博士論文（要約）

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

（魚類筋肉の示す終生成長と遺伝子発現の転写調節機構に関する研究）

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DECLARATION

I, A. K. Shakur Ahammad, hereby declare that the thesis entitled **"Studies on the transcriptional regulatory mechanisms of gene expression mediating indeterminate muscle growth in teleost"** 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.

15 May 2015

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DEDICATION

This thesis is dedicated to my son "Ahmed Shahriar"

-the best gift and inspiration of my life

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Contents

Abbreviations

ANOVA	:	Analysis of variance
ATP	:	Adenosine 5’-triphosphate
bp	:	Base pair
cDNA	:	Complementary DNA
CIP	:	Calf intestinal phosphtase
DAPI	:	diamidine-20-phenylindole dihydrochloride
DNA	:	Deoxyribonucleic acid
dpf	:	Days post fertilization
ECL	:	Erectors and depressors
EGFP	:	Enhanced green florescence protein
HLH	:	Helix-loop helix
HS	:	Horizontal septum
Hh	:	Hedgehog
LS	:	Lateralis superficialis
MADS	:	MCM1, AGAMOUS, DEFICIENS and SRF
MEF2	:	Myocyte enhancing factor 2
MH	:	Mosaic hyperplasia
MPCs	:	Myogenic precursor cells
MYH	:	Myosin heavy chain
MYH	:	Myosin heavy chain gene
MyoD	:	Myoblast determining factor
Myog	:	Myogenin
NFAT		Neoclear Factor of Activated T cell
PBSTw	:	Phosphate-buffered saline with 0.1% tween 20

PCR	:	Polymerase chain reaction
PFA	:	Paraformaldehyde
Pax	:	Paired box protein
RACE	:	Rapid amplification of cDNA ends
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcription PCR
SD	:	Standard deviation
SH	:	Stratified hyperplasia
SPSS	:	Statistical package for social science
TAP	:	Tobacco acid pyrophosphatase
TBSTw	:	Tris-buffered saline with 0.1% tween 20
TEEA	:	The transient embryonic excision assay
TFsearch	:	Transcription factor search

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Abstract

Indeterminate muscle growth provides teleosts with a vast potential to increase their body size. Teleosts are unique amongst vertebrates due to their indeterminate muscle growth. Therefore, fish is an attractive model to study such indeterminate muscle growth mechanisms because in teleost skeletal muscles, both hyperplasia and hypertrophy occur throughout the organism's lifespan. Torafugu myosin heavy chain gene, $MYH_{M2528\text{-}1}$, is a potential target to understand the molecular network involved in indeterminate muscle growth due to its specific expression in neonatal muscle fibers produced by muscle hyperplasia amongst different fish species. In the present study, we examined the promoter activity of the 5′-flanking region of torafugu $MYH_{M2528\text{-}1}$ via an *in vivo* reporter assay using zebrafish and successfully identified 2,100 bp 5'-flanking sequences was sufficient for its expression in skeletal, craniofacial muscles region that had adequate promoter activity (Chapter 2). We also showed this promoter participates in induction of gene expression specifically in neonatal muscle fibers produced by hyperplastic muscle growth at larval and post-larval stages of zebrafish development (Chapter 2). . As well, despite the phylogenetic distance between zebrafish and torafugu, our results clearly indicate that the signaling cascade responsible for the $MYH_{M2528\text{-}1}$ promoter activity is conserved, suggesting that this molecular cascade is conserved among teleosts (Chapter 2). As well, promoter deletion analysis revealed that the-2100~-600bp 5'-flanking sequence of $MYH_{M2528\text{-}1}$ is essential for promoter activity (Chapter 2). This region contains putative binding sites for several myogenic regulatory transcription factors and nuclear factor of activated T-cell (NFAT), a transcription activator involved in regeneration of mammalian adult skeletal muscle. A significant reduction in the promoter activity of the $MYH_{M2528\text{-}1}$ deletion constructs was observed in accordance with a reduction in the number of these binding sites, suggesting the involvement of specific transcription factors in indeterminate muscle growth (Chapter 2). Furthermore, transcriptory regulatory mechanism involved in $MYH_{M2528\text{-}1}$ expression in indeterminate muscle growth remained unknown. Here, we examined the cis-regulatory mechanism of 2,100 bp 5'-flanking region of torafugu $MYH_{M2528\text{-}1}$ using deletion-mutation analysis in zebrafish embryo. Therefore, we revealed that myoblast determining factor (MyoD) binding elements play a key role and participate in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression (Chapter 3). We further discovered that paired box protein (Pax3) are required for promoting $MYH_{M2528\text{-}1}$ expression (Chapter 3) and myocyte enhancer factor-2 (MEF2) binding sites participate in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression in slow/fast skeletal muscles in relation with muscle hyperplasia (Chapter 3). Subsequently, we further demonstrated that the nuclear factor of activated T-cell (NFAT)-like binding sites take part in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression in slow and fast muscles fiber in relation to indeterminate muscle growth (Chapter 3). Finally, we validated that above mention transcription factor in zebrafish and torafugu using gene specific primer through PCR analysis (Chapter 3). These results obviously confirmed that multiple *cis*-elements in the 5'-flanking region of $MYH_{M2528\text{-}1}$ function in the transcriptional regulation of its expression in zebrafish.

List of Publications

The contents of this thesis have been published as follows:

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CHAPTER 1

General Introduction

1.1 General Background

1.1.1 Structural arrangement of teleost myotomal muscle

Teleosts skeletal muscle is structurally arranged into segmental myotomes in a complex sequential fashion. This segmental myotome includes various muscle fibres which increase equally to the body axis even as fibres in deeper region of the myotomes. Therefore, myotomal compartment is associated with the requirement to produce equal reduction of sacromeres at distant body flexures (Alexander, 1969; Rome and Sosnicki, 1990; Johnston et al., 2011). On the contrary, mammallian skeletal muscle enclosed a bundle of muscle fiber, facilitates single muscles in supplying durable, lower force contractions, in connect with primary fracture of movement. In across with mammals, teleost myotomal muscles are structurally segregated into different areas. Regards, fast muscle fibers delineate deeply and major part in the myotome, whereas slow fibers are situate at lateral superficial to the myotome (Fig 1-1) (Van Raamsdonk et al., 1982; Sanger and Stoiber, 2001). Thus, teleost fiber types can be partitioned by the histochemical dissection of SDSase (Fig. 1-1). The fast twitch fibers comprise the greater part of myotomes which are typically larger in diameter than slow muscle fibres in teleosts (Greer-Walker and Pull, 1975; Altringham and Johnston, 1982). The fast muscle fibers hold a low density of mitochondria and have a light capillary network (Johnston, 1982; Egginton and Sidell, 1989; Sanger and Stoiber, 2001). Its contract and fatigue faster than other fibre types (Altringham and Johnston, 1982; Johnston and Salmonski, 1984; Langfeld et al., 1989). They are engaged to power rapid bursts of movement e.g. escape responses when a predator appears (Altringham et al., 1993; Altringham and Ellerby, 1999). Slow muscle fibers are relatively lesser in diameter from other muscle fibres (Greer-Walker and Pull, 1975; Altringham and Johnston, 1982). As well, slow muscle fibres are situated in a thin superficial band adjoining to the lateral line known as lateralis superficialis (LS) with a wedge-shaped condense in the region of the horizontal septum (Fig. 1-1). Besides LS slow muscle, one more type of slow muscle is positioned at the median fins in fish including to the order Tetradontiformes, termed erectors and depressor muscle (ED) (Winterbottom, 1974). Also, slow twitch fibers are extensively appeared to contract slowly (Altringham and Johnston, 1982; Johnston and Salmonski, 1984; Langfeld et al., 1989), contain rich source of mitochondria (Johnston, 1982; Egginton and Sidell, 1989; Sanger and Stoiber, 2001), and are providing with a thick capillary network (Egginton and Sidell, 1989; Sanger and Stoiber, 2001). All these divergent characters of slow muscle fibers imitate their efficient role in stable and constant swimming activities fuelled by aerobic metabolism (Johnston et al., 1977; Rome et al., 1984; Altringham and Ellerby, 1999).

On the other hand, from above major fiber types, some minor fiber 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 ijvestigated in fish. In this regards, intermediate, or pink muscle, is collection in-between twitch muscle fibers, which are typically intermediate in diameter and represent 10%~20% of total teleost myotome cross-sectioned area (Johnston et al., 1977; Langfeld et al., 1989). They are to be found between the fast and slow fiber and contain intermediate contraction speeds and fatigue-resistance contrast to slow and fast muscle fibres (Altringham et al., 1993; Altringham and Ellerby, 1999). Similar to slow fibers, intermediate fibers contain direction similar to the axis of body (Scapolo and Rowlerson, 1987). The additional fiber types for example red rim fibers and tonic fibers form a somewhat little part of myotome and are regard as to contribute less significantly to the swimming of fish.

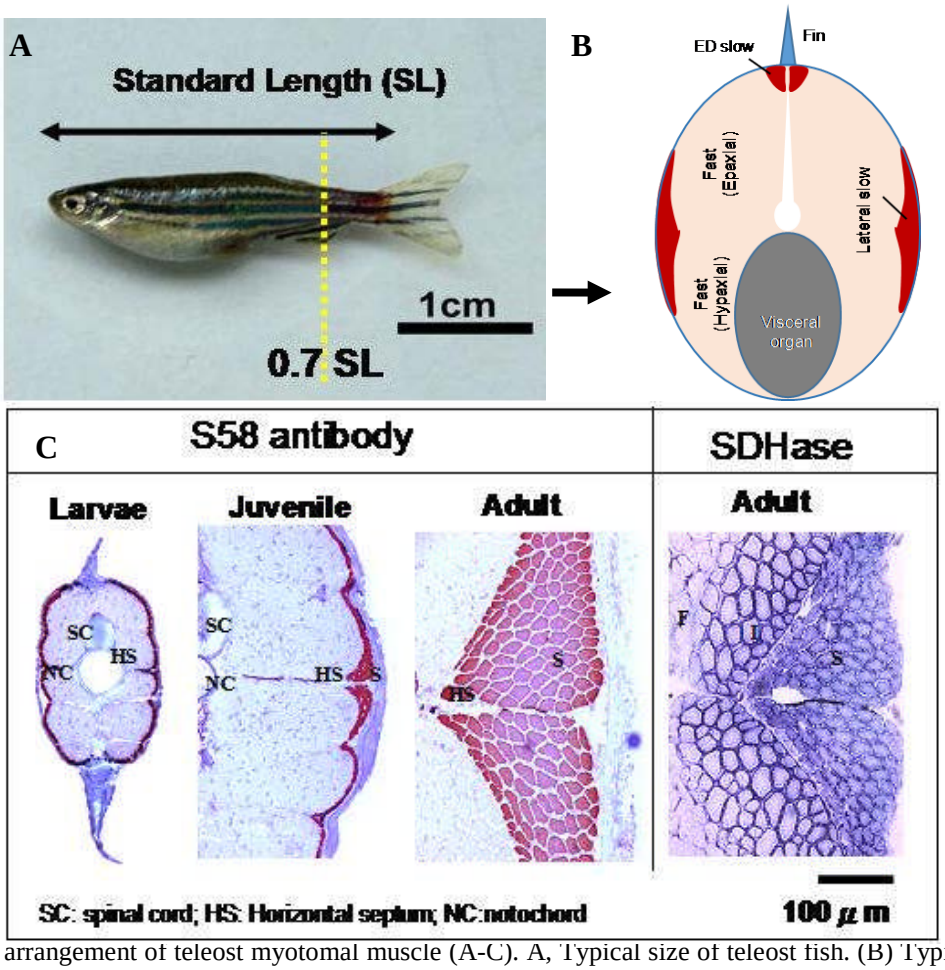

Fig. 1-1. Structural arrangement of teleost myotomal muscle (A-C). A, Typical size of teleost fish. (B) Typical view of slow and fast muscle in zebrafish. (C), Location of slow and fast muscle in zebrafish by S58 antibody and SDSase, respectively (Lee et al., 2010). Slow (S), Intermediate (I), and fast muscle (F).

1.1.2 Teleost myogenesis in relation to indeterminate growth

The main event of myogenesis in teleost skeletal muscle refers to the three stages, embryonic, stratified hyperplasia and mosaic hyperplasia. At each stage, the fundamental processes of myogenesis are ceded concerning steps of determination, proliferation, migration, fusion and differentiation (Fig. 1-2A). Also myoblast-myoblast, myoblast-myotube, and myoblast muscle fibre fusion events took place (Fig. 1-2B). In this regard, myogenetic process necessary for indeterminate muscle growth, which includes the recruitment of newly form muscle fibres (hyperplasia) and development of existing muscle fibres through nuclear accretion (hypertrophy).

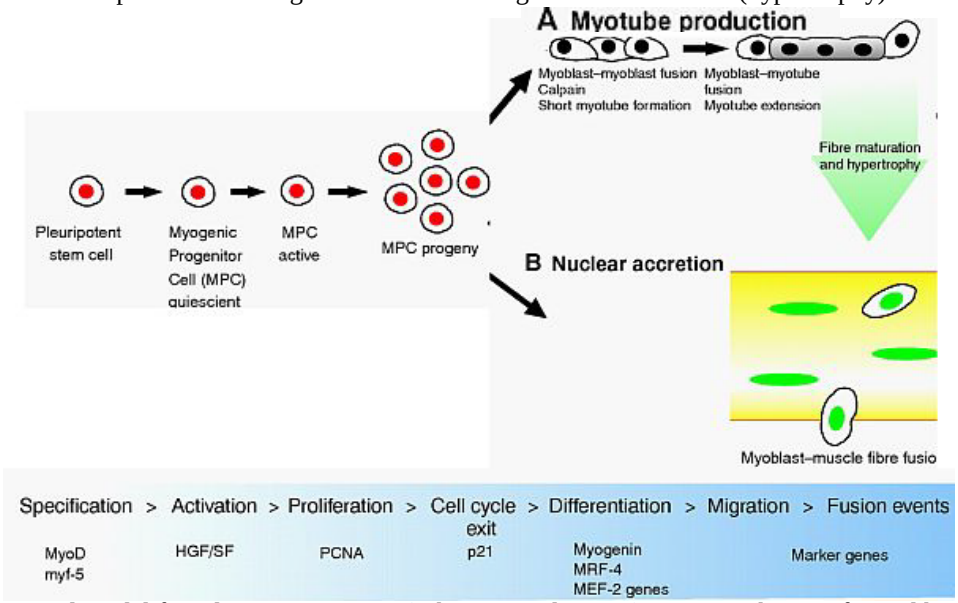

Fig. 1-2: A universal model for teleost myogenesis (Johnston et al., 2006). A, Myotubes are formed by specification, activation, proliferation differentiation process. B, at this stage of growth the MPC progeny can fuse with muscle fiber (myoblast-muscle fiber fusion) in the process of nuclear accretion.

Likely, three major steps of myogenesis found to be noted in teleost, specifically embryonic myogenesis, stratified hyperplasia (SH), and mosaic hyperplasia (MH) (Rowlerson and Veggetti, 2001; Johnston, 2006; Steinbacher et al., 2007; Akolkar et al., 2010; Rescan et al., 2013; Asaduzzaman et al., 2013; Ahammad et al., 2015). Regarding each steps, timing and single events are vary from species to species. As for determinate growth upholding species have miniature of utmost growth such as guppies (*Poecilia reticulate*) (Veggetti et al., 1993; Biga and Goetz, 2006), the first two steps of myogenesis produce the greater part of muscle fibres. Conversely, MH fabricates the bulk of muscle fibres in species which demonstrate indeterminate growth and get to a larger magnitude of body (Johnston, 2006). Therefore, present understanding of three major steps of myogenesis are reviewed in the followining sections.

During embryogenesis skeletal muscle in vertebrates is developed from metameric appearance of somites i.e. segmental blocks of paraxial mesoderm that develop into muscle, axial skeleton and dermis during later development (Noden, 1991; Couly et al., 1992; Elmasri et al., 2004). The somite development in teleosts is identical to that in amphibians, birds, and mammals that is extremely conserved within these vertebrates (Kimmel et al., 1995; Pourquié 2001). After that the paraxial mesoderm, the mesoderm bordering to the central body axis, build up from cells approximately the edge of the germ ring that transfer in the direction of dorsal side, where the somites create and concentrate (Kimmel et al., 1990). As well, additional transformation of the paraxial mesoderm, and its distribution into somites, are exceedingly lying on the axial structures (Lassar and Munsterberg, 1996). The commencement of somitogenesis differs in the direction of epiboly among various species, the progression carry on during a rostral-caudal gradient while the paraxial mesoderm is distributed into membrane-bound blocks of cells (Kimmel et al., 1995; Stickney et al., 2000; Pourquié 2001). During vertebrate embryonic developmental, somites produced as of the presomitic mesoderm and fast form two main substructures, a ventral sclertome section and a dorsally situated epithelial dermomyotome (Pourquié 2001). Contained by the teleost somite, while, the ventral sclerotome, that will become visible the axial bone and cartilage of the embryo, is deeply abridged in contrast with terrestrial vertebrates (Kimmel et al., 1995; Stickney et al., 2000). This may be the sign of the shortened demand intended for behind skeletal framework and an improved locomotory condition for axial muscle compare to appendicular muscle in lymphatic environmental system (Bone, 1966). Inventive work introducing the way of teleost embryonic muscle development was highly executed in zebrafish. According to Devoto et al. (1996) spatial distribution of teleost muscle types occurred in the embryo, wherever slow and fast muscle fibres derived from two distinct subset of muscle pioneers i.e. adaxial cells and lateral presomitic cells in the somites. These two cell type, the adaxial and lateral presomitic cells, can be recognized in the segmental plate on based on their location and structures (Devoto et al., 1996) along with the different gene expression specially *snail* (Thisse et al., 1993) and *myod* (Weinberg et al., 1996). At first, adaxial cells can be differentiated at both side of the notochord earlier to segmentation at the stage of the horizontal septum (HS) as a membrane-like sheet monolayer neighboring all side of the notochord (Devoto et al., 1996). Adaxial cells are predominantly superior and in ordinary shaped than the surrounding lateral presomitic cells that enclose them (Devoto et al., 1996). The arrangement of adaxial cells to chase the slow muscle differentiation program entrusts significantly on inaugural signals from the notochord and floorplate intercedes by members of the hedgehog protein family (Wolff et al., 2003). Previous studies designated that the area before thought to contain lateral presomatic cells (Devoto et al., 1996) are consist of two different cell

types, lateral-anterior and lateral-posterior somite cells (Fig. 1-3A). These three divergent cell types are reorganized during a succession of entire somite replacement events due to produce embryonic slow and fast muscle fibres together with myogenic progenitor cells (MPCs) necessary for following myogenesis. During the entire somite replacement, lateral anterior and posterior cells are reshuffled into the lateral-external and later-medial cell layer (Fig. 1-3B). At late segmentation the 90 degree somite rotation is accomplished foremost to the development of a discrete external cell layer (ECL) foundation from the lateral anterior somite cells (Fig. 1-3C). As well, the adaxial cells begin to move both laterally and radially (Fig. 1-3C). The migrated adaxial cells ultimately turn to monolayer of embryonic slow muscle fibres between ECL and embryonic fast muscle fibres (Fig. 1-3D). Afterward, some cells from ECL move into the myotome while the slow muscle layer lead to develop fast muscle fibres in separate zones as SH (Fig. 1-3E). It has been recommended that ECL plays a vital role in additional successive postembryonic muscle fibre production even though this leftover to be confirmed.

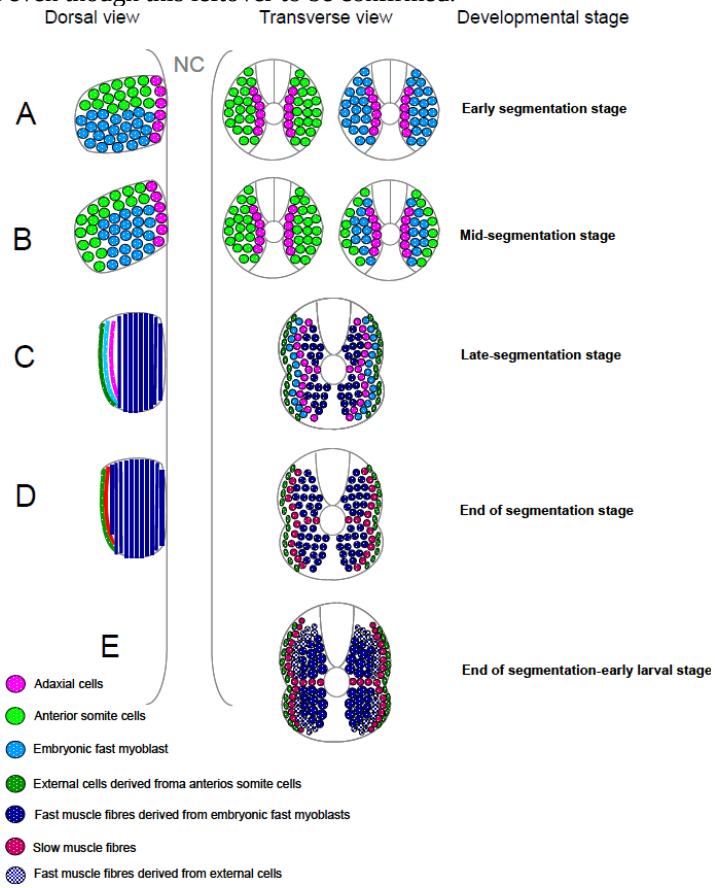

Fig. 1-3: Diagram illustrating the patterning of slow and fast muscle fibres during embryonic and early postembryonic stages (Adapted from Lee, 2010). The transverse views of somites during early and mid segmentation stage designate the distinct origins of anterior somite cells (anterior) and embryonic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

Resulting embryonic myogenesis, new muscle fibres are recruited to from “germinal zones” namely stratified hyperplasia (SH) (Fig. 1-4A). SH has been extensively recognized in many species (review in Rowlerson and Veggetti, 2001) and deliberate the key basis of neomyogenesis during late embryonic and early postembryonic growth. SH produce marked gradients in muscle fibre diameter from the marginal to the deep myotomes (Fig. 1-4) (Rowlerson and Veggetti, 2001). SH took place following the segmentation period in accordance with recruitment of new muscle fibres by their gene expression pattern in zebrafish (Barresi et al., 2001), pearlfish (Steinbacher et al., 2006), brown trout (Steinbacher et al., 2007) and (Rescan et al., 2013). To begin with, newly form slow muscle are frequently incorporated into the dorso-ventral boundary of the accessible monolayer of slow muscle (Barresi et al., 2001) while newly recruited fast muscle fibres are largely added at the side-line of the slow muscle layer, the border of the existing fast muscle and dorso-ventral region of the myotomal muscle (Steinbacher et al., 2006, 2007; Rescan et al., 2013).

After SH, the ultimate phase of hyperplasia took place termed as mosaic hyperplasia (MH) (Rowlerson and Veggetti, 2001). On the contrary to SH, MH includes the increase number of precursor cells which next to migrate and combined to form myotubes on the scaffold of existing fibres to generate a mosaic of muscle fibre diameters in a myotome across section (Fig. 1-4B) (Rowlerson et al., 1995). In fast muscle, muscle fibre development continued up to ~40% of the maximum fork length (Weatherley et al., 1988; Johnston et al., 2003; 2004). In teleost which are greater than ~40% of the maximum fork length, recruitment of new fiber is ceased except the muscle becomes injured (Rowlerson et al., 1997). Subsequently, the conclusion of muscle fibre recruitment all subsequent growth is by fibre hypertrophy alone, which entails the accretion of additional nuclei to maintain the myonuclear domain size within certain limits (Johnston et al., 2003, 2004). On the contrary, slow muscle fibre number maintains to increase with body length to the utmost body size (Johnston et al., 2004).

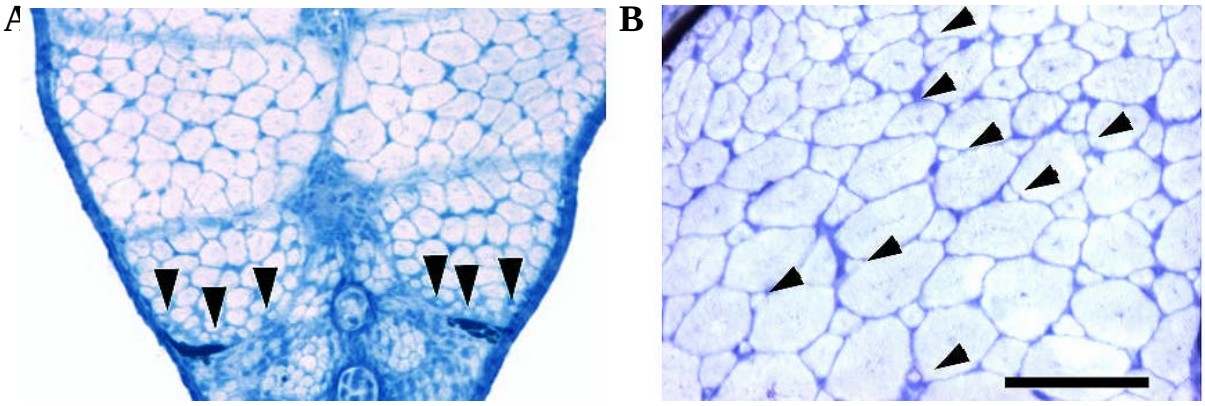

Fig. 1-4: Hyperplastic growth of trunk muscle in zebrafish larvae (Adapted from Lee et al., 2010). A: Methylene blue stained section showed stratified hyperplastic growth. B: Methylene blue stained section showed mosaic hyperplastic growth (red arrowheads).

1.1.3 Fish muscle fiber is an important tool for aging research

Sarcopenia stand for the muscle fiber number continue to decrease with aging of human. As well, it is the relationship between the muscle fiber number and the age of the person, where the number of muscle fibers decreases rapidly from 50 years old (Fig. 1-5) (Lexell et al., 1988). Now-a-days, the prevalence of sarcopenia is tremendously elevated with corresponds to the increasing world's population. Indeed, almost 87 million persons in the United States will be over the age of 65 by the year 2050 (Federal Interagency Forum on Aging-Related Statistics, 2008). This noticeable increase in elderly persons and following diagnoses of sarcopenia cause a momentous challenge to aging researchers (Clark and Manini, 2008, 2010), while the want for efficient treatments for these conditions will be of great significance. In this regard, the most important model organism for aging research is the laboratory mouse where displayed marked senescence as it increases in age. On the other hand, the many intricate variables between mouse and teleosts (e.g., terrestrial vs. aquatic, actinopterygian vs. tetrapod) engender straight coincidence of these species arduous at best. Inspite of many biological differences between mammal and teleost fish, the major issue may be the complementary growth potentials of these two groups of animals. In case of mammals like mouse, attain a definitive size following puberty that is they exhibit an characteristic growth level (Lui and Baron, 2011) and are termed "determinate" growth (Lincoln et al., 1982).

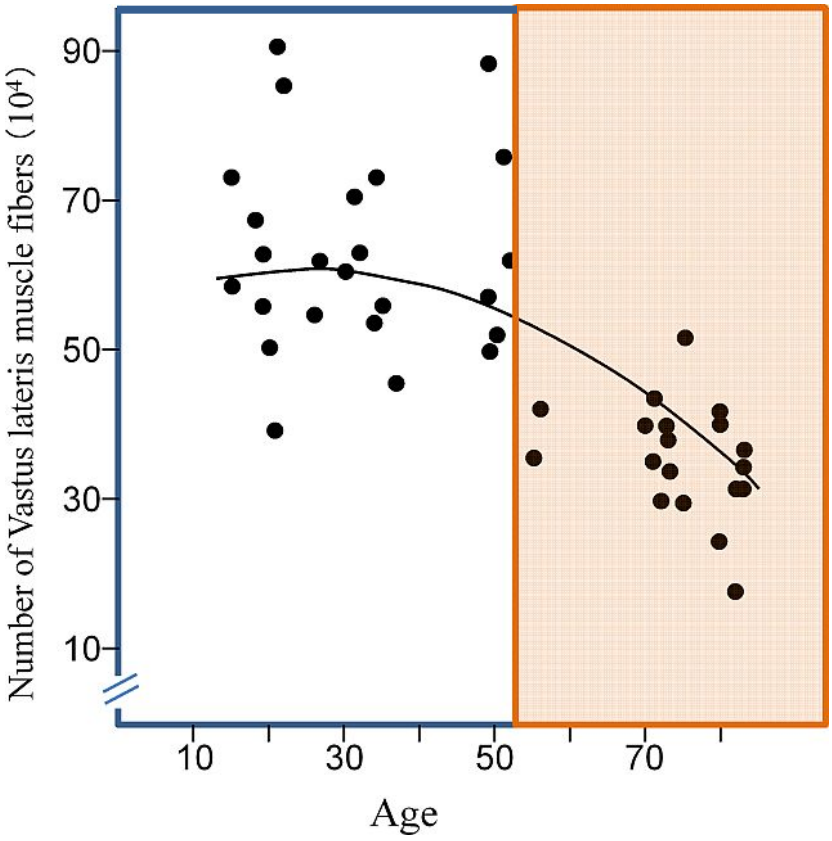

Fig. 1-5: Diagram showing sarcopenia i.e. reduction of muscle mass associated with aging. Sarcopenia indicates the relationship between the muscle fiber number and the age of the person, but the number of muscle fibers decreases rapidly from 50 years old and so on (Adapted from Lexell et al.,1988)

Though, many fish does not show such a strict growth level (Sebens, 1987), as they grow throughout their life cycle, even though at a slower rate. This type of growth, namely "indeterminate," is very frequent among the many fish species. On the contrary to mammals, the indeterminate growth was found to be observing in most fishes is highly slanted by external factors (Sebens, 1987). This pattern is one in which age is extremely analytical of body size (Lincoln et al., 1982), a difference with determinate growth. Regarding skeletal muscle, a tissue with rich metabolic activity representing a large amount of vertebrate muscle mass, the distinction between terrestrial mammals and aquatic piscines persist. Even more fascinatingly, how do fishes carry on to accumulate new muscle fibers even into old age? The answers to such questions are critical for attenuating the effects of sarcopenia in the increasingly large aged population. Lastly, as result of sarcopenia, increasing the risk of diabetes and obesity due to a decrease in basal

metabolism, or overall deterioration in the quality of life due to loss of motor function which are the biggest concerned in world. Therefore, in the therapy of Sarcopenia, it has been attracting attention to study on fish muscle fibers continue to increase lifelong. But the mechanism of fish muscle to grow lifelong does not known at all.

1.1.4 Salient feature of Myosin heavy chain

The most important function of skeletal muscle is to produce movement and maintain postures using the arrangement and protection of an extremely particular mptile machinery. Suchlike contractile functions are essentially ascribed to the main muscle proteins viz. myosin and actin. Myosin is the eukaryotic motor protein that generates the force for cellular movements. It comprises of heavy chains which are involved in locomotion, and light chains which are involved in regulation. A wide spectrum of various myosin motors has recently been categorized into 24 classes based on a phylogenetic analysis of the myosin heavy chains (Foth, et al., 2006). Myosins control many basic cellular functions including protein transport, cell division, apoptosis, adhesion, migration, phagocytosis, exocytosis and contraction (Krendel and Mooseker, 2005). Myosin II is the most important motor protein that regulates actomyosin contractility in both muscle and nonmuscle cells. It is also a hexameric protein complex composed of a pair of myosin heavy chains, a pair of essential and a pair of regulatory light chains (Fig. 1-5A). The myosin heavy chain consists of a conserved motor domain at the N-terminus that drives the movement along actin filaments and a neck domain that serves as a rigid lever arm to generate movement of the motor domain along with a non-conserved helical coiled-coil domain at the C-terminus, which terminates with a short non-helical tail. The light chains bind to the neck domain where the essential light chains provide structural integrity to the motor domain and the regulatory light chains regulate the myosin II ATPase activity (Clark et al., 2007). Likely, myosin II accumulates into bipolar filaments through electrostatic interactions between the coiled-coil domains (Fig. 1-5B) (Hostetter, et al. 2004). The motor domains on each end of the filament associate with oppositely oriented actin filaments. By pulling actin filaments together, myosin II generates cortical tension. Numerous MYHs have been found in vertebrates, which showed strong amino acid sequence homology with each other. The regions that vary in amino acid sequence between MYHs are largely restricted 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.

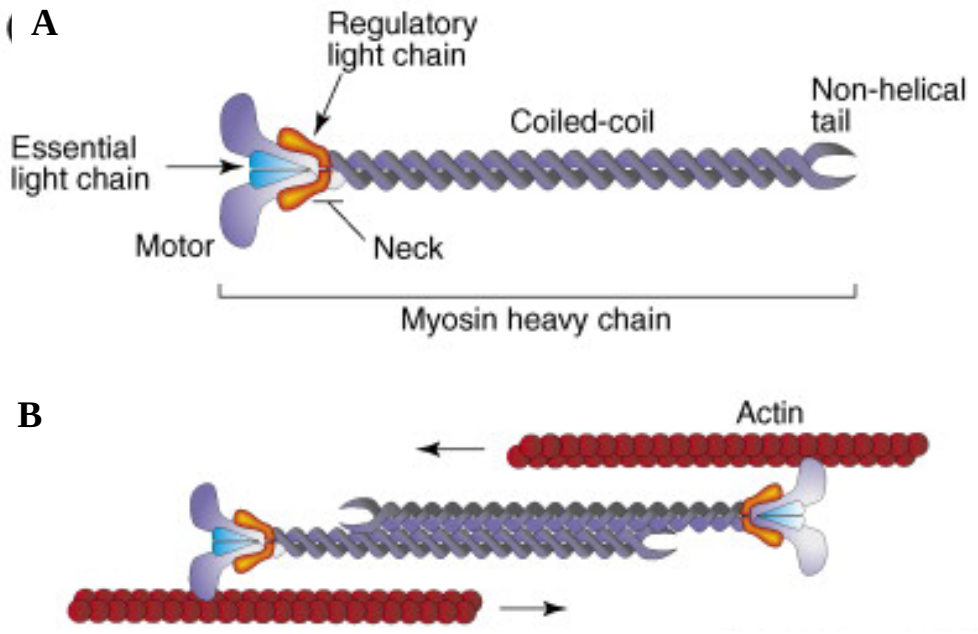

Fig. 1-6: Diagram illustrating the sarcomeric myosin molecule (Adapted from Clark et al., 2007). **(A)** Schematic diagram of a myosin II monomer, depicting the light and heavy chains. The different parts of the heavy chain, including the motor, neck, coiled-coil and nonhelical domains, are indicated. **(B)** Myosin II self-assembles into bipolar filaments through interactions of the C-terminus; the N-terminus binds to actin filaments. Activation of the myosin II motor domain leads to the pulling of actin filaments (in the direction of the arrows) to induce cortical tension.

1.1.5 Expression pattern of myosin heavy chain gene in teleost

Fish are documented to have highly conserved MYH multigene family, although *MYH*s are much more than their higher vertebrate counterparts (Watabe, 2002; Ikeda et al., 2010). Besides, a significant number of *MYH*s expressed in fish during developmental stages resulting in changes of the composition of muscle-fiber type (Liang et al., 2008; Watabe and Ikeda, 2006). Conversly, a minimum of 11 sarcomeric *MYH*s have been recognized in case of mammals, and their development-dependent and tissue-specific expressions contribute to the formation of various muscles such as fast, slow, embryonic and neonatal with different functional properties (Weiss et al., 1999). For instance, 29 fast-type *MYH*s have been recognized in common carp *Cyprinus carpio* (Kikuchi et al., 1999) and 20 sarcomeric *MYH*s in torafugu *Takifugu rubripes* (Ikeda et al., 2007). Previous studies on expressed *MYH*s have been identified as different muscle specific expression that persist throughout the life cycle in fish (Mascarello et al.,

1995; Johanston et al., 1998). *MYH*s are found to be expressed and uttered successively during development in fish. Amongst *MYH* family members, expression patterns of multiple *MYH*s has been characterized during development of particular fish species such as rainbow trout *Onchorhynchus mykiss* (Rescan et al., 2001), zebrafish *Danio rerio* (Bryson-Richardson et al., 2005; Steinbacher et al., 2007; Elworthy et al., 2008; Ahammad et al., 2015), common carp *Cyprinus carpio* (Ennion et al., 1999; Nihei et al., 2006), medaka *Oryzias latipes* (Ono et al., 2006) and torafugu *Takifugu rubripes* (Ikeda et al., 2007; Akolkar et al., 2010; Asaduzzaman et al., 2011, 2013). In rainbow trout, fast-type *MYH* was expressed initially in adaxial cells prior to the expression of slow-type *MYH* (Rescan et al, 2001). Likely, adaxial cells were found to express fast-type MYH, *myhc4*, well before their radial migration in zebrafish (Bryson-Richardson et al., 2005). Elworthy et al. (2008) also showed that adaxial cells are the muscle pioneer cell that initially expresses slow-type MHY, *smyhc1* and migrates towards the lateral surface of zebrafish myotome. Three genes namely *mMYHemb1* in embryos and *mMYHL1* and *mMYHL2* in larvae are predominantly expressed during their development stage of medaka, (Ono et al., 2006). Recently, Jonston 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. In common carp, two fast-type *MYH*s named *MYHemb1* and *MYHemb2* and their homolog *Egg22* and *Egg24* have been categorized during embryonic and larval development (Ennion et al., 1999; Nihei et al., 2006; Ikeda et al., 2010). In torafugu, $MYH_{M743\text{-}2}$ is found be predominantly expressed in fast muscle fibers whereas $MYH_{M86\text{-}2}$ was found to be expressed in slow muscle fibers during embryonic and larval development (Asaduzzzaman et al., 2013). In zebrafish, torafugu *MYHM72528-1* was also found to express both slow and fast muscle in relation with hyperplastic and indeterminate muscle growth (Ahammad et al., 2015). These base line informations point out the *MYH*s demonstrated strictly regulated temporal and spatial expression patterns mediating fish muscle development.

1.1.6 Torafugu and zebrafish are experimental models

The pufferfish known as the Fugu, Torafugu (*Takifugu rubripes*) is very popular in Japan and has a higher market price (Kikuchi et al., 2006). The genome of this species was the first vertebrate genome to be sequenced and made publicly available after the human genome. (Aparicio et al., 2002). Fugu genomes are identified as smallest genome size, only around 400 Mb (approximately an eighth the size of the human genome) due to its compact size (Aparicio et al., 2002). Thefore it became popular as appealing "model" vertebrates for genomic analysis in some way because,

their genomes have essentially the short introns and less percentage of repetitive sequences as other vertebrates (Brenner et al. 1993; Aparicio et al., 2002; Hedges and Kumar, 2002), making this organism an perfect tool for comparative genomics as well as for evolutionary research (Elgar et al., 1996; Venkatesh et al., 2000). The accessibility of the *Takifugu* genome sequence significantly make easy the identification of *MYH*s (Ikeda et al., 2007) and comprehensive expression analysis confirmed their transient and stable expression pattern regulated in tissue- and development-specific manners (Asaduzzaman et al., 2013; Ahammad et al., 2015). However, the regulatory mechanisms involved in the spatio-temporal expression of *MYH*s are mostly unknown.

The zebrafish (*Danio rerio*) is a full-fledged model organism for discovery in developmental biology. At present, the zebrafish has been exploited in studies of muscle genomics, somite formation, myotome development, muscle fiber specification, and muscle differentiation (Sparrow et al., 2008; Buckingham and Vincent, 2009). There are numerous salient features of zebrafish that acquire an ideal experimental model (Fig. 1-6). Therefore, male and female of zebrafish are easily been distinguished due to their external characters (Fig. 1-6A). In experimental purposes, one pair of zebrafish can produce around 30-50 embryos per spawning, likely 2-3 times a week, all over the year depending on the level of maturity (Fig. 1-6A). Zebrafish eggs are translucent and reasonably large (~0.7 mm in diameter) contrast to other teleost of an alike size (Fig. 1-6A). Embryogenesis and organogenesis are quickly develop (Fig. 1-6A). For the period of the first 24 hours of development, the embryos are completely clear, permitting the sighting of developing organs, even deep inside living embryos. The generation time is also comparatively shortened dictating 3-4 months (Fig. 1-6A). Therefore, it was promising to use zebrafish for gene expression study that generated a huge number of stable transgenic lines with a variety of phenotypes (Fig. 1-6B). Consequently, zebrafish is an profitable animal models to create transgenic lines and are simply available to transient reporter analysis for quantification. In addition to its larger size (~8–10 cm), this species exhibits true indeterminate growth, augmenting musculature in the postlarval period through both nascent myofiber recruitment (hyperplasia) (Biga and Goetz, 2006). On top, transgenic zebrafish that conveyed enhanced green fluorescence protein (EGFP) in the control of particular gene are valuable tools for next to cell motility, spatio-temporal gene expression patterns, and dissecting *cis*-regulatory elements in vivo (Long et al., 1997; Motoike et al., 2000; Zhang and Rodaway, 2007).

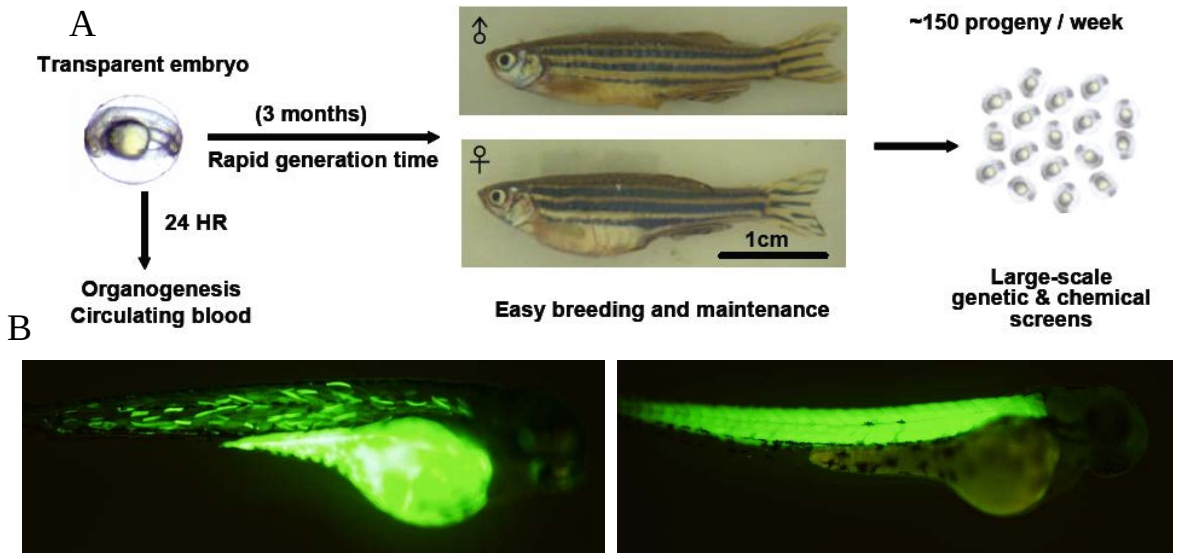

Fig. 1-7: Diagram represents the utilization of zebrafish as model system. (A) General features of zebrafish that make them excellent laboratory models (Adapted from White et al., 2008; Lee et al., 2010; Asaduzzaman et al., 2013). (B) Transgenic approaches in zebrafish embryos are possible (Adapted from Asaduzzaman et al., 2013).

1.6.7 Mechanisms underlying expression of transcription factors in teleost

Myogenic regulatory factors (MRFs) are basic helix-loop-helix (bHLH) transcription factors that regulate myogenesis 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 (Perry and Rudnick, 2000). During myogenesis, the transcription factors Myf-5 and MyoD are essential for the primary determination of the myogenic lineage. In zebrafish, after knockdown of *Myf-5* morpholino has been shown to induce defects in myogenesis (Chen and Tsai, 2002). As well, expression of *myogenin* and *Mrf4* is prompted during myoblast differentiation (Rhodes and Konieczny, 1989; Wright et al., 1989; Miner and Wold, 1990; Edmondson and Olson, 1993; Pownall et al., 2002), and *myogenin* and Mrf4 perhaps have joint functions with MyoD and Myf-5 as transcription factor regulators for the activation of muscle contractile protein genes (Lassar et al., 1991). In addition to MRFs, the paired domain and homeobox-containing transcription factors paired box gene 3 (*Pax3*) and 7 (*Pax7*) control different phases of myogenesis in the embryo and adult. Further 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). An additional important transcription factor hypothesized as having a regulatory role in fiber type-specific gene expression is the NFAT (nuclear factor of activated T cells) (Chin et al., 1998). On the other hand, MEF2 is also activated by NFAT (nuclear factor of activated T cells) (Wu et al., 2000, 2001). So far, transcriptional regulatory regions have been recorded for a few numbers of skeletal muscle-specific *MYH*s in fish.

1.2 Objectives of the study

Many teleost fish represents indeterminate growth and exhibit good models for understanding vertebrate myogenesis, while myotomal muscle fibers are to be found in distinct layer and their development plasticity is continued from early developmental stages to adult stage, (Weatherley and Gill, 1987; Johnston et al., 2003). A significant outcome of indeterminate growth is that the number of fibres needs to increase throughout the life cycle as the muscle mass increases, involving a prolonged period of postembryonic hyperplasia (Greer-Walker, 1970; Stickland, 1983). Indeterminate muscle growth of fish has been mostly examined with zebrafish particularly alarm on the specific genes expression and *cis*-regulatory elements that control the gene expression in connection with myotube production (Johnston et al., 2011). Myosin is the eukaryotic motor proteins that generate the force cellular movements which necessitates various *MYHs* expression during muscle development. In contrast to mammalian skeletal muscles, vertebrate *MYH*s genes are as well expressed in a chronological way during muscle development. The tiger puffer genome has been anticipated as a model organism for fast categorization of vertebrate genes due to its negligible size among vertebrates (Brenner et al., 1993). Thus, the association of the *MYH*s in the fugu genome database has been examined, enlightening that it contains 20 sarcomeric *MYH*s which shaped four clusters on the genome (Watabe and Ikeda, 2006; Ikeda et al., 2007).

Their comprehensive expression analysis of torafugu *MYH*s demonstrated their complicated expression outlines harmonized in tissue specific and hyperplastic manner (Akolkar et al., 2010; Asaduzzaman et al., 2013; Ahammad et al., 2015). Albeit a substantial achievement has been consummated in explicating the molecular genetics covers the developmentally regulated MYHs expression in mammals, miniature is now kenned concerning the molecular mechanisms regarding hyperplastic MYHs expression in fish is mostly remain unknown. Therefore, present study was undertaken in order to comprehend the following concerns in torafugu by using zebrafish embryos for transient and transgenic analysis. Whether or not 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 distinguishing factors regulating the expression of *MYH*s in different muscles in fish to provide clear insight into how these muscles are developed? Whether the similar or dissimilar transcription factors regulate the different fiber type-specific expression of *MYH*s in the same species of fish? Whether or not the *MYH*s are involved in secondary muscle development and indeterminate growth by hyperplasia in fish and how are their expressions regulated transcriptionally? The areas involved in the present study are as follows-

i. To identify the 5'-flanking sequence of torafugu $MYH_{M2528\text{-}1}$ whether could induce *MYH* expression in zebrafish embryos by using *in vivo* reporter assay;

ii. To demonstrate $MYH_{M2528\text{-}1}$ role in the activation of gene expression in neonatal muscle fibers produced by muscle hyperplasia

iii. To identifying cis-acting elements responsible for its expression in relation to indeterminate muscle growth in zebrafish

1.3 Outline of the thesis

This thesis is composed of a general introduction (Chapter 1), two research chapters (Chapters 2 and 3) and a general discussion (Chapter 4). Chapter 1 briefly reviewed the structural arrangement of teleost myotomal muscle, muscle fiber type specification of Teleost, vertebrate skeletal muscle myogenesis- have to highlight sarcopenia, distinctive feature of Myosin heavy chain, expressional regulation of Myosin heavy chain in fish, experimental model vertebrates: Torafugu and zebrafish and mechanisms underlying transcriptional regulatory gene expression in fish. The research (Chapter 2-3) pursued a step-wise approach. The first approach (Chapter 2) documented the regulation of gene expression mediating indeterminate muscle growth in teleosts. Here, we investigate the 2,100 bp to examine the spatial and temporal regulation by using transgenic and transient expression techniques through an *in vivo* reporter assay in zebrafish. The results of this chapter clearly demonstrated promoter involved in teleost indeterminate muscle growth and conserved between large (torafugu) and small (zebrafish) fish. As well, several transcription factors including NFAT may be involved in promoter activity. The next step (Chapter 3) was multiple *cis*-elements in the 5'-flanking region of slow and fast-type of torafugu, $MYH_{M2528\text{-}1}$, function in the transcriptional regulation of its expression. In this chapter, we described the MyoD, Pax3 and MEF2 mediated hyperplastic expression in relation with indeterminate muscle growth in teleost. We further discovered that NFAT binding

elements play a key role in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression. In the general discussion (Chapter 4), major conclusions of the previous chapters were incorporated and concluded, strength and weaknesses of the pursued approaches were outlined and suggestions for further studies were given.

CHAPTER 2

Regulation of gene expression mediating indeterminate muscle growth in teleosts

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Abstract

Teleosts are unique amongst vertebrates due to their indeterminate muscle growth, i.e., continued production of neonatal muscle fibers until death. However, the molecular mechanism(s) underlying this property is unknown. Here, we focused on the torafugu (*Takifugu rubripes*) myosin heavy chain gene, $MYH_{M2528\text{-}1}$, which is specifically expressed in neonatal muscle fibers produced by indeterminate muscle growth. We examined the flanking region of $MYH_{M2528\text{-}1}$ through an *in vivo* reporter assay using zebrafish (*Danio rerio*) and identified a 2100bp 5'-flanking sequence that contained sufficient promoter activity to allow specific gene expression. The effects of enhanced promoter activity were observed at the outer region of the fast muscle and the dorsal edge of slow muscle inzebrafish larvae. At the juvenile stage, the promoter was specifically activated in small diameter muscle fibers scattered throughout fast muscleand in slow muscle near the septum separating slow and fast muscles. This spatio-temporal promoter activity overlapped with known myogenic zones involved in teleost indeterminate muscle growth. A deletion mutant analysis revealed that the-2100~-600bp 5'-flanking sequence of $MYH_{M2528\text{-}1}$ is essential for promoter activity. This region contains putative binding sites for several representative myogenesis-related transcription factors and nuclear factor of activated T-cell (NFAT), a transcription activator involved in regeneration of mammalian adult skeletal muscle. A significant reduction in the promoter activity of the $MYH_{M2528\text{-}1}$ deletion constructs was observed in accordance with a reduction in the number of these binding sites, suggesting the involvement of specific transcription factors in indeterminate muscle growth.

2.1 Introduction

Skeletal muscle comprises a large portion of the mass of vertebrates. The bulk of vertebrate growth, therefore, depends on an increase in skeletal muscle mass during a species's lifespan. Skeletal muscles display two types of growth patterns, hypertrophy and hyperplasia. The former is characterized by an increase in the size of existing muscle fibers (myocytes) while the latter results in an increase in the number of muscle fibers. In mammals, however, the contribution of hyperplasia to muscle growth is quite small in the postnatal period and further growth primarily depends on hypertrophy (Rowe &Goldspink, 1969), resulting in limited growth and a definitive body size. Production of new muscle fibers after the neonatal period in mammals is observed only in the regeneration of injured muscle (reviewed by Dhawan & Rando, 2005). Conversely, in teleost skeletal muscles, both hyperplasia and hypertrophy occur throughout the organism's lifespan (Mommsen, 2001; Johnston et al., 2001). This 'indeterminate' muscle growth provides teleosts with a vast potential to increase their body size, in some cases from a few milligrams to a hundred kilograms (Johnston, 2001). In addition, the degree of muscle growth is highly variable amongst teleost species, resulting in a magnitude of differences in adult body size. Thus, the indeterminate production of muscle fibers is an important phenomenon that dictates teleost growth.

The mechanisms underlying indeterminate muscle growth are also relevant to understanding age-related muscular disorders in mammals. Mammalian skeletal muscles undergo marked senescence called sarcopenia, the loss of muscle mass due to an age-associated decrease in the number and size of muscle fibers. Sarcopenia in humans is a severe problem globally, associated with increasing age (Clark & Manini, 2008, 2010). Various studies using mammalian models such as mice and rats have identified several genes involved in senescence, with relevant genetic modifications resulting in a marked delay in the senescence of various organs, including skeletal muscle (Froehlich et al., 2013a). However, these modified mammalian models merely display a delay in senescence and eventually still achieve a severe sarcopenia phenotype. In this regard, teleosts are an attractive model because the naturally negligible senescence of their skeletal muscles presents a potentially powerful system through which a method to inhibit sarcopenia can be discovered (Froehlich et al., 2013a). However, the molecular mechanisms responsible for the indeterminate muscle growth found in teleosts are completely unknown.

Myosin heavy chain (MYH) is a subunit of myosin, the most abundant protein in skeletal muscle. Many isoforms of MYH exist, and their variation in expression is the primary determinant of the differential physiological properties of muscle fibers, such as slow vs. fast twitch (Weiss et al., 1999). The expression patterns of MYH isoforms also change along with the progression of growth stages such as embryonic, neonatal, and adult (Berg et al., 2001). Interestingly, several studies have reported that new muscle fibers (neonatal muscle fibers) produced by post-embryonic hyperplasia express specific MYH isoforms in common carp (Ennion et al., 1995), sea bream (Rowlerson et al., 1997), and zebrafish (Rowlerson et al., 1997). Our previous studies also identified a MYH gene (*MYH*), $MYH_{M2528\text{-}1}$, in the torafugu (*Takifugu rubripes*) and is expressed in neonateal muscle fibers produced by muscle hyperplasia at the larval, juvenile and adult stages (Akolkar et al., 2010; Asaduzzaman et al., 2013). This recent study (Asaduzzaman et al., 2013) reported that at larval stages of torafugu, $MYH_{M2528\text{-}1}$ is expressed in dorsal and ventral extreme regionby stratified hyperplasia and subsequently the generation of fast fiber with small diameter by mosaic hyperplasia in a sequential fashion at the juvenile stages (Asaduzzaman et al., 2013). In the case of adult *T. rubrifes*, both fast and slow muscles expressed different *MYH*s among which $MYH_{M2528\text{-}1}$ was expressed in juvenile fast fibres with relatively small diameters and slow fibres with relatively large diameters, implying that this gene is associated with muscle hyperplasia (Akolkar et al., 2010).Therefore, the better understanding the mechanisms of $MYH_{M2528\text{-}1}$ transcription regulation will provide a basis to dissect the molecular network involved in the production of neonatal muscle fibers through hyperplasia in fish. To the best of our knowledge, there are no published report on the isolation and characterization of 5'-flanking region of any *MYH* functioning in the formation of neonatal muscle fibers to understand the molecular mechanism responsible for its transcriptional regulation in fish to date. Therefore, in the present study, we examined the torafugu$MYH_{M2528\text{-}1}$ promoter via an *in vivo* reporter assay using zebrafish and demonstrated its role in the activation of gene expression specifically in neonatal muscle fibers produced by larval and post-larval muscle hyperplasia amongst different fish species.

2.2 Materials and methods

2.2.1. Experimental fish

The dorsal fin of an adult torafugu *T. rubripes* (body mass ~ 1 kg) was used for the extraction of genomic DNA. Torafugu larvae at 10 days post-fertilization (dpf) were supplied by the Oshima Fishery Hatchery Co., Ltd, Nagasaki, Japan.Adult zebrafish *D. rerio* were raised at the zebrafish rearing facility at the Department of Aquatic Bioscience,

The University of Tokyo. Fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark in small aquariums supplied with continuous freshwater in a recirculatory system. Spawning of zebrafish was carried out by placing a pair of males with a pair of females. Embryos and larvae were maintained at 28°C as described previously (Westerfield, 1995).

2.2.2. Determination of the transcription start site of *MYH*$_{M2528\text{-}1}$

The 5'-flanking sequence of *MYH*$_{M2528\text{-}1}$ retrieved from the Ensemble Fugu Genomic Database (FUGU4.0). A GeneRacer kit (Invitrogen, Carlsbad, CA, USA) was used to define the transcriptional start site of *MYH*$_{M2528\text{-}1}$ through RNA ligase-mediated rapid amplification of 5'cDNA ends (RLM-RACE). Total RNA from torafugu larvae at 10 dpf was extracted with ISOGEN solution (Nippon Gene, Tokyo, Japan) and treated with calf intestinal phosphatase (CIP). Dephosphorylated RNA was then decapped using tobacco acid pyrophosphatase (TAP) and ligated with GeneRacer RNA oligomers according to the manufacturer's instructions. The 5'cDNA end was amplified by PCR using a *MYH*$_{M2528\text{-}1}$-specific reverse primer (Table 2-1) with the GeneRacer 5'primer (Invitrogen) included in the kit. Amplified 5'cDNA fragments were subcloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced with an ABI 3100 genetic analyzer (Applied Biosystems, CA, USA) after labeling with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Screening of putative transcription binding sites in the 5'-flanking region was carried out by Genomatix (http://www.genomatix.de), the TFSEARCH program (www.cbrc.jp/research/db/TFSEARCH.html) and the manual identification.

2.2.3. Construction of reporter vector

To identify the minimal promoter, a series of 5'deletion constructs within the 5,000 bp flanking region from the start codon of *MYH*$_{M2528\text{-}1}$ was generated by PCR using torafugu genomic DNA as a template. A total nine forward primers and one reverse primer were designed (Table 2-1) to amplify the 5,000 bp and a series of 5'distal deletion regions. All of these amplified PCR products were inserted individually into the *Bam*HI-*Xho*I site of the Tol2-EGFP reporter vector named pT2AL200R150G (courtesy of Dr. Koichi Kawakami) by In-Fusion Advantage PCR Cloning (Clontech, CA, USA). The plasmid DNA for microinjection was isolated from each deletion construct using the GenElute^{TM} plasmid mini-prep kit (Sigma-Aldrich, Steinheim, USA). These constructs were named as P5000, P4000, P3000, P2500, P2300, P2100, P1500, P1000, and P600, where the numbers refer to the nucleotide positions

upstream of the $MYH_{M2528\text{-}1}$ start codon (Fig. 1). All constructs contained a $MYH_{M2528\text{-}1}$ flanking region conjugated with the EGFP reporter gene, SV40 polyA signal, and Tol2 transposase binding sites.

Table 2-1: Neocletide sequence of oligonucleotide primers for various experiments

Experiment	Primer Name	Neolceotide Sequence (5′……..…3′)	Length (bp)
Generation of deletion constructs	P5000F	TTGGGCCCGGCTCGAGGACCAGTGCGGAGGGACAGA	36
	P4000F	TTGGGCCCGGCTCGAGTGTAGGATTCCAACCTATTTGGTCT	41
	P3000F	TTGGGCCCGGCTCGAGCCACTGTGATACTGAATAATAAGGG	41
	P2500F	TTGGGCCCGGCTCGAGGAAGATACATAAGATGTCCCTGACT	41
	P2300F	TTGGGCCCGGCTCGAGCAAGGGGCAAACCTCCAGCACT	38
	P2100F	TTGGGCCCGGCTCGAGGCTGCAGAATTAGTGTGAATGACATAT	43
	P1500F	TTGGGCCCGGCTCGAGATTATATCTTGCTGGTAATCACTTCAGAATTTC	49
	P1000F	TTGGGCCCGGCTCGAGTACTGCCAAAGAGCATAAAAGAGATGC	43
	P600F	TTGGGCCCGGCTCGAGTGCACAAGCGCAGCACAACCC	37
	Reverse	GGCGACCGGTGGATCCGATGGCTCTTTACTGCACAAGCACAAA	43
Transcriptional start site	$MYH_{M2528\text{-}1}$ specific reverse primer	GAA GAT TTC ATC GTC TTT CAC AGT G	25
Insert Check PCR	Tol2 Insert F	TTTACGTCGCCGTCCAGCTC	20
	Tol2 Insert R	TGGGCTTGCTGAAGGTAGGG	20

2.2.4. *In vivo* reporter assay

Each EGFP reporter construct was diluted to 100 ngµL^{-1}with sterile distilled water containing 0.025% phenol red and injected into fertilized zebrafish eggs at one- to two-cell stages. Microinjection was performed using the IM300 microinjector (Narishige, Tokyo, Japan). Embryos were reared at 28°C and EGFP expression patterns were observed under a MVX10 macro-zoom microscope (Olympus, Tokyo, Japan) and a FV1000 confocal laser scanning microscope (Olympus). Fish were anesthetized with 0.6 µM tricainemethane-sulfonate (Sigma-Aldrich) to inhibit movement during observation.

2.2.5. Generation of theTg:$MYH_{M2528\text{-}1}$:EGFP transgenic line

RNA encoding a functional Tol2 transposase enzyme was transcribed *in vitro* from pCS-TP vector (Kawakami et al., 2000, 2004). The Tol2-based construct (P2100) was co-injected with transposase mRNA into one- to two-cell stage embryos. At 8-10 h post-microinjection, embryos were subjected to Transient Embryonic Excision Assay (TEEA) to confirm whether the excision occurred properly. Following a successful TEEA, the EGFP-positive embryos were

identified at 1 dpf and then transferred into the fish rearing unit at the Department of Aquatic Bioscience, The University of Tokyo, until sexual maturity. Individual founder 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 intercrossing F1 fish.

2.2.6. Immunohistochemistry and antibodies

For immunohistochemistry, larvae and juveniles of the transgenic line were fixed with 4% PFA in Tris-buffered saline (25 mMTris-HCl[pH 7.4], 137 mMNaCl, 2.7 mMKCl) containing 0.1% Tween 20 (TBSTw) overnight at 4°C. Fixed samples were washed with TBSTw and blocked using a 1.5% blocking reagent (Roche Applied Science) in TBSTw. Transverse sections were prepared at a thickness of 16 µm with a cryostat Tissue-Tek Cryo3 (Sakura Finetech, Tokyo, Japan) at -20°C before the first immunoreactions. The primary antibodies used in this study were as follows: the EGFP antibody (Clontech, CA, USA) was used at a dilution of 1:1,000 in the blocking solution, and F310 (fast muscle fiber-specific), F59 (slow muscle fiber-specific), and MF20 (striated muscle-specific) antibodies supplied by Developmental Studies Hybridoma Bank (Iowa city, IA, USA) at 1:20. Immunoreaction with the primary antibody was performed overnight at 4°C. After incubation, embryos were washed with TBSTw and labeled with the secondary antibodies, anti-mouse IgG Alexa Fluor 555 and anti-rabbit IgG Alexa Fluor 488 (Invitrogen), at a dilution of 1:250 overnight at 4°C. The embryos were subsequently washed with TBSTw and labeled with DAPI (Invitrogen). EGFP was also observed using staining of the tissue section with 5 mM BODIPY TR ceramide (Molecular Probes) at room temperature for 30 min. The signals in the cryosection samples were viewed using an Olympus FluoView1000 confocal laser scanning microscope (Olympus).

2.2.7. Cyclopamine treatment

Tg:MYH$_{M2528\text{-}1}$:EGFP embryos at two- to four-cell stages were transferred into 2, 4, 6, and 10µg mL^{-1} cyclopamine solution (Wako, Otsu, Japan) containing 0.2%, 0.4%, 0.6% and 1.0% ethanol and incubated at 28.5°C. Control embryos were developed in 0.02, 0.04, 0.06 and 0.1% ethanol, respectively, containing water without cyclopamine. EGFP expression in cyclopamine-treated and untreated control embryos was observed from 2–3dpf using a MVX10 macro-zoom microscope (Olympus)

2.2.8. Data and statistical analyses

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

2.3 Results

2.3.1. Determination of the *MYH*$_{M2528\text{-}1}$ transcription start site

We first determined the transcription start site to characterize the 5'flanking region of *MYH*$_{M2528\text{-}1}$. Based on the 5'RACE, the transcription start site was determined to be 502 bp from the start codon (Fig. 2-1). Exons 1 and 2 are transcribed as an untranslated region, and the start codon is located in exon3 (Fig.2-1).

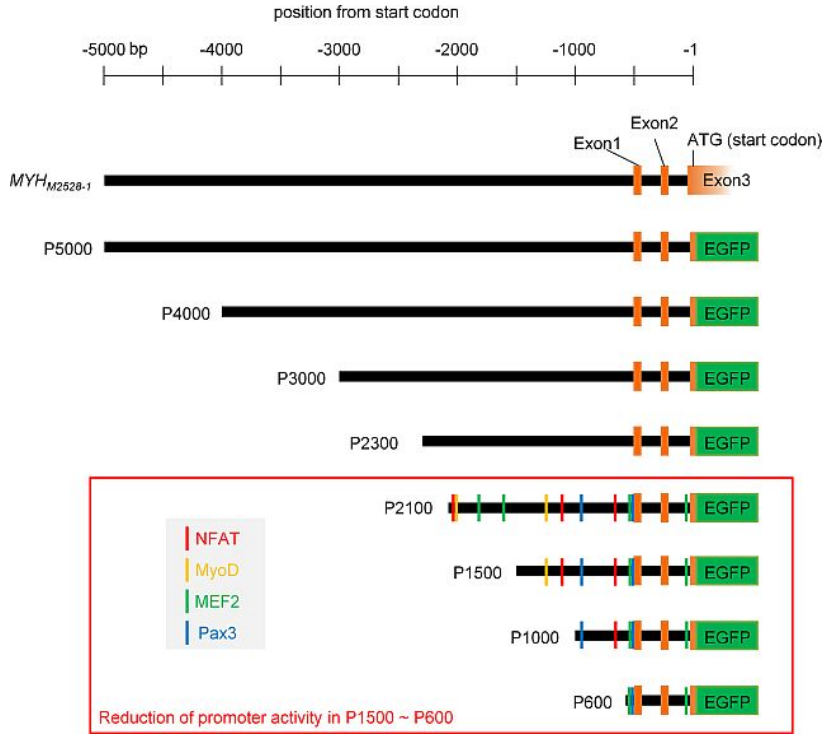

Fig. 2-1. Schematic of reporter constructs used in this study where a genomic region located 5000 bp upstream from the start codon of torafugu *MYH*$_{M2528\text{-}1}$ amplified by PCR and inserted into an EGFP reporter vector (P5000). Serial deletion constructs containing 4000 (P4000), 3000 (P3000), 2500 (P2500), 2300 (P2300), 2100 (P2100), 1600 (P1600), 1000 (P1000), and 600 (P600) bpof the 5'-flanking sequence from the start codon, respectively, were also constructed. All constructs contain the 5' untranslated region of *MYH*$_{M2528\text{-}1}$ encoded by exons one, two, and part of three. Putative binding sites of representative muscle differentiation-related transcription factors, nuclear factor of activated T-cell (NFAT), MyoD, myocyte enhancer element 2 (MEF2), and paired box 3 (Pax3), are plotted on the P2100 ~ P600 constructs.

2.3.2. The 2100bp 5′-flanking region of torafugu *MYH*$_{M2528\text{-}1}$ is the minimal promoter necessary to induce gene expression in zebrafish skeletal muscle

To map the minimal promoter necessary to induce expression of *MYH*$_{M2528\text{-}1}$, a series of 5'distal deletion constructs of the flanking sequence of *MYH*$_{M2528\text{-}1}$, namely P5000, P4000, P3000, P2500, P2300, P2100, P1500, P1000, and P600, respectively, were microinjected into fertilized eggs of zebrafishas an *in vivo* reporter assay. For P5000, ~ 97% of the injected embryos displayed strong EGFP expression along skeletal muscle fibers (Fig. 2-2A-B and Fig. 2-3A). The EGFP expression was detected at 1 dpf and continued to be expressed in the whole myotomal region of larva at 2dpf (Fig. 2-4A-E). At 3 dpf, EGFP was found to be expressed in the craniofacial and myotomal muscles (Fig. 2-2K, Fig. 2-4E). In the myotomal region, both slow and fast muscle fibers expressed EGFP (Fig. 2-5).

Although almost the same expression pattern was observed in zebrafish larvae injected with P5000 through P600 (Fig. 2-2), 5'flanking regions shorter than 2,100bp resulted in a significant reduction in EGFP expressionin the myotomal muscle fibers (Fig. 2-2M-R, Fig. 2-4). As shown in Fig. 2-3A, the ratio of EGFP-expressing larvae per injected larvae of P1500 ~ P600 was significantly reduced compare with those of P5000 ~ P2100 injected larvae. In addition, the number of EGFP-positive muscle fibers per embryo was markedly reduced in P1500 ~ P600 injected larvae (Fig. 2-3B, Fig. 2-4F-N). This finding indicates that *cis*-acting element(s) in between -2,100 to -600 bpin the 5'flanking region participate in the promoter activity of *MYH*$_{M2528\text{-}1}$. We screened for the putative binding sites of several myogenesis-related transcription factors within the 2100bp sequence and found two MyoD, four myocyte enhancer element 2 (MEF2), two paired box 3 (Pax3), and three nuclear factor of activated T-cell (NFAT) binding sites (Fig. 2-1). The reduced promoter activity (Fig. 2-3) for the successive deletion of 5' flanking region from P2100 indicating that any or combination of these *cis*-elements might be involved in the transcriptional regulation of *MYH*$_{M2528\text{-}1}$.

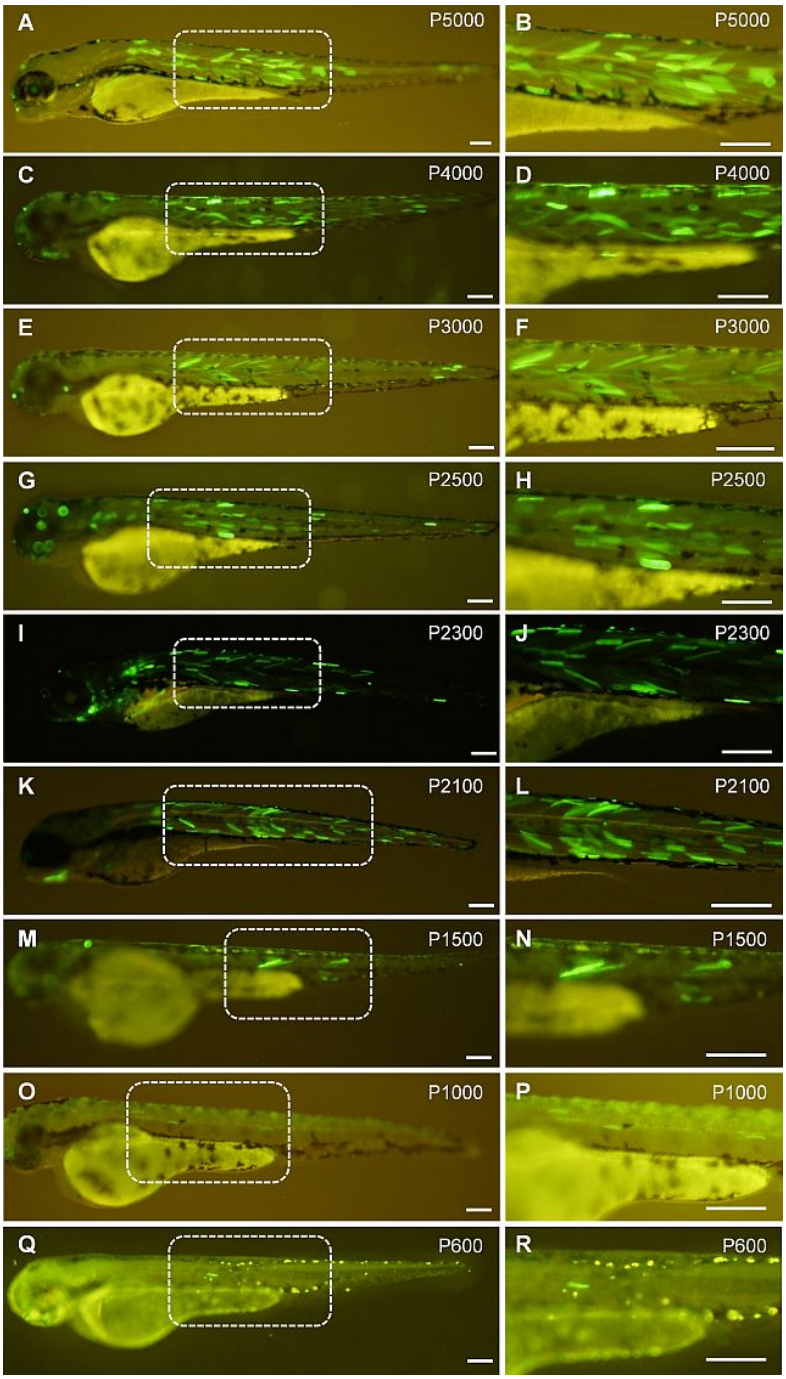

Fig. 2-2. EGFP expression in zebrafish larvae injected with *MYH*$_{M2528\text{-}1}$ reporter constructs. A-R, EGFP expression in myotomal skeletal muscle of zebrafish embryos injected with reporter constructs, P5000 (A,B), P4000 (C,D), P3000 (E,F), P2500 (G,H), P2300 (I,J), P2100 (K,L), P1500 (M,N), P1000 (O,P) and P600 (Q,R). Head to left in all panels. Right side panels are the magnified view of boxed areas of the left side panels. Scale bars: 100 μm.

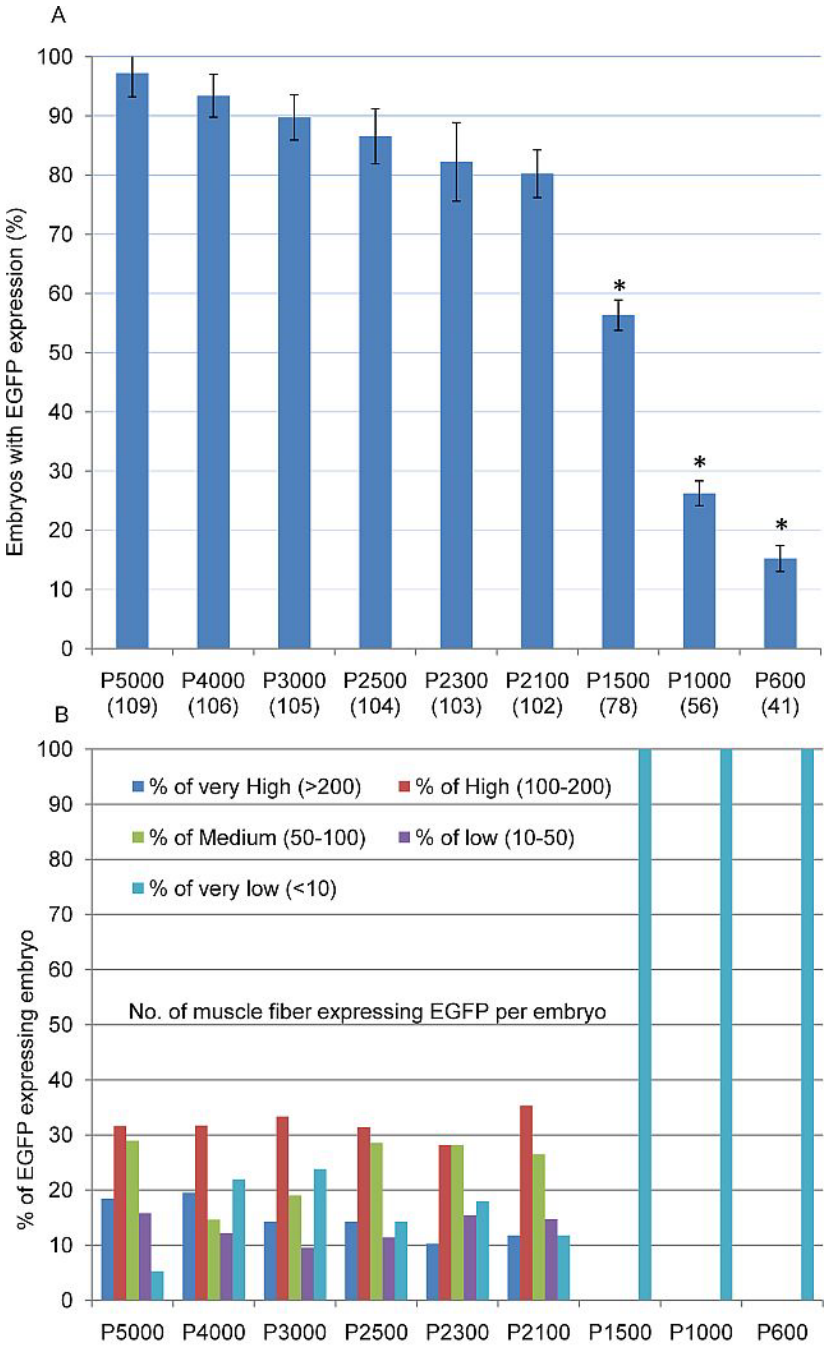

Fig. 2-3. Bar graph showing the promoter activity of the reporter constructs where EGFP expression was observed in the myotomal compartments of zebrafish larvae at 3dpf. A, percentages of larvae that express EGFPin the myotomal compartments following injection of each construct and the total number of larva injected with each construct are shown in parentheses. Differences are significant via ANOVA followed by Tukey's test at **P*<0.05.B,the number of EGFP-expressing muscle fibers per larva in different constructs. Fiber numbers per larva are categorized into 5 classes (very high ~ very low) by colors.

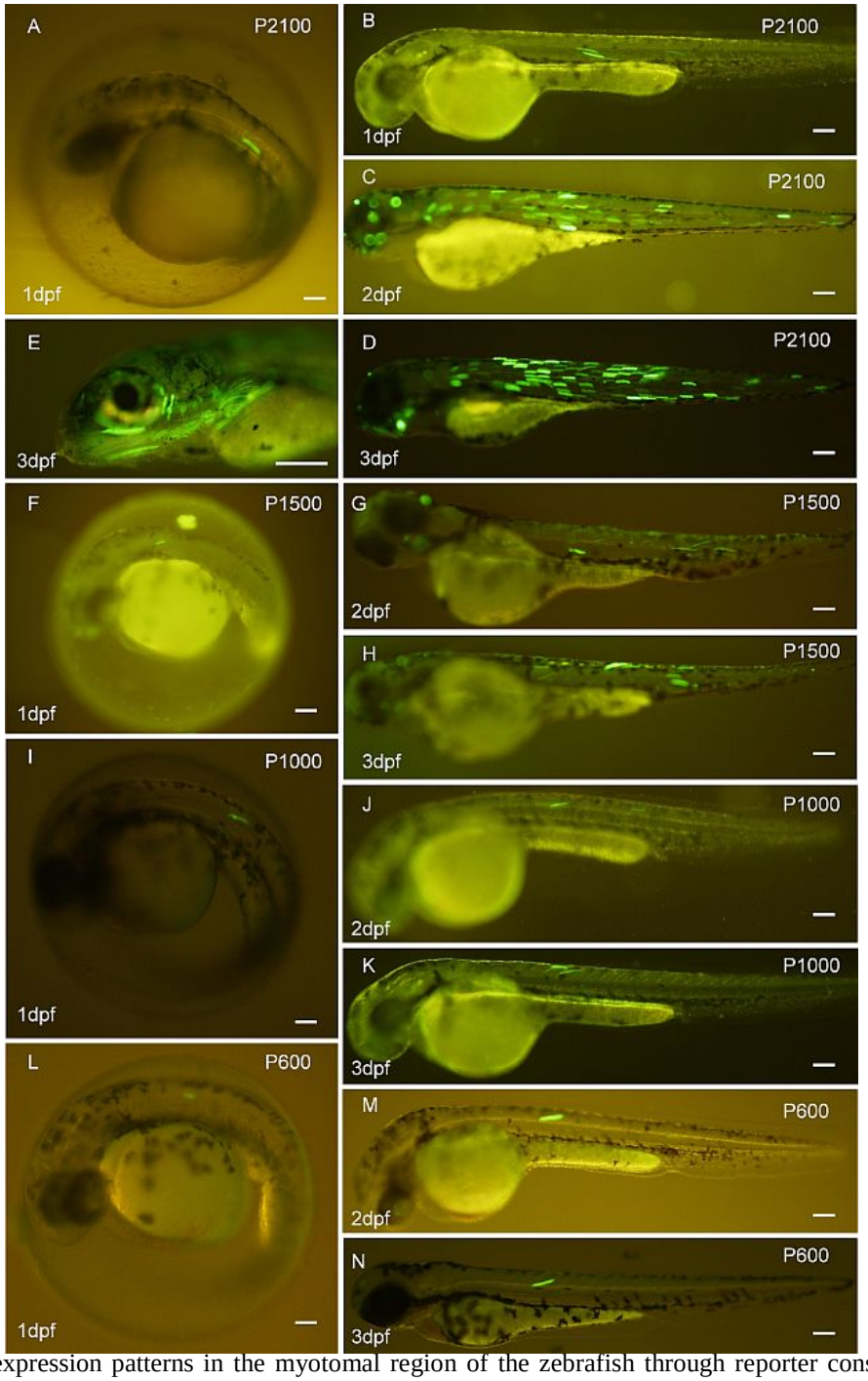

Fig. 2-4. EGFP expression patterns in the myotomal region of the zebrafish through reporter construct (A-N). A zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C; 3dpf, D) injected with the P2100 reporter construct. D-E, EGFP expression patterns in craniofacial muscles in zebrafish larvae at 3 dpf. As well, zebrafish embryo (1dpf, F), (1dpf, I), (1dpf, L) and larva (2dpf,G; 3dpf,H), (2dpf, J; 3dpf,K), (2dpf,M; 3dpf,N) injected with P1500, P1000, P600 reporter constructs, respectively. Scale bars: 100 μm.

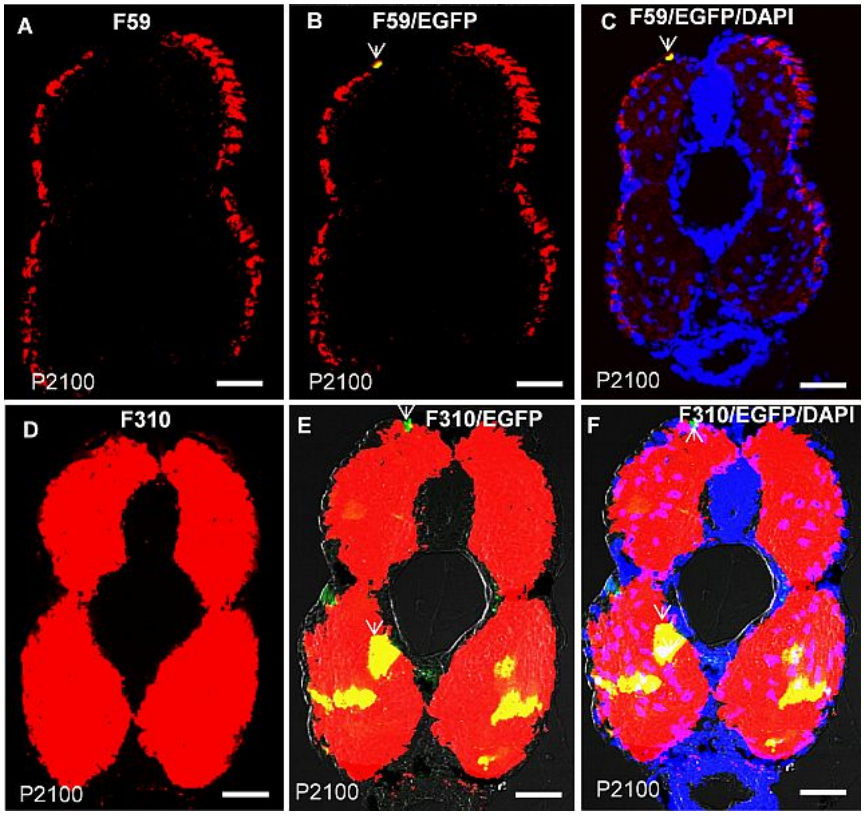

Fig. 2-5. Immunohistochemistry localizing EGFP expression in both fast and slow muscle fibers of P2100-injected zebrafishlarvae. A-C, slow muscle fibers expressing EGFP as reacted with F59 antibody (A, F59 antibody view; B, F59 with EGFP; and C, F59 and EGFP with DAPI) in a P2100-injected larva at 3 dpf. D-F,fast muscle fibers expressing EGFP as reacted with F310 antibody in a P2100-injected larva at 3 dpf (D, F310 antibody stained view; E, F310 with EGFP; F, F310 and EGFP with DAPI). Scale bars: 50 μm.

2.3.3. *MYH*$_{M2528\text{-}1}$ promoter activity in zebrafish larvae

To confirm the specific activity of the *MYH*$_{M2528\text{-}1}$ promoter in post-embryonic muscle hyperplasia, a stable transgenic zebrafish line, Tg:MYH$_{M2528\text{-}1}$:EGFP, was established using the P2100 construct and temporal and spatial EGFP expression was analyzed. Similar to the *in vivo* reporter assay (Fig. S2), Tg:MYH$_{M2528\text{-}1}$:EGFP displayed EGFP expression at 1 dpf (Fig. 2-6A-B). After hatching at 2dpf, EGFP continued to be expressed in the whole myotomal region of the larva (Fig. 2-6C). Observation with a fluorescent microscope also confirmed that Tg:MYH$_{M2528\text{-}1}$:EGFP expressed EGFP in the whole myotomal region (Fig. 2-6D) and craniofacial muscles (Fig. 2-6E) at 3 dpf.

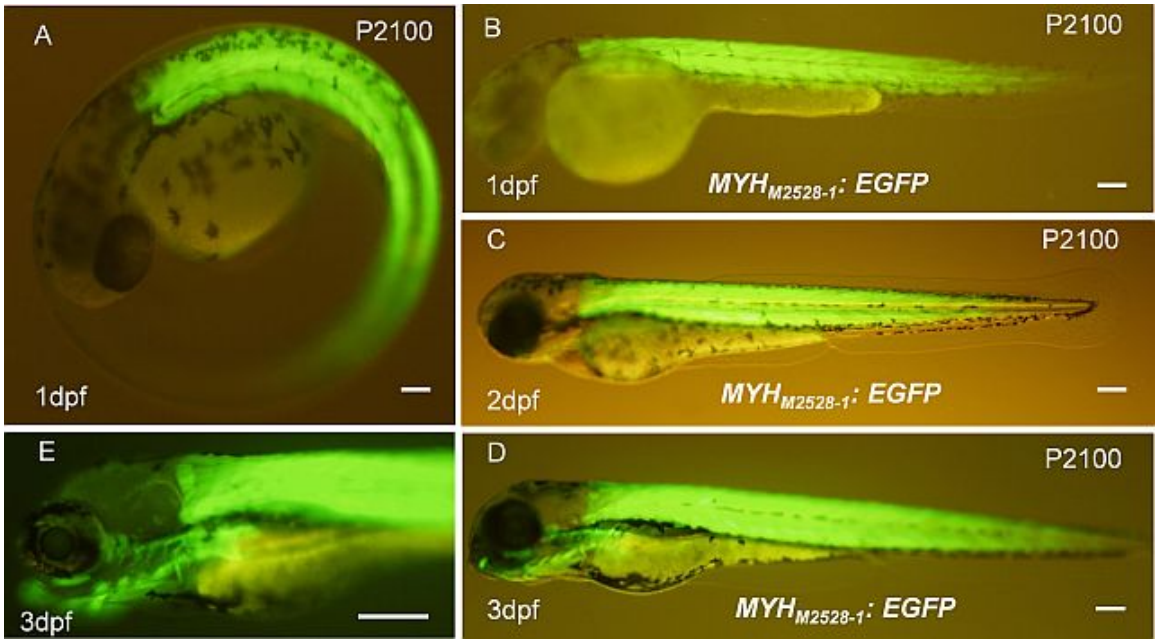

Fig. 2-6. Expression patterns of EGFP in the*Tg:MYH$_{M2528-1}$:EGFP* stable transgenic line embryo and larva. A stable line was established by injecting the P2100 construct. EGFP expression was observed in the whole myotomalregion at 1dpf (A,B), 2dpf (C), and 3dpf (D). In addition to myotomal skeletal muscle, the craniofacial muscle also expresses EGFP at 3dpf (E). Scale bars: 100 μm.

In teleosts, slow and fast muscle fibers occupy distinct regions of the myotomal skeletal muscle (Bone, 1978). Fast muscle fibers comprise the deep portion of the myotome, which makes up most of the trunk musculature. Slow muscle fibers are segregated into a wedge-shaped region of the myotome surface at the lateral end of the horizontal myoseptum. Furthermore, fast and slow muscles have distinct developmental lineages (Devoto et al., 1996) and patterns of post-embryonic growth. In fast muscles of teleost larva, the apical surface region actively produces neonatal muscle fibers via hyperplasia (Rowlerson et al., 1994). On the other hand, the slow muscles of teleost larva form a monolayer at the myotome surface, and the dorsal and ventral edge of the layer produce neonatal muscle fibers via hyperplasia (Baressi et al., 2001).

Figure 2-7 shows the immunohistochemistry of Tg:MYH$_{M2528-1}$:EGFP at the larval stage (3dpf) to clarify the type and position of EGFP-positive muscle fibers. Slow muscle fibers at this stage formed a monolayer at surface of myotome (Fig. 2-7A-C) as described above. Most slow muscle fibers were EGFP negative, but a fiber at the dorsal edge expressed EGFP (Fig. 2-7D-F, arrowhead). In fast muscle, many muscle fibers expressed EGFP, but their

distribution was predominant in the apical surface region (Fig. 2-7G-I). In Fig. 2-7K and 2-7L, the slow muscle fibers at the dorsal edge (not stained in red by F310 antibody) also expressed EGFP, as indicated by arrowheads. Consequently, the distribution of EGFP-expressing muscle fibers was consistent with the above-mentioned myogenic zone via hyperplasia at the teleost larval stage.

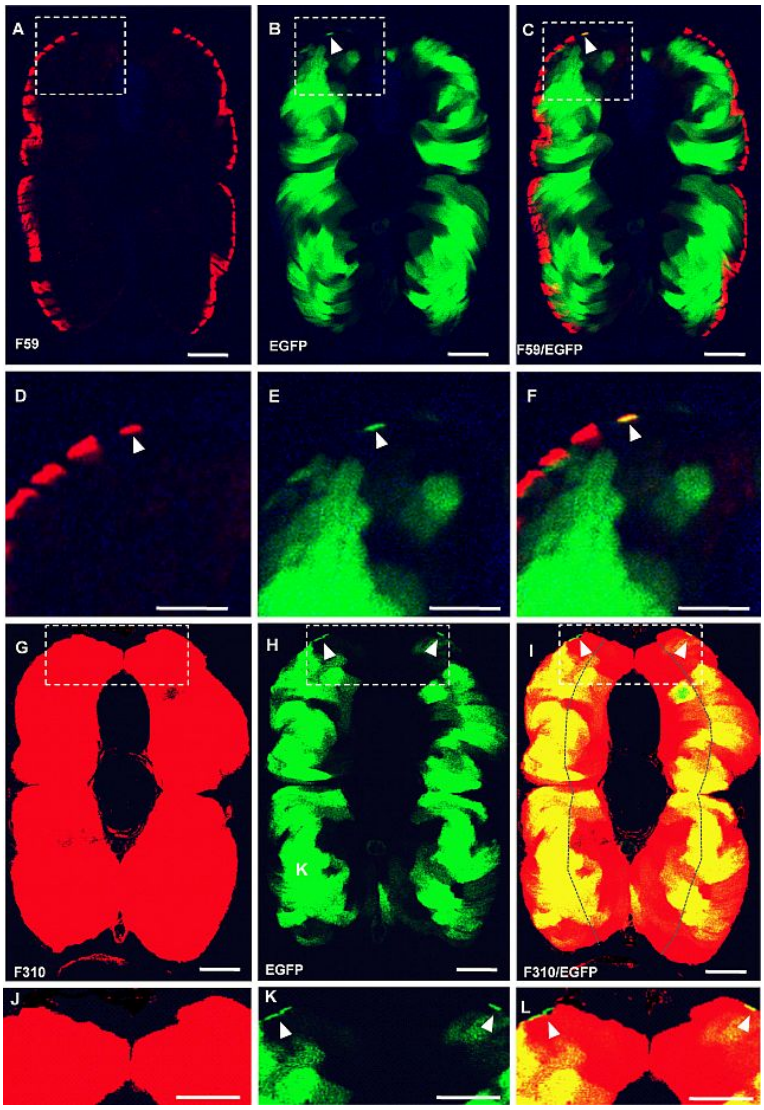

Fig. 2-7. Localization of EGFP-expressing myotomal muscle fibers in *Tg:MYH$_{M2528\text{-}1}$:EGFP* larvae (A-L). A-F, transverse section of myotomal region of *Tg:MYH$_{M2528\text{-}1}$:EGFP* at 3dpf. A-C, slow muscle fibers were stained red with a F59 antibody (A, F59 antibody; B, EGFP; C, merged view). D-F, the magnified view of boxed areas of panels A-C. G-L, fast muscle fibers were identified with a F310 antibody (G, F310 antibody; H, EGFP; I, merged view). The dotted line in panel I indicates the middle of the myotomal compartment. EGFP-positive fast muscle fibers are predominantly distributed in the outer region. J-L, the magnified view of boxed areas of panels G-I. EGFP-expressing slow muscle fibers are indicated by arrowheads in panels A-F,H,I,K,L Scale bars: 50 μm

2.3.4. *MYH*$_{M2528\text{-}1}$ promoter activity in juvenile and adult zebrafish

After the larval stage, fast and slow muscles of teleosts still show different hyperplastic growth patterns. In fast muscle, myogenic cells scattered amongst existing muscle fibers produce neonatal muscle fibers. This growth pattern is termed mosaic hyperplasia (Rowlerson et al., 1995; Rowlerson & Veggetti, 2001). On the other hand, in slow muscle, the myogenic region is positioned near the septum between slow and fast fibers and produces neonatal slow muscle fibers (Rowlerson et al., 1995). Figure 2-8A illustrates the distribution of slow and fast muscles in a transverse section of the teleost trunk. It should be noted that the duration of post-embryonic muscle hyperplasia also differs between fast and slow muscles. Recruitment of neonatal muscle fibers in fast muscle ceases at a definitive size (Weatherley, 1988; Johnston et al., 2001; Fernandes et al., 2005). In contrast, the number of slow muscle fibers continually increases with fish length (Johnston et al., 2001). In the case of zebrafish, post-larval muscle hyperplasia in fast muscle stops at approximately 17mm standard length (SL) (Fig. 2-8B) (Lee, 2010).

Immunohistochemistry was performed to clarify the position of EGFP-positive muscle fibers at post-larval (juvenile) and adult stages of Tg:MYH$_{M2528\text{-}1}$:EGFP. At the early juvenile stage (20 dpf, 10mm SL), EGFP expression in fast muscle was observed in small diameter fibers between large existing muscle fibers (Fig. 2-8C,D,G,H). In the lateralis slow and elector-depressor (ED) slow muscles of both the early and late juvenile stages of zebrafish development, EGFP expression was observed near the septum of slow and fast muscles (Fig. 2-8E-F). The distribution of EGFP-positive muscle fibers overlaps the above mentioned myogenic regions at the post-larval stage, as well as the expression pattern of endogenous *MYH*$_{M2528\text{-}1}$ in the torafugu (Akolkar et al., 2010; Asaduzzaman et al., 2013).

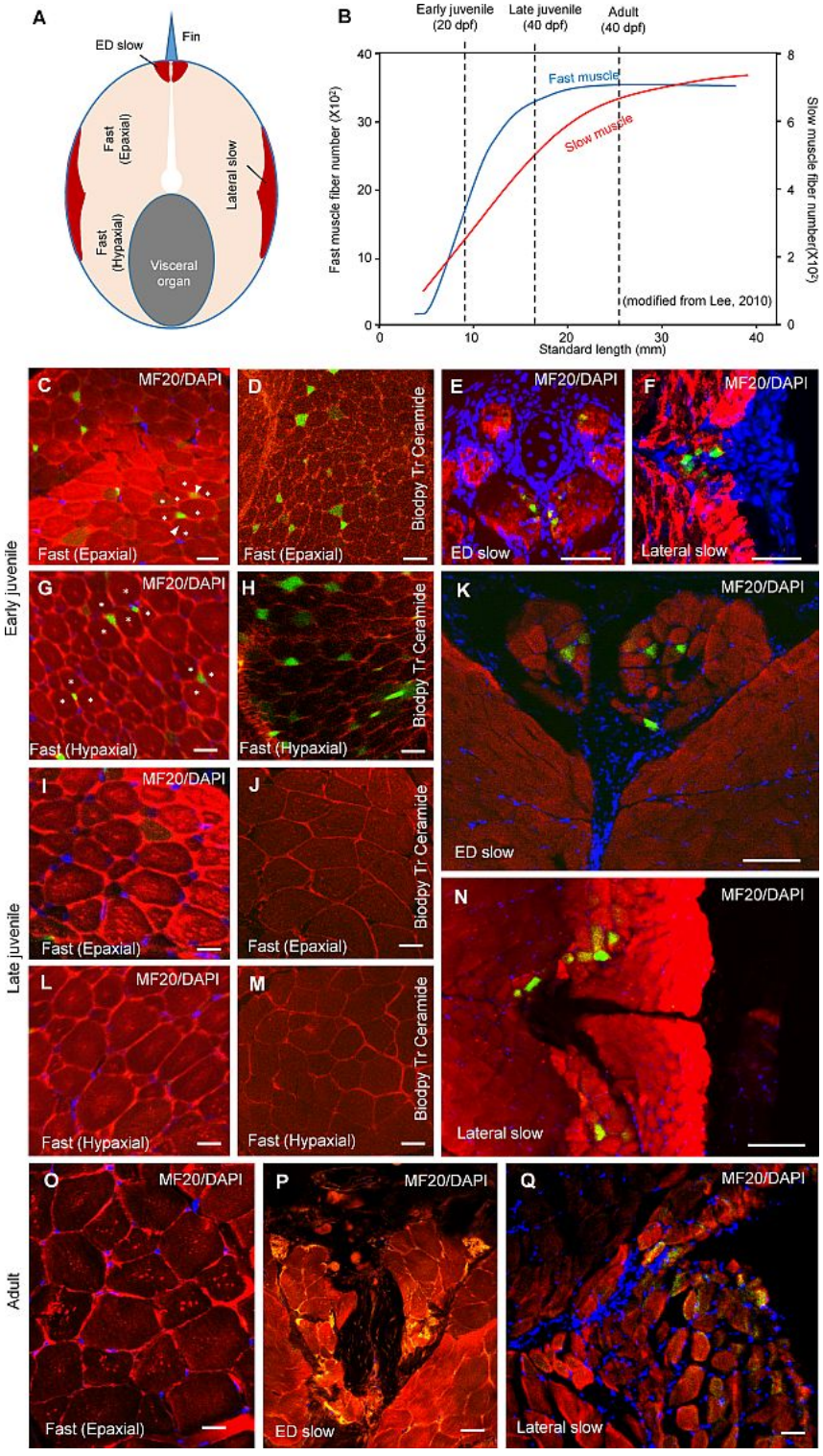

Fig. 2-8. Localization of EGFP-expressing myotomal muscle fibers in *Tg:MYH*$_{M2528\text{-}1}$*:EGFP* zebrafish (A-Q). (A) Cross-section of zebrafish. (B) body size-related increase in muscle fiber numbers in slow and fast muscles of zebrafish. Data cited from Lee (2010). (C-H) early, (I-N) late juvenile & (O-Q) adult stages. Scale bar: 50 μm

Consistent with the difference in the growth pattern between slow and fast muscles (Fig. 2-8B), promoter activity was not observed in fast muscle (Fig. 2-8I,J,L,M,O) but in slow muscle (Fig. 2-8K,N,P, Q) at the late juvenile (40 dpf, 17mm SL) and adult stage (60dpf, 25mm SL), respectively. Taken together with the EGFP expression pattern in the larvae and juveniles of Tg:$MYH_{M2528\text{-}1}$:EGFP, we concluded that 2100bp from the start codon of $MYH_{M2528\text{-}1}$ is enough functional promoter to allow gene expression specifically in neonatal muscle fibers produced by larval and post-larval muscle hyperplasia.

2.3.5. $MYH_{M2528\text{-}1}$ promoter works in secondary myogenesis

In zebrafish embryo, adaxial cells to be found adjacent to the axial midline which form superficial momolayer of slow muscle fibers through primary myogenesis (Devoto et al., 1996). Here, we observed that Tg:$MYH_{M2528\text{-}1}$:EGFP transgenic fish did not show any EGFP in the adaxial cells meaning that it is not involved in primary myogenesis (Fig. 2-9).

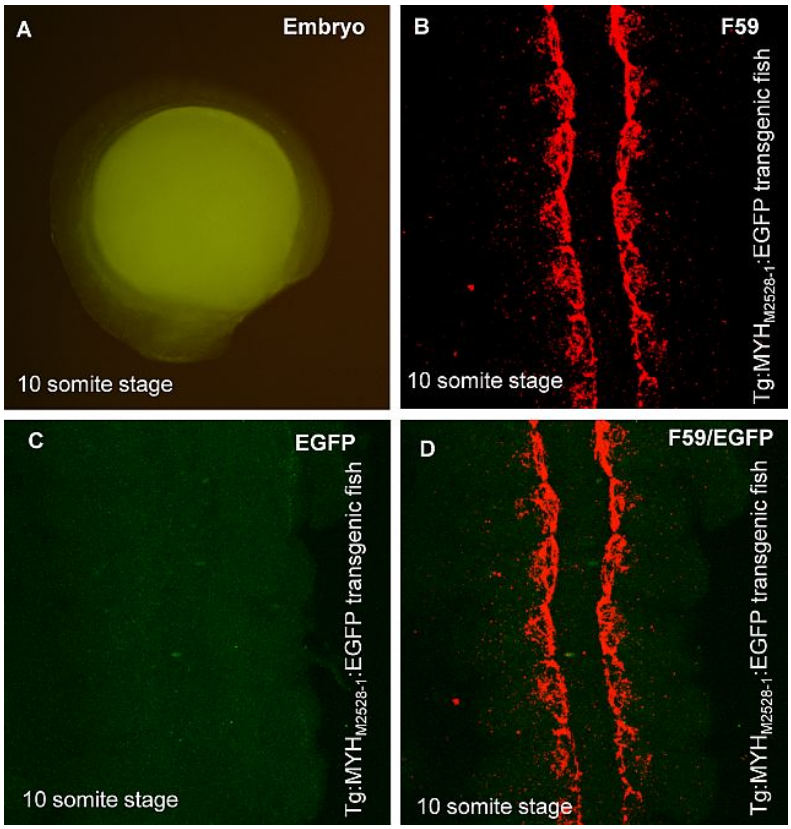

Fig. 2-9. Immunohistochemistry of Tg:$MYH_{M2528\text{-}1}$:EGFP transgenic embryo at 10 somite stage. A-D, adaxial cell does not expressed EGFP as not reacted with F59 antibody (A, 10 somite stage Tg:$MYH_{M2528\text{-}1}$:EGFP transgenic embryo; B, F59 antibody view; C, EGFP; and D, F59 and EGFP) in a at 10 somite stage embryo. Scale bars: 50 μm.

As well, Hedgehog (Hh) signaling is important for slow muscle development in vertebrates. In zebrafish embryos and larva, fast muscle fibers formed in absence of Hh signaling and slow muscle fibers are subdivided into two components, according to their dependence on Hh signaling (Elworthy et al., 2008). One component consists of primary slow muscle fibers that differentiated from adaxial cells at embryonic development and require Hh signaling. After formation of the primary slow muscle, secondary slow muscle fibers are produced without Hh signaling via post-embryonic muscle hyperplasia. We examined the Hh signaling dependency of EGFP-expressing muscle fibers in Tg:$MYH_{M2528\text{-}1}$:EGFP larvae to confirm whether the fibers are produced by secondary slow muscle formation. Cyclopamine is a well-known inhibitor of Hh signaling (Incardona et al., 1998; Chen et al., 2002). After treatment with cyclopamineat 2.0, 4.0, 6.0 and 10μg mL^{-1}, 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. 2-10D). Cyclopamine treated embryos showed very little change of EGFP expression compared to the control wild embryos treated with only ethanol (Fig. 2-10B,C,E,F). Depending on the dose, the rate of embryos showing EGFP expression in the muscle fibers varies from 47.39% (2.0 μg/ml), 47.09% (4.0 μg/ml), 44.88% (6.0 μg/ml) and 42.35% (10.0 μg/ml) compared to 49.71% in those without cyclopamine treatment (Fig. 2-10G).

Furthermore, immunohistochemistry was performed on the cross section by using 10μg mL^{-1} cyclopamine treated larvae to confirm its expression in the secondary slow muscle fibers. In 10μg mL^{-1} cyclopamine treated larvae, all primary muscle fibers along the lateral superficial regions disappeared and only secondary slow muscle fiber stained with F59 antibody (Fig. 2-10H,J). The EGFP expressing fiber was detected at only dorsal extreme of the myotome and is secondary slow muscle fiber (Fig. 2-10I,K). Finally, we can conclude that Tg:$MYH_{M2528\text{-}1}$:EGFP larvae treated with 10μg mL^{-1} cyclopamine showed EGFP in secondarily derived slow fiber, indicating that $MYH_{M2528\text{-}1}$ is involved in by secondary myogenesis.

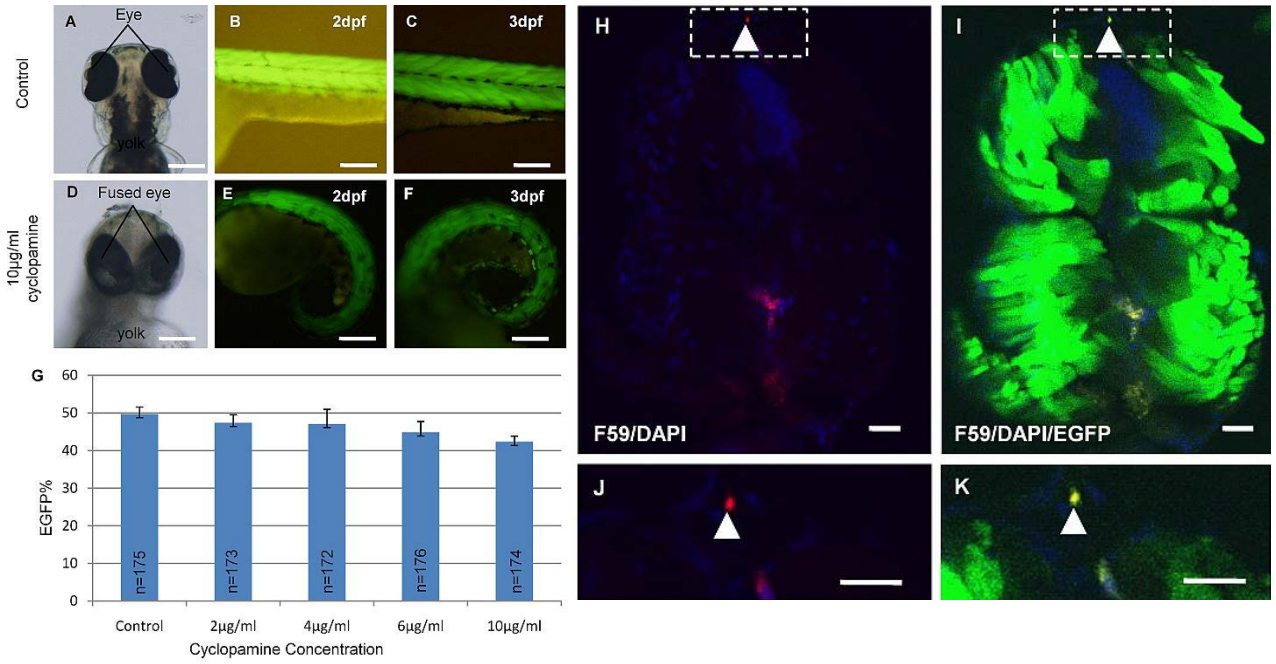

Fig. 2-10. Influence of Hedgehog (Hh) signaling pathway on the *MYH*$_{M2528\text{-}1}$ promoter and immunohistochemistry (A-K). Hh signaling was inhibited by cyclopamine treatment. Fertilized eggs of Tg:MYH$_{M2528\text{-}1}$:EGFP were transferred into 2 – 10.0μg mL^{-1}cyclopamine solution. Control embryos were developed without cyclopamine. A-F, morphology and EGFP expression of control (A-C) and 10.0μg mL^{-1} cyclopamine-treated (D-F) transgenic line. A,D, ventral views of head of zebrafish larvae at 3dpf. Inhibition of Hh signaling resulted in a fused-eye phenotype (D). EGFP expression in larvae at 2 dpf (B,E) and 3 dpf (C,F). Cyclopamine-treated larvae displayed a curled body phenotype resulting from Hh signal inhibition but maintained normal EGFP expression in fast and slow muscle fibers. G, the rate of zebrafish embryos expressing EGFP in the larvae at 3dpf with and without cyclopamine treatment. H-K, transverse section of myotomal region of 10.0μg mL^{-1} Cyclopamine-treated larvae of *Tg:MYH*$_{M2528\text{-}1}$*:EGFP* at 3dpf. H, all primary slow muscle fibers disapper and only secondary muscle fiber stained with F59 antibody. I, merged figure showed reacted with F59 antibody and EGFP expressing fibers are located at the dorsal extreme region. J-K, the magnified view of boxed areas of panels H-I. Scale bars: 100 μm.

2.4 Discussion

Skeletal muscles are not only important as the primary organ involved in mobility but are also being recognized as a prominent tissue with the capacity to influence aging and lifespan (Demontis et al., 2010, 2013). It has been shown

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