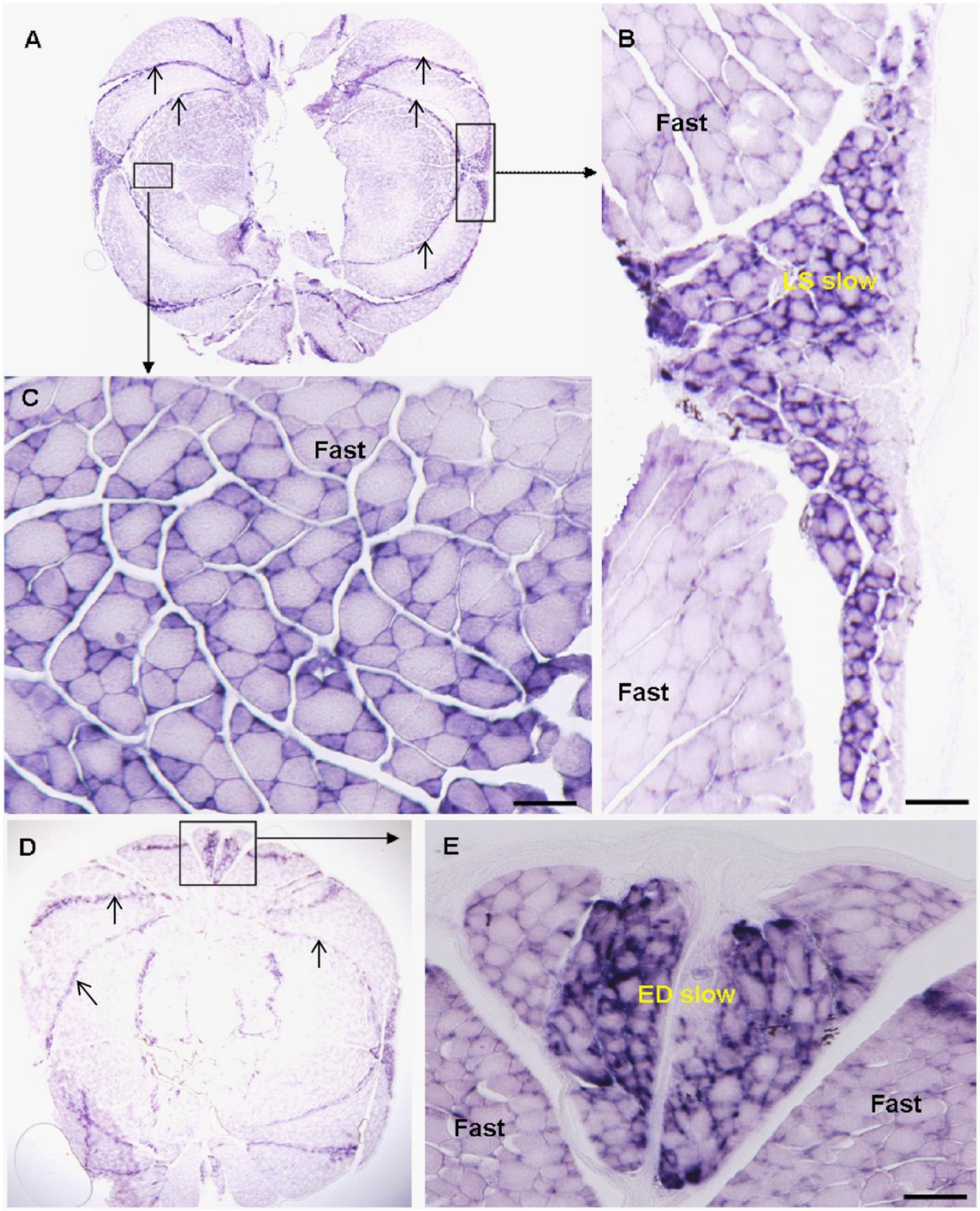

Fig. 4-2: The expression of *MYH*$_{M2528\text{-}1}$ in juvenile torafugu (body weight 4.5 g). Panel A, the cross-sectional view of the whole trunk muscle at the level of relatively anterior position, showing the expression of *MYH*$_{M2528\text{-}1}$. Panel B, the magnified area marked in panel A showing the transcripts of *MYH*$_{M2528\text{-}1}$ localized in inner parts of lateralis superficial (LS) slow muscle fibers. Panel C, the magnified area marked in panel A, showing the transcripts of *MYH*$_{M2528\text{-}1}$ localized only in fast muscle fibers with small diameters. Panel D, the cross-sectional view of the whole trunk muscle at the level of relatively posterior position. Panel E, the magnified area marked in panel D. Arrows indicate the positive signals in fibers around myosepta. Scale bars = 100 μm.

4.4 Discussions

4.4.1 *MYH$_{M2528\text{-}1}$* is involved in hyperplastic muscle growth in torafugu

MYH$_{M2528\text{-}1}$ is a gene of special interests since we have recently demonstrated its involvement in the generation of fast-twitch fibers with small diameters possibly by mosaic hyperplasia in the adult trunk fast muscle (Akolkar et al., 2010). *MYH$_{M2528\text{-}1}$* is structurally an adult fast type (Akolkar et al., 2010). However, this gene was expressed not only in adult torafugu but also in embryos and larvae. The expression pattern of *MYH$_{M2528\text{-}1}$* provides a functional similarity of fish muscle growth with regeneration in mammalian skeletal muscles which is known to occur either after injury in skeletal muscle or when provoked experimentally or occurring as a part of a disease process (for details, see Schiaffino and Patridge, 2008). Such regenerating fibers in mammalian skeletal muscles transiently express embryonic *MYHemb* and perinatal *MYHperi*, which are replaced later by adult-specific ones (Sartore et al., 1982; Matsuda et al., 1983; Whalen et al., 1990). Although such regulation is thought to be a common feature of vertebrate skeletal muscles, no direct evidence has been provided at the molecular level regarding the involvement of embryonic *MYH* in generating fibers in adult skeletal muscle of fish. Earlier, Ennion et al. (1995) showed that new fibers generated by hyperplasia tend to express distinct *MYHFG2* in adult fast skeletal muscle of common carp. However, the transcripts of *MYHFG2* were undetected during development as well as in slow skeletal muscles of carp in a sharp contrast with the expression of torafugu *MYH$_{M2528\text{-}1}$*. Recently, Johnston et al. (2009) have reported the up-regulation of fast-type *myhz1* in small diameter fibers of zebrafish fast muscle that were still recruiting myotubes. The present finding regarding the expression of *MYH$_{M2528\text{-}1}$* in embryos and larvae during development has a high significance with regards to the establishment of hypothesis that the fibers generated by MH in fast muscle of torafugu tend to express structurally adult, but expressionally larval and adult, fast-type *MYH* which is later replaced by adult-specific

MYH. Such finding in torafugu suggests the equivalent mechanisms by which expressional regulation in a part of fiber generation is likely to occur in fish.

Initial myogenesis in the embryo results in the formation of a superficial monolayer of slow fibers and underlying presumptive fast-white fibers. Further growth in fish skeletal muscles is established by the recruitment of new fibers in distinct zones by SH in post-embryonic myotome and in all over myotome of adult fast muscle by MH (Stickland, 1983; Rowlerson et al., 1985; Rowelerson and Veggetti, 2001), accompanied by the increase in fiber volume by hypertrophy. Interestingly, the transcripts of $MYH_{M2528\text{-}1}$ were localized to the dorsal and ventral extremes of larval myotome (see Fig. 4-1C-D). The dorso-ventral extremes of larval myotome are considered as the main growth engines for recruitment of new fibers by SH (Rowlerson and Veggetti, 2001). The appearance of $MYH_{M2528\text{-}1}$ transcripts before hatching further suggests the onset of SH in late embryonic stage prior to hatching in torafugu as also demonstrated in other fish (Stickland et al., 1988; Steinbacher et al., 2006; Steinbacher et al., 2007). During SH, fibers are also formed in a layer between superficial slow and lateral fast muscles (Rowlerson and Veggetti, 2001; Steinbacher et al., 2007). The fibers located between LS slow and fast muscles with intermediate oxidative potentials contained the transcripts of $MYH_{M2528\text{-}1}$ (Akolkar et al., 2010). Such fibers possibly appear either in late larval development (Scapolo et al., 1988) or at the onset of exogenous feeding that corresponds to the appearance of intermediate fibers (van Raamsdonk et al., 1982). Both slow and fast fibers are generated during the SH process. Interestingly, in zebrafish SH growth of slow muscle occurs even in mutant lacking hedgehog signaling pathway (Barresi et al., 2001). This indicates that the mechanisms specifying the identity of new slow muscle fibers during the SH process are different from those specifying the slow fate of adaxial cells in the early embryonic stage.

Generally, in fish which grow to a large final size, the vast majority of muscle fibers are formed in a long lasting hyperplastic process disseminated throughout the entire myotome. Both the phenotypic properties of new fibers and the age of the onset of the mosaic hyperplastic phase vary widely between species. Unfortunately, there are few studies about the onset of MH which focused on the muscle growth right through from embryonic stages to adult life. Apart from the involvement of *MYH*$_{M2528\text{-}1}$ in SH of torafugu larvae, its involvement in MH was further confirmed in juvenile torafugu where the transcript of *MYH*$_{M2528\text{-}1}$ were expressed in fast fibers with small diameters as well as inner part of LS and ED slow fibers (see Fig. 4-2). MH muscle growth principally occurs during juvenile life for most of the commercially important aquaculture fish species (reviewed in Kiessling et al. 1991). In some species, MH starts after the previous phase has ceased, but in others it may initially overlap with stratified growth (Stoiber and Sanger, 1996; Johnston et al., 1998). In general, the intensity of MH growth is greatest in early juvenile fish (Rowlerson et al., 1995). It is interesting to note that the novel function of *MYH*$_{M2528\text{-}1}$ in fibers that are generated by SH and MH in torafugu suggests distinct functions of *MYH*s in different phases of muscle growth. Thus, to focus on a large final body size with a marked distinction of phases of muscle growth and functional specialization of members of *MYH* family, torafugu is considered to be an ideal fish for studies on the mechanisms underlying embryonic and post embryonic muscle growth.

CHAPTER 5

General Discussion

The formation and growth of skeletal muscle in vertebrates proceed via successive phases of myogenesis that occur during embryonic and fetal development under the influence of various signals (Shi and Garry, 2006; Buckingham, 2007). A complex and sequential expression pattern of *MYH*s is observed during muscle fiber development in fish. The comprehensive analysis of the torafugu whole genome database by comparative genomics revealed over 20 sarcomeric *MYH*s (Ikeda et al., 2007). In this context, several *MYH*s were cloned from torafugu embryos, larvae and adult by our research group (Ikeda et al., 2007; Akolkar et al., 2010; Asaduzzaman et al., 2013). Furthermore, several *MYH*s were found to be expressed in fiber-type specific manner during early development or involved in hyperplastic muscle growth of torafugu (Ikeda et al., 2007; Akolkar et al., 2010; Asaduzzaman et al., 2013). However, our knowledge about factors that regulate fiber-type and developmental-stage dependent expression of *MYH*s in fish is still limited. In this study, a step-wise approach (Chapters 2-4) was followed to understand the transcriptional regulatory mechanisms involved in the *MYH*s expression during skeletal muscle development in fish. In this chapter, major findings of the previous chapters are integrated, interpreted and broadly discussed, and suggestions for further studies are given.

5.1 The 5'-flanking region of torafugu *MYH*s functioned similarly in zebrafish embryos

The expression analysis showed that $MYH_{M743\text{-}2}$ and $MYH_{M86\text{-}2}$ were expressed in fiber-type specific manners only in embryonic and larval stages of torafugu (Ikeda et al., 2007; Asaduzzaman et al., 2013). However, the regulatory mechanisms involved in the spatio-temporal expression of $MYH_{M743\text{-}2}$ and $MYH_{M86\text{-}2}$ have remained unknown. For the above purposes, regulatory sequences were isolated and identified as the first step to examine the spatial and temporal regulation of $MYH_{M743\text{-}2}$ and $MYH_{M86\text{-}2}$. *In vivo* reporter analysis to understand the regulatory mechanism of these genes expression in torafugu were found to be largely limited by the difficulties of routinely rearing and breeding them in the laboratory compared to other

experimental teleost model such as zebrafish (Alestrom et al., 2006). Therefore, zebrafish was used as a model in this study because transgenic fish expressing fluorescent protein under the control of *MYH*s promoters are useful tools for identifying the regulatory transcription elements and visualizing muscle fiber-specific gene expression patterns in live embryos (Long et al., 1997; Motoike et al., 2000; Zhang and Rodaway, 2007). The data showed that the 2,075 bp 5'-flanking region of $MYH_{M743\text{-}2}$ (Chapter 2) and 2,614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ (Chapter 3) were sufficient to direct spatial and temporal muscle-specific *EGFP* expression in zebrafish embryos. The results from the transient assays were also confirmed by generating stable transgenic zebrafish lines. The expressions of the transgenes in a stable line ($MYH_{M743\text{-}2}$:*EGFP* and $MYH_{M86\text{-}2}$:*EGFP*) were consistent with the transient and endogenous expression patterns of $MYH_{M743\text{-}2}$ (Chapter 2) and $MYH_{M86\text{-}2}$ (Chapter 3). The zebrafish expressing muscle fiber-specific fluorescence developed normally and produced offspring that stably expressed EGFP.

Several unique features of fish skeletal muscles make them an excellent model for studies on muscle development. The most important feature is the anatomical separation of fast and slow fibers in fish. Both the slow and fast muscle fiber contains characteristic MYHs which can be shown by immunostaining with MYH specific antibodies (Rowlerson et al., 1985; Scapolo and Rowlerson, 1987). The present study reported that $MYH_{M743\text{-}2}$ is highly expressed in fast muscle fibers (Chapter 2) and $MYH_{M86\text{-}2}$ is expressed in slow muscle fibers in torafugu embryos and larvae (Chapter 3). To determine whether or not the expression of EGFP was consistent with their endogenous mRNA expression patterns, EGFP-expressing embryos were characterized by whole mount antibody staining and immunohistological observations. In consistent with the endogenous mRNA expression in torafugu, transient and stable transgenic line zebrafish embryos showed consistent EGFP expression in fast muscle fiber for $MYH_{M743\text{-}2}$ (Chapter 2) and slow muscle fibers for $MYH_{M86\text{-}2}$ (Chapter 3). These findings confirmed that torafugu *MYH*s promoters can be analyzed *in vivo* in zebrafish embryos.

5.2 The activity of slow muscle-specific *MYH*$_{M86\text{-}2}$ promoter depends on hedgehog signals

In teleost, hedgehog (Hh) has been shown to play critical roles in muscle development. Hh is a secreted signaling protein from notochord that acts through at least two proteins, Patched (Ptc) and Smoothened (Smo). Ptc negatively regulates Hh signaling by inhibiting Smo. Hh binding to Ptc relieves this inhibition and allows Smo to transduce the signal (Ingham & McMahon 2001). Hh activity is necessary and sufficient to induce slow muscle and muscle pioneer cells both *in vivo* and *in vitro* (Weinberg et al., 1996; Du et al., 1997; Norris et al., 2000; Wolff et al., 2003). In zebrafish, the differentiations of adaxial cell-derived primary slow muscle fibers and a subset of secondary slow muscle fibers depend on the Hh signaling. The findings of this study (Chapter 3) suggested that EGFP expressing cell may correspond to Hh signaling dependent slow muscle fibers in zebrafish. However, further studies are required to confirm whether the EGFP expressing fibers under the control of *MYH*$_{M86\text{-}2}$ promoter are Hh dependent primary and/or a subset of secondary slow muscle fibers.

5.3 The regulatory elements involved in the transcriptional regulation of *MYH*s expression in fish have been previously reported to perform similar function in mammals

Interesting physiological difference exists between teleost fish and mammalians vertebrates. The same basic genes are present and appear functional; however slightly different regulatory mechanisms exist in the regulation of fish muscle development and growth. Fish musculature is much different from mammalian muscles in terms of segregated distribution of fast and slow muscle fibers. In mammals, most of the skeletal muscles contain a mixture of various fiber types, enabling individual muscle in providing long-lasting, low-intensity contractions together with fast burst activity. In contrast to mammals, different fiber-types are separated in fish. Fish fast muscle fibers locate deeply in the myotomal muscle whereas slow twitch muscle fibers are found in a narrow mid-lateral layer just under the skin. Based on these contrast arrangement patterns of

different fiber-types, it would be really interesting to investigate whether or not the similar transcriptional regulatory mechanisms of *MYH*s expression exists among fish and mammalian vertebrates.

The formation of different types of muscle fibers depends on differential expression of a suite and contractile and metabolic proteins specialized for each fiber type. Characterization of the *cis*-acting sequences involved in the expression of muscle fiber type-specific genes should shed light on the factors involved in the establishment of different fiber types. A growing number of regulatory sequences involved in the pattern of expression of fast and/or slow muscle-specific genes have been characterized in both mammals and fish; among them, the most extensively studied ones are the MRF family of transcriptional activators, which includes MyoD, Myf5, myogenin, and MRF-4 (Bergstrom et al., 2002; Berkes and Tapscott, 2005; Allen et al., 2005). These "master" transcription factors play a central role in regulating muscle development and growth in mammals (Tapscott, 2005). Another transcription factor MEF2 was found to function predominantly in expression of muscle-specific *MYH*s in mammals (Swoap, 1998; Lakich et al., 1998; Croissant et al., 2000). In consistent with mammals, the present study also demonstrated that MyoD (Chapter 2) and MEF2 (Chapter 2 and 3) binding sites participates in the transcriptional regulation of *MYH*s in fish.

SRF, a MADS box transcription factor related to MEF2, also regulates skeletal, as well as cardiac and smooth muscle genes by binding a DNA sequence known as a CArG box in mammals (Norman et al., 1988; Miano, 2003; Davis et al., 2008). A previous study showed that a SRF binding site residing in the -120 to -80 bp promoter region act as an enhancer of *MyHC-IIb* transcriptional activity predominantly in fast muscle of adult mice (Allen et al., 2005). Combined deletion and site-directed mutagenic analyses revealed that the low transcriptional activity of human *MyHC-IIb* is largely the result of reduced SRF binding within the proximal promoter region (Harrison et al., 2011). Although the role of SRF in transcriptional control of *MYH*

expression has been reported in mammals (Allen et al., 2005; Harrison et al., 2011), the underlying roles of SRF in transcriptional regulation of muscle-specific expression of *MYH*s have been poorly reported in teleost. In consistent with the previous studies in mammals, the combined mutation and deletion studies also showed that SRF plays a pivotal role in the transcriptional regulation of fast muscle-specific expression of $MYH_{M743\text{-}2}$ in fish (Chapter 2). Therefore, this study provided the new insight that SRF binding sites within the upstream promoter regulate the transcriptional activity of fast-type *MYH* in fish as like mammals (Chapter 2).

In two evolutionarily distant organisms, mice and zebrafish, Sox6 is involved in specifying the muscle fiber-type (Hagiwara et al., 2007; von Hofsten et al., 2008). Therefore, it was of interest to assess the role of the Sox6 binding sites in the regulation of slow muscle-specific $MYH_{M86\text{-}2}$ promoter. In zebrafish and mice, the loss of Sox6 activity led to the up-regulation of numerous slow muscle fiber–specific genes, suggesting that Sox6 functions as a transcriptional suppressor of slow muscle fiber-specific genes (Hagiwara et al., 2007; von Hofsten et al., 2008). In addition, over-expression of Sox6 resulted in a decrease in expression of slow fiber-specific genes in mice as well as zebrafish (van Rooij et al., 2009; von Hofsten et al., 2008). In mice, it directly represses the transcription of multiple myosin and sarcomeric genes by binding to conserved *cis*-elements in their promoters (Quiat et al., 2011). The present study also demonstrated that deleting Sox6 binding elements significantly increased the reporter gene mRNA expression (Chapter 3). Thus, in addition to fast muscle-specific repressive function, Sox6 may also function as a transcriptional suppressor of $MYH_{M86\text{-}2}$ promoter activity in fish as like mammals.

The role of NFAT binding sites in the transcriptional regulation of slow *MYH*s promoters has been well documented in avian and mammals (Calvo et al., 1999; Jiang et al., 2004; Giger et al., 2004; Calabria et al., 2009; Naya et al., 2010; Meissner et al., 2011) muscles cells but not in the fish. In consistence with the other studies on avian and mammalian muscles, our study also demonstrated an important role of NFAT binding sites in the transcriptional regulation of slow

muscle-specific *MYH* expression in fish (Chapter 3). Most of the regulatory elements identified in our study in fish were previously reported to be involved in the transcriptional regulation of *MYH*s expression in mammals.

5.4 Different regulatory elements involved in the transcriptional regulation of fiber types-specific *MYH*s expression in fish

Skeletal muscle fibers are classified based on two functional characteristics: contractile speeds and metabolic activity. These two functions are interrelated as the speed of contraction reflects how fast and by which mechanisms the cell metabolizes ATP. The fast-twitch fibers are characterized by low myoglobin and mitochondrial contents but rich in glycogen, making them suitable for intense short-burst activity. In contrast, slow oxidative fibers are high in mitochondria and myoglobin, making them suitable for longer sustained activity, or endurance acitivity (Goldspink et al., 2001; Sanger and Stoiber, 2001). Slow- and fast-twitch myofibers of skeletal muscles express unique sets of muscle-specific genes, and these distinctive programs of gene expression are controlled by variations in motor neuron activity. Several axes of regulation exist for the members of the *MYH*s family, including tissue-specific (muscle *versus* non-muscle), muscle type-specific (striated *versus* smooth muscle), fiber type-specific (fast *versus* slow). More recently, progress has been made in elucidating the *cis*-regulatory sites that contribute to slow *versus* fast muscle gene expression.

Several mediators and transcription factors have been identified for the nerve dependent fiber type regulation in skeletal muscle in mammals. It is well established that, as a consequence of more frequent neural stimulation, slow fibers maintain higher levels of intracellular free calcium than fast fibers. Therefore, slow muscle-specific genes are often regulated by calcineurin -dependent signaling mechanisms. Calcineurin-dependent signaling mechanisms have been characterized extensively in the activation of cytokine gene expression in T and B lymphocytes

responding to stimuli that elevate intracellular free calcium concentration (Rao et al., 1997). Binding of calcium to a calmodulin-calcineurin complex stimulates serine/ threonine phosphatase activity of calcineurin, the major substrates of which are NFAT transcription factors. Dephosphorylation of NFATs by calcineurin promotes their translocation from the cytoplasm to the nucleus, where they bind a cognate nucleotide recognition sequence (Rao et al., 1997) and stimulate transcription of target genes. In the nucleus, NFAT proteins bind DNA in conjunction with other transcriptional regulators, including MEF2, binding sites for which are clustered in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow-fiber program. In fast fibers, high-amplitude calcium transients stimulated by infrequent, phasic firing of the motor nerve are of insufficient duration to maintain calcineurin in the active state, so NFAT proteins remain phosphorylated and are excluded from the nucleus. When NFAT proteins are unavailable for DNA binding and protein–protein interactions at target promoters, the slow-fiber-specific program is down-regulated, and genes encoding fast-fiber-specific proteins are transcribed. $MYH_{M86\text{-}2}$ is selectively expressed in slow, oxidative skeletal muscle fibers (Chapter 3), whereas $MYH_{M743\text{-}2}$ is expressed in fast, glycolytic myofiber subtype in torafugu embryos and larvae (Chapter 2). In consistent with the above discussion, the present study also suggested that the expression of slow muscle-specific $MYH_{M86\text{-}2}$ is largely regulated by NFAT and MEF2 binding elements in torafugu (Chapter 3).

Sox6, a member of the SoxD family, transcription factors exhibits dual functions as a transcriptional activator or repressor (Wilson and Koopman, 2002; Han and Levebvre, 2008). Sox6 transcription factors play an important role in terminal differentiation of skeletal muscle by suppressing the transcription of mostly slow muscle fiber genes (Quiat et al., 2011). Recent study also showed that fast fiber-specific gene expression in Sox6 knock-out skeletal muscle reduced dramatically but the reason was unknown (An et al., 2011). In this study, Sox6 was also found to be functional as a transcription repressor of slow muscle-specific $MYH_{M86\text{-}2}$ (Chapter 3) but its

role was not investigated in fast muscle-specific *MYH*$_{M743\text{-}2}$. The present study also described a novel extension of the roles of Sox6 binding elements in regulating muscle fiber type-specific differentiation in fish (Chapter 3). Previous studies reported that SRF, although not strictly involved in the transcriptional regulation of fiber-type specific expression of *MYH*s as like calcineurin dependent NFAT elements, participates in the regulation of fast muscle-specific *MYH*s in mammals (Allen et al., 2005; Harrison et al., 2011). In this study, SRF was also found to play an important role to direct fast muscle-specific *MYH*$_{M743\text{-}2}$ expression in fish (Chapter 2). Basic, bHLH proteins of the MyoD family and several other varieties of transcription factors collaborate to establish myogenic cell lineages in the embryo, and are essential for muscle-specific gene transcription (Olson et al. 1995), but it seems difficult to characterize these proteins in fiber type-specific gene expression.

5.5 Diversity of involvement of *MYH*s in newly formed fibers in teleost fish and mammalian vertebrates

In mammalian vertebrates, the definitive number of skeletal muscle fibers is pre-established in early development or shortly after the birth. Following birth, growth occurs through the enlargement of existing muscle fibers by increase in length, diameter and nuclear content, a process known as hypertrophic muscle growth. However, during the process of regeneration of mammalian skeletal muscles after injury or upon stress, hyperplastic growth occurs, thereby generating new fibers (Goldspink 1972). In contrast to mammals, the evidence suggest that fish species with a large final body size and rapid growth exhibit a second phase of muscle growth immediately after primary myogenesis by increasing of number of muscle fiber, a process known as hyperplastic muscle growth. These results suggest that the onset of the second and third phases of myogenesis occurs simultaneously and follow the first phase of growth, uninterrupted, in fish characterized by indeterminate growth.

Fish express different *MYH*s during this indeterminate muscle growth and studies have shown greater diversity in *MYH*s in fish than mammals. A complete shift in *MYH*s expression occurs during the development of muscle fibers. Histochemical and immunohistochemical analyses implicated that a different *MYH* was expressed in small-diameter fibers in the white myotomal region in carp (Rowlerson et al., 1995). In terrestrial vertebrates, new regenerative fibers have been reported to express embryonic- and neonatal-type *MYH*s (Sartore et al., 1982; Whalen et al., 1990). In this context, no direct evidence on the mechanisms involved in the expression of embryonic *MYH*s in new fibers generated by hyperplastic growth has been reported in fish. Utilizing a carp genomic library and previously identified different lambda clones containing *MYH*s sequences, a novel *MYH* was isolated from small-diameter muscle fiber in carp, the *FG2* (Ennion et al., 1995). Interestingly FG2 was not expressed in slow or fast muscle fibers. *In situ* hybridization study revealed that *FG2* expression in restricted to only small diameter white fibers in fast growing carps (Ennion et al., 1995). Ennion et al. (2005) showed that *FG2* is expressed transiently during the differentiation of satellite cells into muscle fibers, suggesting a specific role in muscle cell recruitment. Various embryonic and neonatal *MYH*s are expressed in differentiating mammalian satellite cells following injury or trauma. However, carp *FG2* is not expressed in juvenile, fry or developing embryos of carp, demonstrating that it is not an embryonic or neonatal isoform, but a more specific "growth isoform gene". As equivalent growth isoform gene has not been identified in any terrestrial vertebrates, which highlights the unique growth attributes seen in fish. More interestingly, torafugu $MYH_{M2528\text{-}1}$ is only *MYH* in fish that has been found to be involved in both SH and MH to date (Chapter 4). Unlike Carp *FG2*, torafugu $MYH_{M2528\text{-}1}$ was found to be expressed in late embryonic, larval and juvenile stages in addition to adult stages, but its expression was restricted to only small diameter fibers, not like the expression of common embryonic and larval type *MYH*s in fish. In addition, torafugu $MYH_{M2528\text{-}1}$ was also found to be expressed in inner part of the LS and ED slow muscle fibers (Chapter 4). New fibers formation and the age at the onset of SH and MH vary widely between

species (Rowelerson and Vigeetti, 2001). Less has been studied especially on the onset of SH and MH during development of fish by using *MYH* as a marker. By observing the expression pattern of $MYH_{M2528\text{-}1}$, it was revealed that SH started immediately after hatching of torafugu around 10 dpf and MH present in juvenile stages of torafugu at 4.5 g body weight (Chapter 4). The novel function of $MYH_{M2528\text{-}1}$ in fibers that are generated by SH and MH in torafugu suggests distinct functions of *MYH*s in different phases of muscle growth.

Conclusion and future prospective

Finally, the present study provides greater insight into complex transcriptional regulation of *MYH*s that are specifically expressed in fast and slow muscle fibers during embryonic and larval development, and newly formed fibers during post-embryonic hyperplastic growth in fish. In most cases, expressions of muscle-specific *MYH*s in fish are transcriptionally regulated by multiple regulatory sequences in their 5'-flanking regions. Different *cis*-elements in the 5'-flanking regions of *MYHs* are participated in the transcriptional regulation of slow and fast muscle-specific expression in fish. Although some of the previous studies reported the transcriptional regulatory mechanisms underlying the fast muscle-specific *MYH*s, to the best of our knowledge this study (Chapter 3) is the first report about the transcriptional regulation of slow muscle-specific *MYH* expression in fish. A greater understanding of factors responsible for the transcriptional regulation of *MYH*s expression in different muscle fibers-types would provides a greater insight into how these muscle fibers are established and maintained in fish. In this study, most of *cis*-elements are identified by deletions and mutations analysis in live zebrafish embryos. However, deletions of complete putative binding sites may affect the binding of other transcription factors and a putative consensus binding sequence may not necessarily bind the predicted transcription factors. Therefore, further studies are needed to investigate whether and how the predicted transcription factors bind to the promoters and whether the predicted transcription factors interact with other cofactors to activate *MYH* expression in fish. Regulation

of *MYH*s expression by multiple regulatory sequences appears to be a common mechanism for the control of muscle-specific gene expression. Therefore, combined deletion of multiple *cis*-elements of NFAT and MEF2 binding sites would provide more clear insight about transcriptional regulation of $MYH_{M86\text{-}2}$. In teleost fish, there are no conclusive reports about the transcriptional regulatory mechanism of *MYH*s involved in hyperplastic muscle growth. Therefore, it would be really interesting to derive conclusive evidence from analysis of the promoter and the 5'-upstream region of torafugu $MYH_{M2528\text{-}1}$ in future.

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Summary and Conclusion

Vertebrate skeletal muscle consists of heterogeneous tissues including various types of muscle fibers and the fiber-type specification is crucial for muscle development. Myosin is the major structural and functional muscle protein in the contractile apparatus. Terminal differentiation and specification of various types of muscle cells during development require specific expression of myosin heavy chain (MYH) isoforms; thereby MYH is thought to be a key molecule on the functional diversity of muscles. As in mammalian skeletal muscles, the members of the MYH genes (*MYH*s) of fish are expressed in a complex, sequential fashion during development and growth. Although a considerable progress has been made in elucidating the molecular genetics underlying the development-dependent, muscle-specific expression of *MYH*s in mammals, little is currently known regarding comparable issues in fish. In this study, the 5'-flanking sequences of development-dependent, fast- and slow-type *MYH*s of torafugu *Takifugu rubripes*, $MYH_{M743\text{-}2}$ and $MYH_{M86\text{-}2}$, respectively, were cloned and analyzed for their transcriptional regulation in zebrafish *Danio rerio* embryos using transient and transgenic approaches. The present study also focuses on the expressional regulation of *MYH*s, which are supposed to be associated with hyperplastic muscle growth in fish.

1. Transcriptional regulation of fast muscle-specific $MYH_{M743\text{-}2}$ expression

$MYH_{M743\text{-}2}$ is highly expressed in fast muscle fibers of torafugu embryos and larvae. When the 2075-bp fragment of $MYH_{M743\text{-}2}$ 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 EGFP expression 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 jaw, eye and pectoral fin muscles. The expression of the transgene in embryos and larvae from the stable transgenic line of zebrafish was consistent with

that of zebrafish embryos microinjected the 2075 bp construct. While the injected 2075 bp construct showed EGFP expression in both fast and slow muscle fibers of larvae as revealed by immunohistochemical analysis, embryos from the stable transgenic line expressed EGFP in fast muscle fibers only. These results clearly demonstrated that the 2075 bp 5'-flanking region of $MYH_{M743\text{-}2}$ contains essential *cis*-regulatory sequences for myogenesis that are conserved among torafugu and zebrafish.

Using the MatInspector and TFsearch programs, the 2075-bp 5'-flanking region from the start codon of $MYH_{M743\text{-}2}$ was found to contain putative transcription factor-binding sites for myocyte enhancing factor 2 (MEF2), myogenic determining factor (MyoD) and serum response factor (SRF), all of which have been implicated in the expressional regulation of muscle-specific genes during development. Embryos microinjected a modified 2075 bp construct, where the total three SRF binding sites were deleted, showed a significantly reduced EGFP expression. However, the deletion of all SRF binding sites failed to eliminate $MYH_{M743\text{-}2}$ expression completely. Moreover, the minimum 468-bp basal promoter region from the start codon containing the third SRF binding site from the 5'-end was found to direct EGFP expression in the myotomal compartment. Mutations of this SRF binding site in the 468-bp construct also reduced $MYH_{M743\text{-}2}$ expression in the myotomal compartments, suggesting that this site is necessary for mediating the basal expression of $MYH_{M743\text{-}2}$. In this regard, the expression of two zebrafish SRFs, *srf1* and *srf2*, was observed as early as at 2 hours post fertilization and continued to be expressed throughout the successive developmental stages. Such expression pattern of *srf*s was consistent with their potential roles in regulating $MYH_{M743\text{-}2}$ expression in zebrafish embryos.

There were three MyoD and eight MEF2 binding sites within the 2075-bp 5'-flanking region of $MYH_{M743\text{-}2}$. Functional analyses revealed that deletions of multiple sites of these factors significantly reduced the $MYH_{M743\text{-}2}$ promoter activity in skeletal muscles. Such reductions were also validated by real-time PCR analysis. Deletion mutation analyses also suggested that MyoD,

but not MEF2, binding sites are crucial for *MYH*$_{M743\text{-}2}$ promoter activity in craniofacial and pectoral fin muscles. However, the deletion of all MyoD- or MEF2-binding sites failed to eliminate *MYH*$_{M743\text{-}2}$ expression completely. Taken together, it is indicated that multiple transcription factors including SRF, MyoD and MEF2 participate in the expression of *MYH*$_{M743\text{-}2}$.

Torafugu *MYH*$_{M743\text{-}2}$ showed homology over 55% on the rVISTA analysis in the 5' flanking region from −664 to −364 bp with the corresponding region of a green spotted pufferfish *Tetraodon nigroviridis* orthologous gene, *MYH13.* Embryos microinjected the 2075-bp construct excluding this conserved region completely abolished the EGFP expression, suggesting that the conserved regulatory *cis*-elements in this region regulate the transcriptional activity of *MYH*$_{M743\text{-}2}$. Further studies are required to precisely map responsible *cis*-elements in this region.

2. Transcriptional regulation of slow muscle-specific *MYH*$_{M86\text{-}2}$ expression

MYH$_{M86\text{-}2}$ transcripts are highly expressed in slow muscle fibers of torafugu embryos and larvae. To examine whether or not the 5'-flanking sequence of torafugu *MYH*$_{M86\text{-}2}$ would function similarly in zebrafish, we constructed a reporter vector containing the 2614 bp 5'-flanking region fused to *EGFP* in pT2AL200R1150G vector. The microinjection of the 2614 bp construct in zebrafish embryos exhibited EGFP expression in slow muscle fibers at 1dpf and after hatching (2dpf) continued to be expressed in the whole myotomal region in larvae. The transient superficial slow muscle-specific expressions of *MYH*$_{M86\text{-}2}$ were further confirmed by generating stable transgenic zebrafish lines. Apart from the slow muscle-specific expression, both the endogenous *MYH*$_{M86\text{-}2}$ and the reporter gene were also found to be expressed in pectoral fin muscles. These data suggest that the 2614-bp 5'-flaking region contained the necessary regulatory elements for *MYH*$_{M86\text{-}2}$ expression in slow muscle fibers.

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*$_{M86\text{-}2}$: *EGFP*, were treated with cyclopamine (2.5, 5 and 10 μg/ml), a well-known hedgehog signaling inhibitor. Depending on the dose, cyclopamine-treated embryos showed reduction or complete elimination of EGFP expression in slow muscle fibers, suggesting that *MYH*$_{M86\text{-}2}$ promoter activity depends on the hedgehog signaling.

To identify the key regulatory region for the slow muscle-specific expression, a deletion analysis was performed within the 5.5-kb 5'-flanking region of *MYH*$_{M86\text{-}2}$. The deletion of the 5'-flanking region up to -2614 bp upstream of the *MYH*$_{M86\text{-}2}$ promoter had no or marginal effect on the activity and slow muscle-specificity of the promoter. The deletion of a distal 500 bp region (-2000/-1500) caused ectopic *MYH*$_{M86\text{-}2}$ expression in fast muscle fibers. *In silico* analysis revealed five putative Sox6 binding sites which spanned the regions at -1953/-1947 (Sox6_1), -1525/-1519 (Sox6_2), -808/-802 (Sox6_3), -792/-786 (Sox6_4) and -41/-35 (Sox6_5) in the 2,614 bp 5'-flanking region of *MYH*$_{M86\text{-}2}$, including first two Sox6 binding sites (Sox6_1 and Sox6_2) in the 500 bp distal region. Notably, the deletion of these Sox6 binding sites (except Sox6_5) individually or in various combinations caused ectopic EGFP expression in the fast muscle fibers. In mice and zebrafish, Sox6 directly represses the transcription of slow muscle fiber-enriched genes by binding to conserved *cis*-regulatory elements. In consistent with such finding, the deletion of these Sox6 binding elements (except Sox6_5) significantly increased the reporter gene mRNA expression. Thus, in addition to fast muscle-specific repressive function, Sox6 elements may also function as a transcriptional suppressor of *MYH*$_{M86\text{-}2}$ promoter activity.

Promoter dissection study also showed that the deletion of 614 bp spanning the region from -2614 to -2001 significantly reduced the number of EGFP expressing muscle fibers. It suggests that 614 bp region may contain positive *cis*-elements required for slow muscle-specific expression of *MYH*$_{M86\text{-}2}$. Several studies on various model system have reported that

calcineurin/nuclear factor of activated T cells (NFAT) pathways appear to be involved in the transcriptional regulation of slow muscle-specific gene expression including those of slow *MYH*s. *In silico* analysis identified four putative NFAT binding sites spanning the region at -2258/-2254 (NFAT_1), -1968/-1964 (NFAT_2), -1604/-1600 (NFAT_3) and -306/-302 (NFAT_4) bp in the 2,614 bp 5'-flanking region of $MYH_{M86\text{-}2}$, including first one (NFAT_1) in the 614 bp distal region. Embryos microinjected these NFAT binding sites deletion constructs (except NFAT_2) showed significantly reduced EGFP expression in slow muscle fibers, and elimination of multiple and all the sites of NFAT elements showed more pronounced effects. However, the deletion of all NFAT binding sites could not completely abolish the EGFP expression, suggesting that *cis*-elements other than NFAT binding sites require for directing the $MYH_{M86\text{-}2}$ expression.

Members of myogenic regulatory factors (MyoD, Myogenin, myf5 and MRF4) and MEF2 have all been shown to regulate the transcription of numerous muscle-specific genes. Analysis of the 2614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ by Genomatix MatInspector program identified two putative MyoD and three MEF2 binding sites. The deletion of both two MyoD binding sites did not significantly reduce EGFP expression percentages in slow muscle fibers. However, the deletion of multiple MEF2 binding sites significantly reduced EGFP expression in slow muscle fibers. These data suggest that MEF2, but not MyoD, participates in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression.

3. *MYH*s expression associated with hyperplastic muscle growth in fish

The relationship of *MYH*s expression with muscle fiber formation has not been well understood in fish. In torafugu, the transcript of $MYH_{M2528\text{-}1}$ appeared in embryos at 6 dpf and continued to be expressed in later embryonic and larval development successively as well as in adult fast and slow skeletal muscles. The dorso-ventral extremes of larval myotome are considered as the main growth engines for recruitment of new fibers by stratified hyperplasia. Interestingly, the

transcripts of *MYH*$_{M2528\text{-}1}$ were localized specifically in the dorsal and ventral extremes of larval myotome, suggesting its involvement in stratified hyperplasia. Apart from the involvement in stratified hyperplasia in torafugu larvae, *in situ* hybridization study showed that *MYH*$_{M2528\text{-}1}$ is also involved in mosaic hyperplasia in juvenile torafugu where its transcripts were expressed in fast fibers with small diameters as well as inner parts of superficial slow muscle fibers.

Conclusion

The present study provides a greater insight about the transcriptional regulatory mechanisms involved in *MYH*s expression during skeletal muscle development in fish. This study is the first report about the transcriptional regulatory mechanisms involved in the slow muscle-specific *MYH* expression in fish. A greater understanding of factors responsible for the transcriptional regulation of *MYH*s expression in different muscle fibers-types would provides a greater insight into how these muscle fibers are established and maintained in fish. In this study, the *cis*-elements are identified by deletions and mutations analyses in zebrafish embryos. However, a putative consensus binding sequence may not necessarily bind the predicted transcription factors and deletions of putative binding sites may affect the binding of other transcription factors. Therefore, further studies are needed to investigate whether and how the predicted transcription factors bind to the promoters and whether the predicted transcription factors interact with other cofactors to activate *MYH*s expression in fish.