博士論文

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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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

A

Thesis submitted to

Graduate School of Agricultural and Life Sciences

The University of Tokyo

in partial fulfillment of the requirement

for the degree of

Doctor of Philosophy

in

Department of Aquatic Bioscience

By
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September, 2015

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 fibers which increase equally to the body axis even as fibers 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, mammalian skeletal muscle encloses a bundle of muscle fiber, facilitates single muscles in supplying durable, lower force contractions, in connection with primary fracture of movement. In contrast with mammals, teleost myotomal muscles are structurally segregated into different areas. In this regard, fast muscle fibers delineate major part in the myotome, whereas slow fibers are situated at lateral superficial to the myotome (Fig 1-1) (Van Raamsdonk et al., 1982; Sanger and Stoiber, 2001). These muscle fibers can be partitioned by the histochemical dissection of succinic dehydrogenase (SDHase) (Fig. 1-1). The fast muscle fibers comprise the greater part of myotomes which are typically larger in diameter than slow muscle fibers 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). They contract and fatigue faster than slow muscle fibers (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 lesser in diameter from fast muscle fibers (Greer-Walker and Pull, 1975; Altringham and Johnston, 1982).

Slow muscle fibers 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 those of the order Tetradontiformes, and termed erectors and depressor muscle (ED) (Winterbottom, 1974). Also, slow muscle fibers 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 andStoiber, 2001), and are providing 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, besides above major fiber types, some minor fibers 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 rim fibers (van Raamsdonk et al., 1980) have been investigated 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 provide intermediate contraction speeds and fatigue-resistance (Altringham et al., 1993; Altringham and Ellerby, 1999). Similarly to slow fibers, intermediate fibers have orientation parallel 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 regarded to contribute less significantly to the swimming of fish.

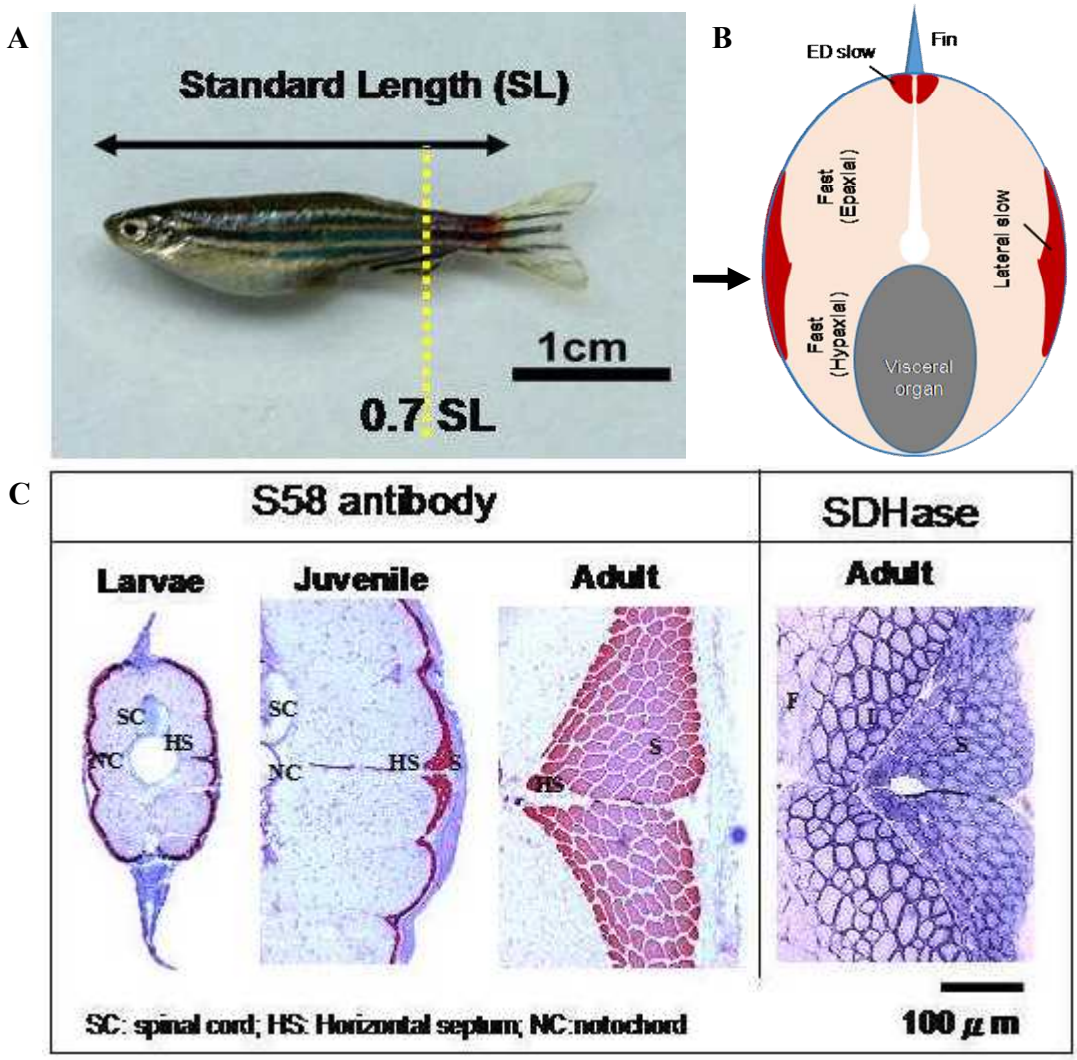

Fig. 1-1. Structural arrangement of teleost myotomal muscle (A-C). A, Typical size of teleost fish. (B) Typical view of slow and fast muscle in zebrafish. (C), Location of slow and fast muscle in zebrafish by S58 antibody and SDHase staining, respectively (Lee et al., 2010). Slow (S), Intermediate (I), 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. Hyperplasia means the increase in muscle fiber numbers, at each stage, the fundamental processes of myogenesis are included the steps of determination, proliferation, migration, fusion and differentiation (Fig. 1-2A). Also myoblast-myoblast, myoblast-myotube, and myoblast muscle fiber fusion events took place (Fig. 1-2B). In this regard, myogenesis necessary for indeterminate muscle growth, which includes the recruitment of newly form muscle fibers (hyperplasia) and development of existing muscle fibers through nuclear accretion (hypertrophy).

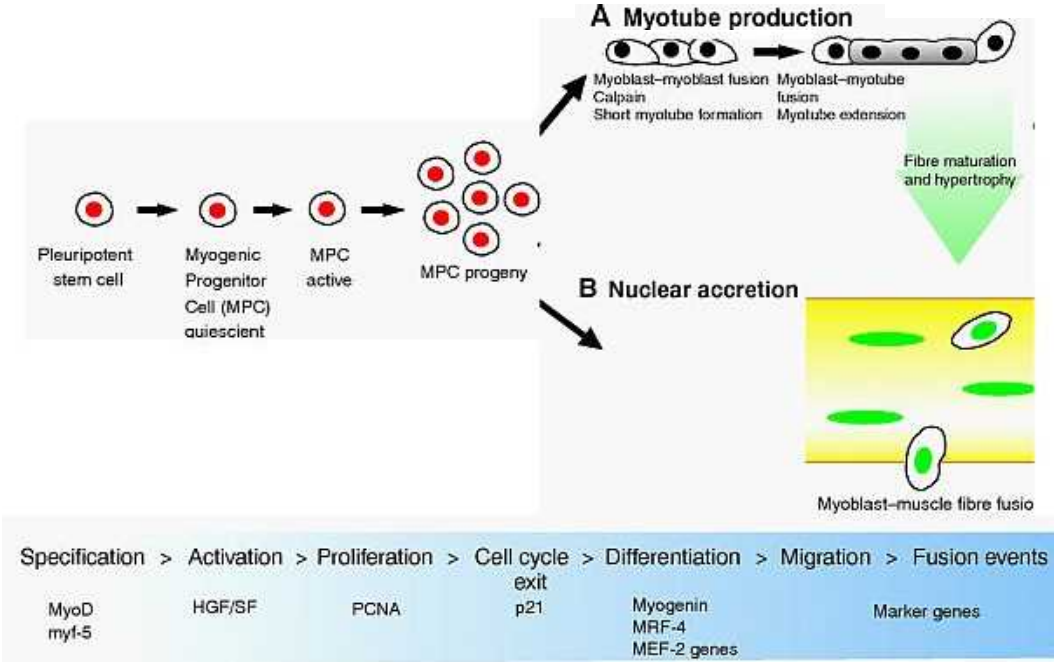

Fig. 1-2: A universal model for teleost myogenesis (Johnston et al., 2006). A, Myotubes are formed by specification, activation, proliferation differentiation process. B, at this stage of growth the MPC progeny can fuse with muscle fiber (myoblast-muscle fiber fusion) in the process of nuclear accretion.

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 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 fibers. Conversely, MH fabricates the bulk of muscle fibers 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, exceedingly lying on the axial structures

(Lassar and Munsterberg, 1996). The initiation of somitogenesis differs with direction of epiboly among various species and their progression continued where the paraxial mesoderm is distributed into membrane-bound blocks of cells (Kimmel et al., 1995; Stickney et al.,2000; Pourquié 2001). During vertebrate embryonic development, somites produced as of the presomitic mesoderm and fast form two main substructures, a ventral sclerotome section and a dorsally situated epithelial dermomyotome (Pourquié 2001). In the teleost somite, 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 frame work 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 fibers derived from two distinct subset of muscle pioneers i.e. adaxial cells and lateral presomitic cells in the somites. These two cell types, 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 floor plate intercedes by members of the hedgehog (Hh) 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 fibers 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 fibers between ECL and embryonic fast muscle fibers (Fig. 1-3D). Afterward, some cells from ECL move into the myotome while the slow muscle layer lead to develop fast muscle fibers in separate zones as SH (Fig. 1-3E). It has been recommended that ECL plays a vital role in additional successive postembryonic muscle fiber production even though this leftover to be confirmed.

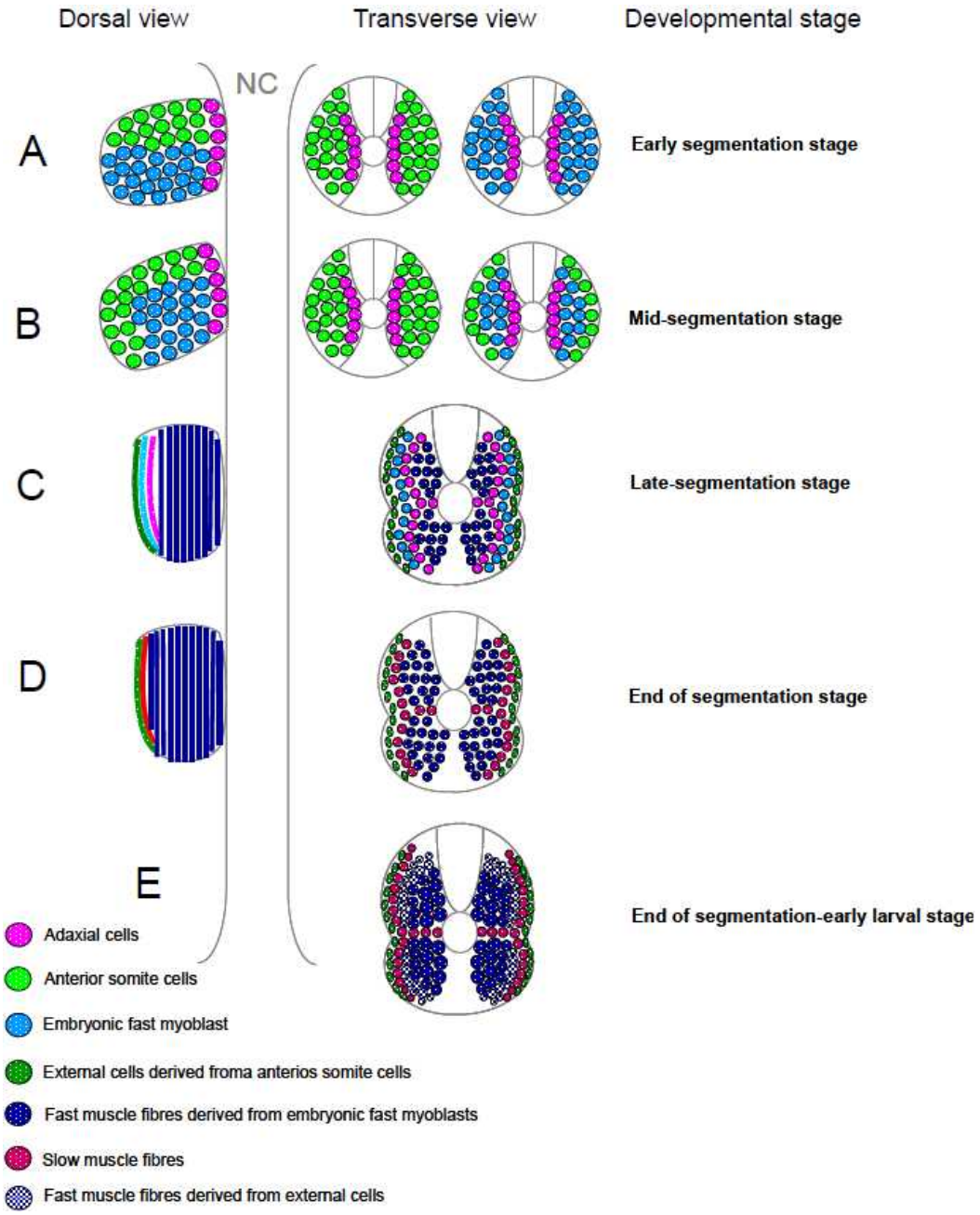

Fig. 1-3: Diagram illustrating the patterning of slow and fast muscle fibers during embryonicand early postembryonic stages (Adapted from Lee, 2010). The transverse views of somite during early and mid-segmentation stage designate the distinct origins of anterior somite cells (anterior) and embryonic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

Resulting embryonic myogenesis, new muscle fibers 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 fiber 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 fibers by their gene expression pattern in zebrafish (Barresi et al., 2001), pearlfish (Steinbacher et al., 2006), brown trout (Steinbacher et al., 2007) and rainbow trout (Rescan et al., 2013). To begin with, newly formed 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 fibers 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 fibers to generate a mosaic of muscle fiber diameters in a myotome across section (Fig. 1-4B) (Rowlerson et al., 1995). In fast muscle, muscle fiber 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 fiber recruitment all subsequent growth is by fiber 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 fiber number maintains to increase with body length to the utmost body size (Johnston et al., 2004).

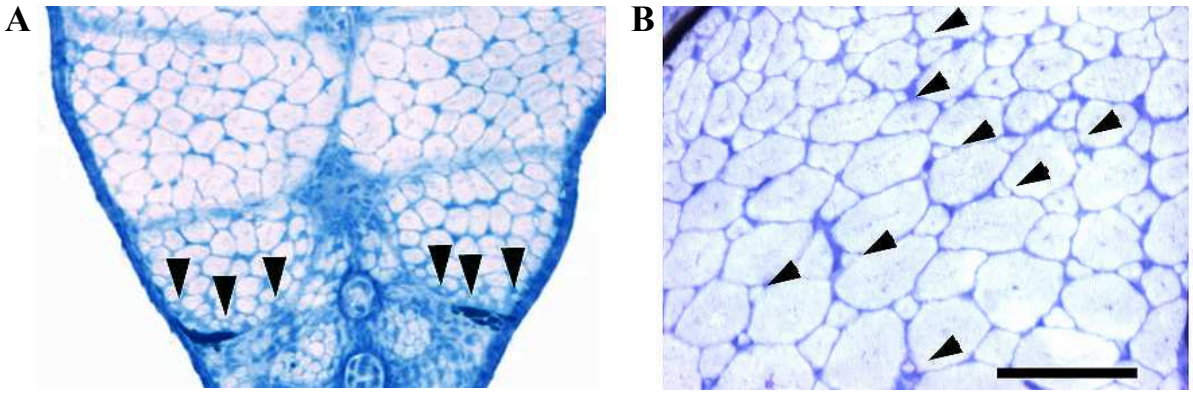

Fig. 1-4: Hyperplastic growth of trunk muscle in zebrafish larvae (Adapted from Lee et al., 2010). A: Methylene blue stained section showed stratified hyperplastic growth. B: Methylene blue stained section showed mosaic hyperplastic growth (red arrowheads).

1.1.3 Fish muscle fiber is an important tool for aging research

Sarcopenia represents the muscle fiber number continues 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 peoples will be over the age of 65 by the year 2050 in United States of America (Federal Interagency Forum on Aging-Related Statistics, 2008). This noticeable increase in aged peoples and following diagnoses of sarcopenia cause a momentous features to aging scientists (Clark and Manini, 2008, 2010), while the want for efficient medications for these conditions will be of great significance. In this regard, the most important animal model intended for aging study is the laboratory mice which displays

noticeable aging as it increases in age. On the other hand, the many intricate traits among mouse and teleosts (e.g., telluric vs. aquatic, actinopterygian vs. tetrapodus) engender straight coincidence of above animals arduous to a certain extent. In spite of many genetical differences among mammal and fish, the major issue possibly the complementary development of mammal and fish. In case of mammals like mouse, attain a ultimate body mass subsequent to reach sexual maturity and become capable of reproduction which display an distinctive growth level (Lui and Baron, 2011) and are termed "determinate" growth (Lincoln et al.,1982).

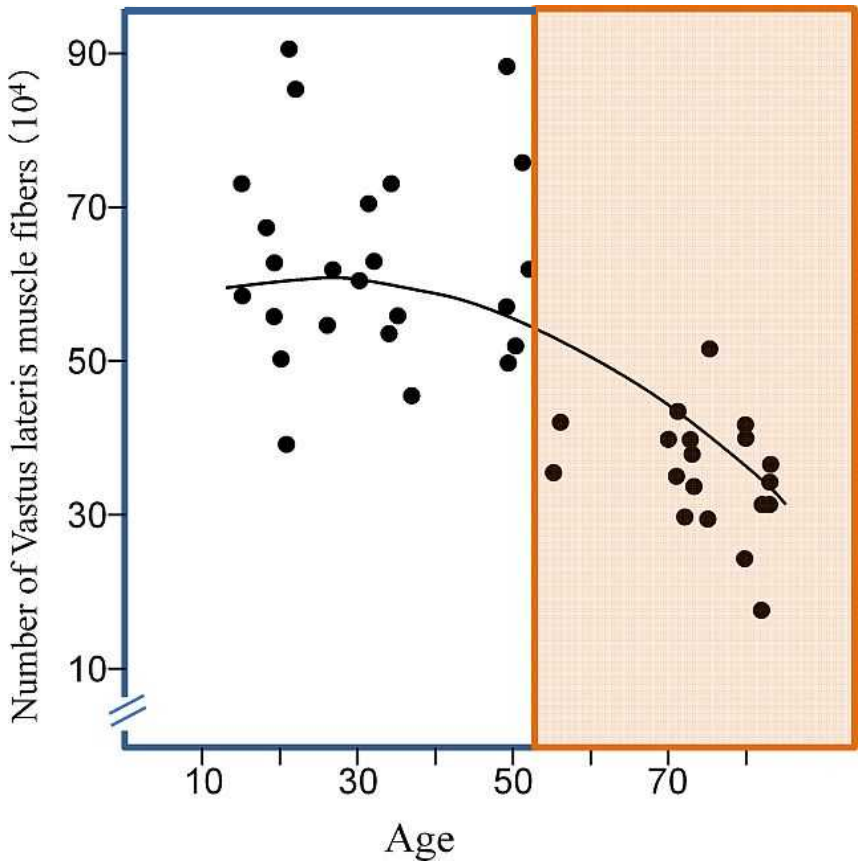

Fig. 1-5: Diagram showing sarcopenia i.e. reduction of muscle mass associated with aging. Sarcopenia indicates the relationship among the muscle fiber number and the aged person, but the number of muscle fibers decreases rapidly from 50 years old and so on (Adapted from Lexell et al.,1988).

Though, many fish does not show like a severe growth level (Sebens, 1987), while them mature all over their life cycle, even at passive rate. This kind of growth, namely "indeterminate," is incredibly frequent amongst fishes. On the contrary to mammals, the indeterminate growth was found to be observing in teleost is greatly influenced by exterior distinctiveness (Sebens, 1987). This pattern is disagreed with determinate growth (Lincoln et al., 1982) where age is extremely analytical of body size. Regarding skeletal muscle include metabolic activity representing a huge amount of vertebrate muscle, the distinction among terrestrial mammals and fishes persist. All the more fascinatingly, how do fishes carry on to build up new muscle fibers alike into old age? The response to those aforesaid questions is significant for mitigating the effects of sarcopenia in progressively more large aged population (Froehlich et al., 2013b). 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 motile machinery. Such like 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 (myosin heavy chain, MYH) which are concerned in locomotion, and light chains which are implicated in regulation. According to phylogenetic tree, 24 classes of

various ranges of myosin proteins has been classified (Foth, et al., 2006). Myosins have power over numerous essential cellular functions together with transporting protein, cell division, cell death, bonding, movement, phagocytosis, cellular excretion and contraction (Krendel and Mooseker, 2005). Among myosin family members, myosin II is the most important motor protein domain that controls actomyosin contraction in various muscle and non muscle cells. It is also one kind of hexamer protein compound that consist of a pair of MYHs i.e. one pair of essential and one pair of regulatory light chains (Fig. 1-5A). The MYH encompasses a conserved motor and major domain by the side of the N-terminus that force the movement alongside with actin filaments and a neck or narrow domain so as to present as a inflexible pedal section to produce movement of the major domain beside with a non-conserved twisted coiled-coil domain at the C-terminus region, which come to an end with a small non-helical tail. As well light chains combined to the narrow domain wherever the essential light chains supplied structural strength and integrity to the motor domain, and the regulatory light chains control the myosin II ATPase activity (Clark et al., 2007). Likely, myosin II accumulates into bipolar filaments during electrostatic communications between the coiled-coil domains (Fig. 1-5B) (Hostetter et al. 2004). The motor domains lying on every ending of the filament connect with oppositely leaning actin filaments. As a result of hauling actin filaments in connect with myosin II group engender cortical tension. Moreover, MYHs are encoded by multigene family and numerous MYH isoforms 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.

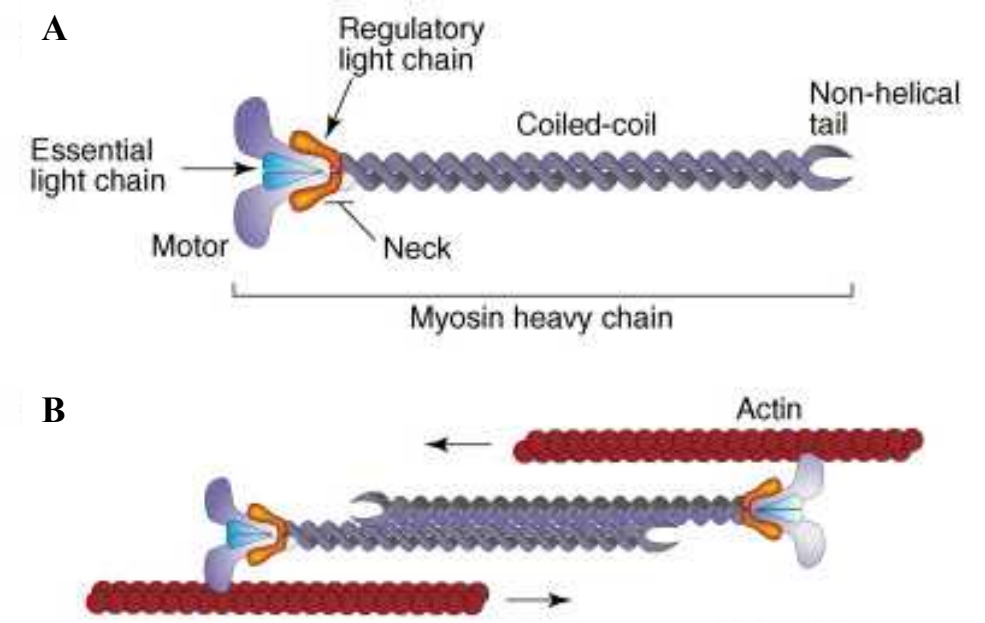

Fig. 1-6: Diagram illustrating the sarcomeric myosin (myosin II) molecule (Adapted from Clark et al., 2007). (A) Illustrative diagram of a myosin II monomer, representing the light and heavy chains. The various parts of the heavy chain, together with the motor, neck, coiled-coil and nonhelical domains, are designated. (B) Myosin II self-assembles into bipolar filaments through contacts of the C-terminus; the N-terminus attachs to actin filaments. Commencement of the myosin II motor domain directs to the hauling of actin filaments (in the course of the arrows) to persuade 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 number of *MYH*s are much more than their higher vertebrate counterparts (Watabe, 2002; Ikeda et al., 2010). Besides, a significant number of *MYH*s are expressed in fish during developmental stages resulting in changes of the composition of muscle-fiber type (Watabe and Ikeda, 2006; Liang et al., 2008). Conversely, 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 have revealed that *MYH*s show different muscle specific expressions 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 (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 named *mMYHemb1* in embryos and *mMYHL1* and *mMYHL2* in larvae, are predominantly expressed during their development stage of medaka, (Ono et al., 2006). 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). These base line information points out the *MYH*s demonstrated strictly regulated temporal and spatial expression patterns mediating fish muscle development.

1.1.5 Expression of myosin heavy chain gene and teleost indeterminate muscle growth

Regulation of *MYHs* is also important to understand teleost indeterminate muscle growth. Jonston et al. (2009) have reported the up-regulation of fast type *MYH* named *myhz1* in small diameter fibers of zebrafish fast muscle that were still recruiting myotubes. In torafugu, a fast-type *MYH* named $MYH_{M2528\text{-}1}$, was also found to be expressed in newly formed small diameter muscle fibers at adult stage mosaic hyperplasia (MH) (Fig. 1-7) (Akolkar et al., 2010; Asaduzzzaman et al., 2013). Expression of $MYH_{M2528\text{-}1}$ was also investigated at myogenic zone during stratified hyperplasia (SH) (Fig. 1-8) (Asaduzzzaman et al., 2013). These lines of information clearly indicate that specific *MYH* expression is activated at newly formed muscle fibers in relation to teleost indeterminate muscle growth

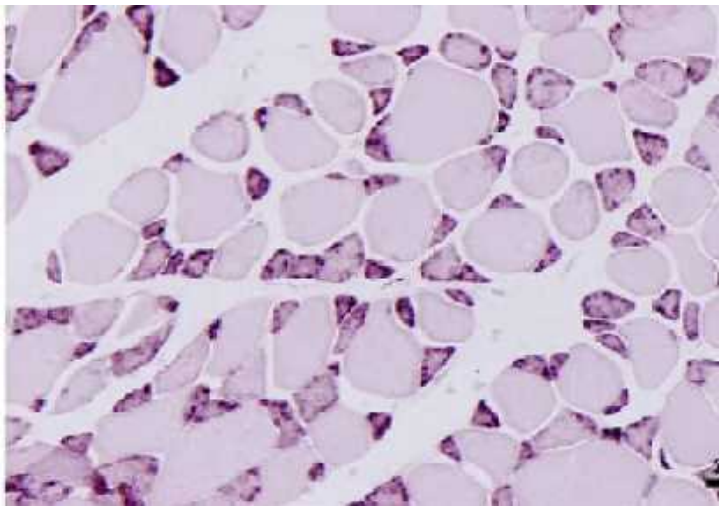

Fig. 1-7: *In situ* hybridization of adult torafugu $MYH_{M2528\text{-}1}$ in fast muscle (Adapted from Akolkar et al., 2010; Asaduzzzaman et al., 2013).

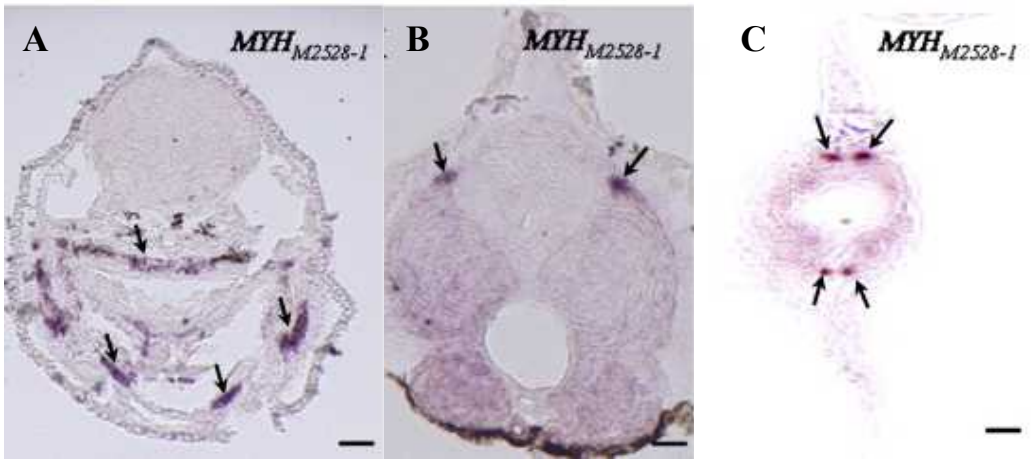

Fig. 1-8: *In situ* hybridization of larval torafugu *MYH*$_{M2528-1}$ in craniofacial muscle (A), dorsal extreme (B) and dorsal and ventral extreme (C) (Adapted from Akolkar et al., 2010; Asaduzzaman et al., 2013).

1.1.7 Torafugu and zebrafish are experimental models

The pufferfish known as the fugu, torafugu 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). Therefore 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 attractive 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 (Akolkar et al., 2010; Asaduzzaman et al., 2013; Ahammad et al., 2015).

The zebrafish 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. 1-9). Therefore, male and female of zebrafish are easily distinguished due to their external characters (Fig. 1-9A). In experimental purposes, one pair of zebrafish can produce around 30-50 embryos per spawning, likely once a week, all over the year depending on the level of maturity (Fig. 1-9A). Zebrafish eggs are translucent and reasonably large (~0.7 mm in diameter) contrast to other teleost of an alike size (Fig. 1-9A). Embryogenesis and organogenesis are quickly develop (Fig. 1-9A). 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. 1-9A). 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. 1-9B). Consequently, zebrafish is a profitable animal models to create transgenic lines and are simply available to transient reporter analysis for quantification. 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). Actually, promoter analysis of some MYH gene have been successfully conducted by using zebrafish (Asaduzzaman et al., 2013).

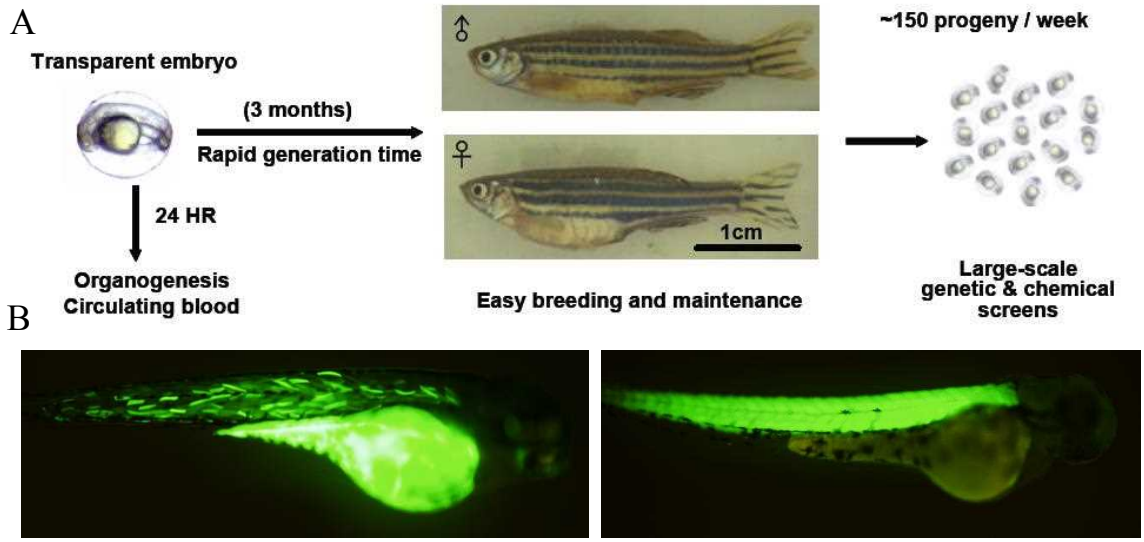

Fig. 1-9: Diagram represents the utilization of zebrafish as model system. (A) General features of zebrafish thatmake them excellent laboratory models (Adapted from White et al., 2008; Lee et al., 2010; Asaduzzaman et al., 2013). (B) Transgenic approaches in zebrafish embryos are possible (Adapted from Asaduzzaman et al., 2013).

1.6.7 Transcription factors in teleost myogenesis

Transcription factor is primary regulator for gene expression. Myogenic regulatory factors (MRFs) are basic helix-loop-helix (bHLH) transcription factors that regulate myogenesis. MRFs include myogenic factor 5 (Myf5), myogenic differentiation 1 (Myod1, also known as MyoD), Myf6 (also known as Mrf4) and myogenin 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 by 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 unique model for understanding vertebrate myogenesis. A significant outcome of indeterminate growth is that the number of fibers needs to increase throughout the life cycle as the muscle mass increases, involving a prolonged period of postembryonic hyperplasia (Greer-Walker, 1970; Stickland, 1983). However, molecular mechanisms involved in the indeterminate growth is completely unknown. On the other hand, myosin is the eukaryotic motor proteins that generate the force for cellular

movements which necessitates various *MYHs* expression during muscle development. $MYH_{M2528\text{-}1}$ is promising target to resolve mechanisms underlying the indeterminate muscle growth because of its coordinate expression with larval and post-larval muscle hyperplasia: new muscle fibers produced by hyperplasia specifically express the $MYH_{M2528\text{-}1}$ (Akolkar et al., 2010; Asaduzzaman et al., 2013).

The areas involved in the present study are as follows-

i. To identify torafugu $MYH_{M2528\text{-}1}$ promoter which can induce gene expression in stratifiedand mosaic hyperplasia by zebrafish *in vivo* reporter assay.
ii. To establish $MYH_{M2528\text{-}1}$:EGFP transgenic line in zebrafish and examine the EGFP expression pattern in comparison with torafugu endogenous $MYH_{M2528\text{-}1}$ expression.
iii. To identifying *cis*-acting elements responsible for $MYH_{M2528\text{-}1}$ expression in relation to indeterminate muscle growth

Based on above experiments, present study deals with following concerns: Whether does the 5'-flanking sequences of torafugu *MYH* work in zebrafish which is phylogenically far from torafugu in teleost lineage? How is the *MYH* expression regulated transcriptionally? It is also noted that marked difference in body size between torafugu and zebrafish. The body size difference should be related to skeletal muscle mass because majority of vertebrate body is comprised of the skeletal muscle. Finally, most important question is 'what is the responsible mechanism which dictates determinate and indeterminate growth?'

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 MYH, expressional regulation of MYH 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 isolation of the $MYH_{M2518\text{-}1}$ promoter mediating indeterminate muscle growth in teleosts. Here, we investigated promoter activity of the 5' flanking region of $MYH_{M2518\text{-}1}$ to examine the spatial and temporal regulation by using transgenic and transient expression techniques through *in vivo* reporter assay in zebrafish. The results of this chapter clearly demonstrated that promoter involved in teleost indeterminate muscle growth is conserved between large (torafugu) and small (zebrafish) fish.

The next step (Chapter 3) documented identification of multiple *cis*-elements in the $MYH_{M2528\text{-}1}$ promoter, which function in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression. In this chapter, we described the MyoD, Pax3 and MEF2 mediated the gene expression in relation with indeterminate muscle growth. 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

Identification of the promoter which activates gene expression mediating indeterminate muscle growth in teleost

The content of this chapter was published as:

A. K. Shakur Ahammad, M. Asaduzzaman, S. Asakawa, S. Watabe and S. Kinoshita. 2015. Regulation of gene expression mediating indeterminate muscle growth in teleosts. Mechanism of Development, 137: 53-65.

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 in zebrafish larvae. At the juvenile stage, the promoter was specifically activated in small diameter muscle fibers scattered throughout fast muscle and 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 '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). On the other hand, 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). Therefore, 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 and is expressed in neonatal 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 region by 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 fibers with relatively small diameters and slow fibers 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 10days 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).

2.2. 3. Infomatics

Transcription factor-binding sites in the 5'-flanking region of *MYH$_{M2528\text{-}1}$* was carried out by Genomatics (www.genomatics.de), the TFSEARCH program (www.cbrc.jp/research/db/TFSERCH.html) and manual identification.

2.2. 4. 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: Nucleotide sequence of oligonucleotide primers for various experiments

Experiment	Primer Name	Nucleotide 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. 5. *In vivo* reporter assay

Each EGFP reporter construct was diluted to 100 ng/µl 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 tricaine methane-sulfonate (Sigma-Aldrich) to inhibit movement during observation.

2.2. 6. 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. 7. Immunohistochemistry and antibodies

For immunohistochemistry, larvae and juveniles of the transgenic line were fixed with 4% PFA in Tris-buffered saline (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 2.7 mM KCl) 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. 8. 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. 9. 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, Fig. 2-2). Exons 1 and 2 are transcribed as an untranslated region, and the start codon is located in exon3 (Fig.2-1, Fig. 2-2).

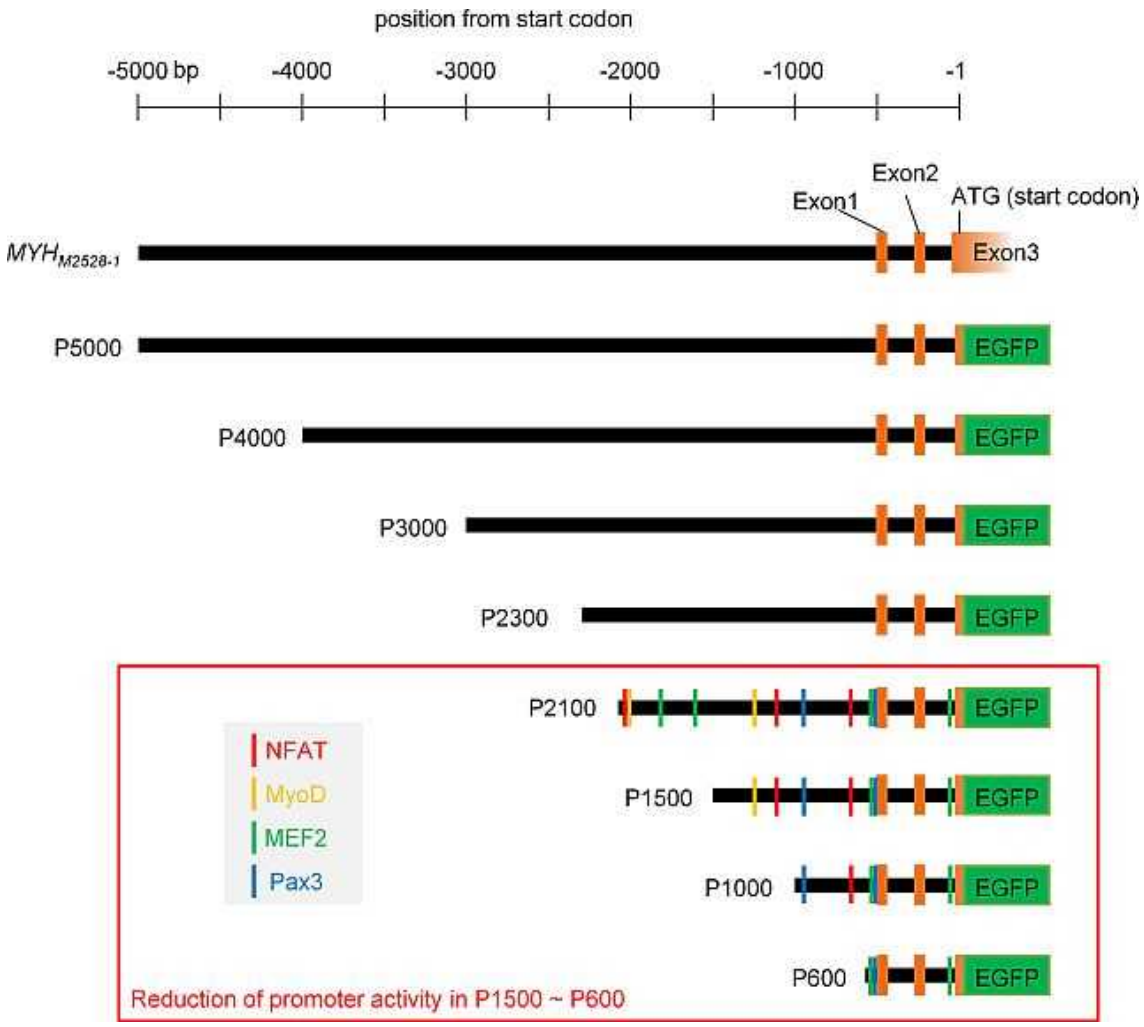

Fig. 2-1. Schematic of reporter constructs used in this study. A genomic region located 5000 bp upstream from the start codon of torafugu*MYH*$_{M2528\text{-}1}$ amplified by PCR and inserted into an EGFP reporter vector (P5000). Serial deletion constructs containing 4000 (P4000), 3000 (P3000), 2500 (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.

Fig. 2-2. The DNA sequence of the 2100 bp 5'-flanking region from the start codon of *MYH*$_{M2528\text{-}1}$. The transcription initiation site determined by 5'-RACE is indicated by a bold G. Based on the transcription factor binding site analysis via the Genomatix and TFSEARCH programs, MyoD, MEF2, Pax3, and NFAT-like binding motifs and exons are indicated by boxes. The translation start codon located on the third exon is underlined.

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

zebrafish has an *in vivo* reporter assay. For P5000, ~ 97% of the injected embryos displayed strong EGFP expression along skeletal muscle fibers (Fig. 2-3A-B and Fig. 2-4A). The EGFP expression was detected at 1 dpf and continued to be expressed in the whole myotomal region of larva at 2dpf (Fig. 2-5A-E). At 3 dpf, EGFP was found to be expressed in the craniofacial and myotomal muscles (Fig. 2-3K, Fig. 2-5E). In the myotomal region, both slow and fast muscle fibers expressed EGFP (Fig. 2-6). Although almost the same expression pattern was observed in zebrafish larvae injected with P5000 through P600 (Fig. 2-3), 5'flanking regions shorter than 2,100bp resulted in a significant reduction in EGFP expression in the myotomal muscle fibers (Fig. 2-3M-R, Fig. 2-5). As shown in Fig. 2-4A, 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. 2-4B, Fig. 2-5F-N). This finding indicates that *cis*-acting element(s) in between -2,100 to -600 bp in 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 2100 bp 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, Fig. 2-2). The reduced promoter activity (Fig. 2-4) 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 $MYH_{M2528\text{-}1}$.

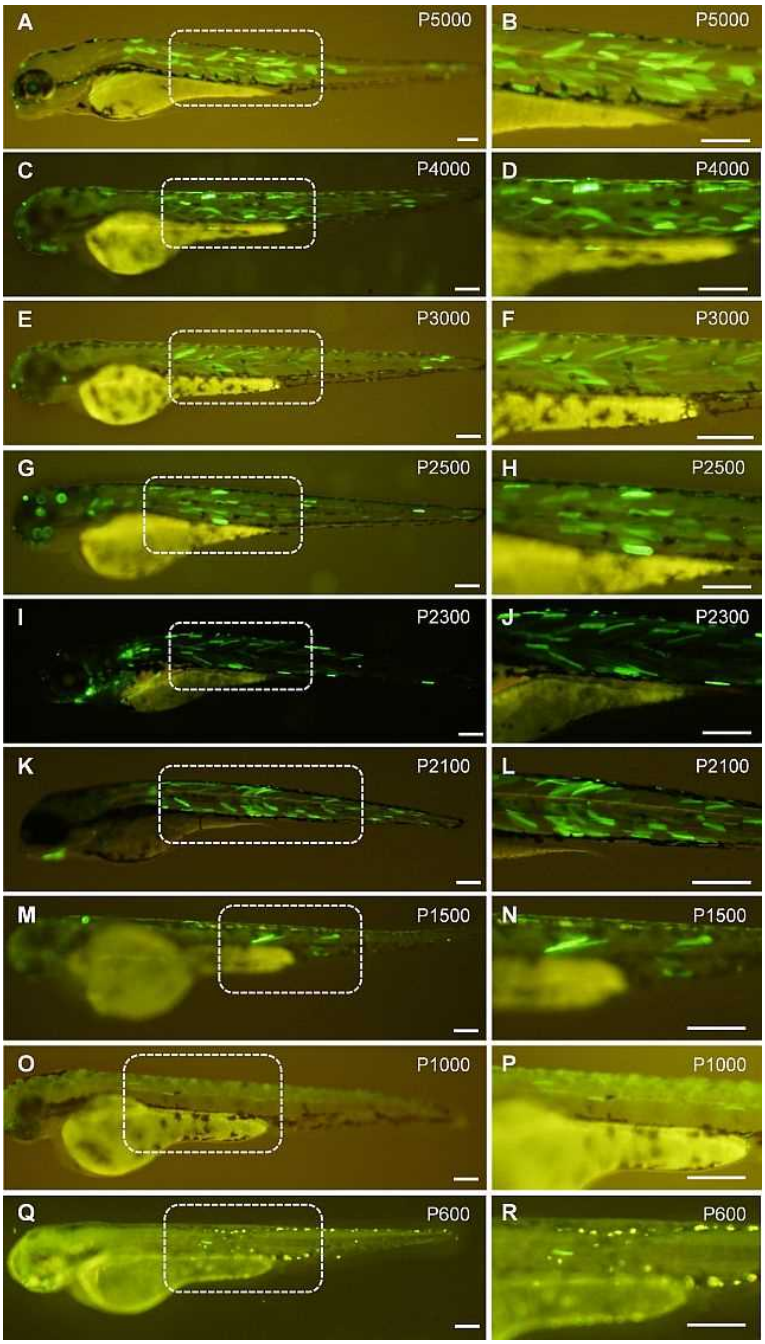

Fig. 2-3. EGFP expression in zebrafish larvae injected with*MYH*$_{M2528\text{-}1}$ reporter constructs. A-R, EGFP expression in myotomal skeletal muscle of zebrafish embryos injected with reporter constructs, P5000 (A,B), P4000 (C,D), P3000 (E,F), P2500 (G,H), P2300 (I,J), P2100 (K,L), P1500 (M,N), 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.

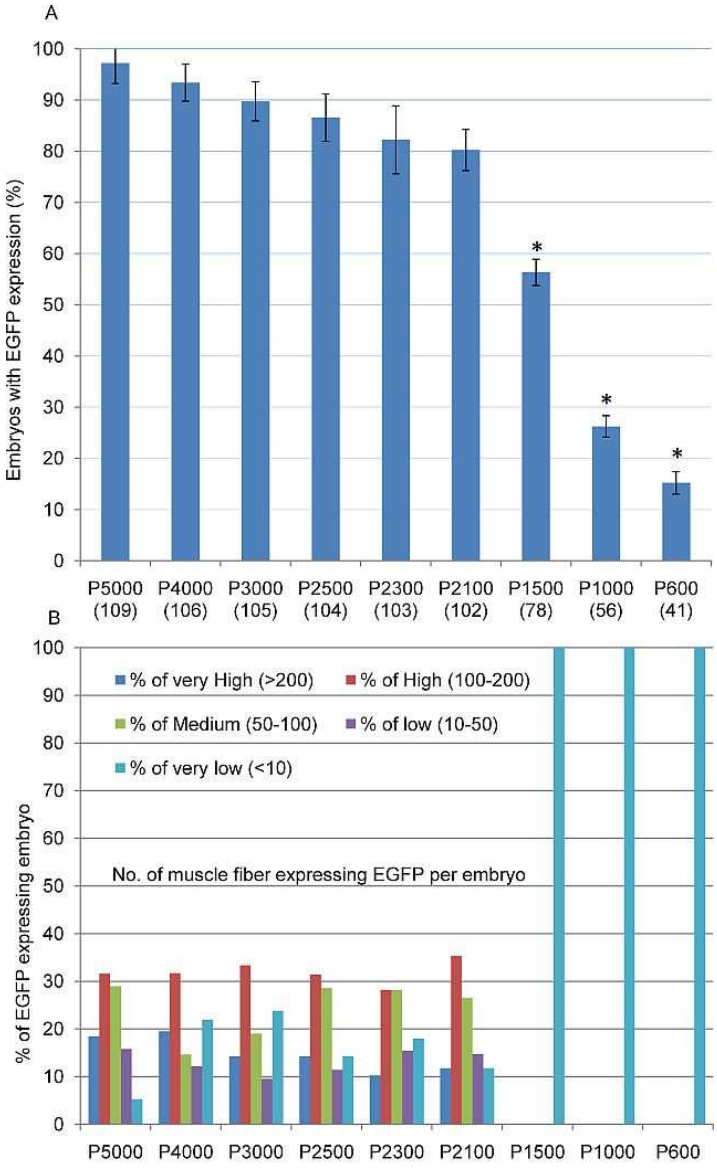

Fig. 2-4. Bar graph showing the promoter activity of the reporter constructs where EGFP expression was observed in the myotomal compartments of zebrafish larvae at 3dpf. A, percentages of larvae that express EGFP in the myotomal compartments following injection of each construct and the total number 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.

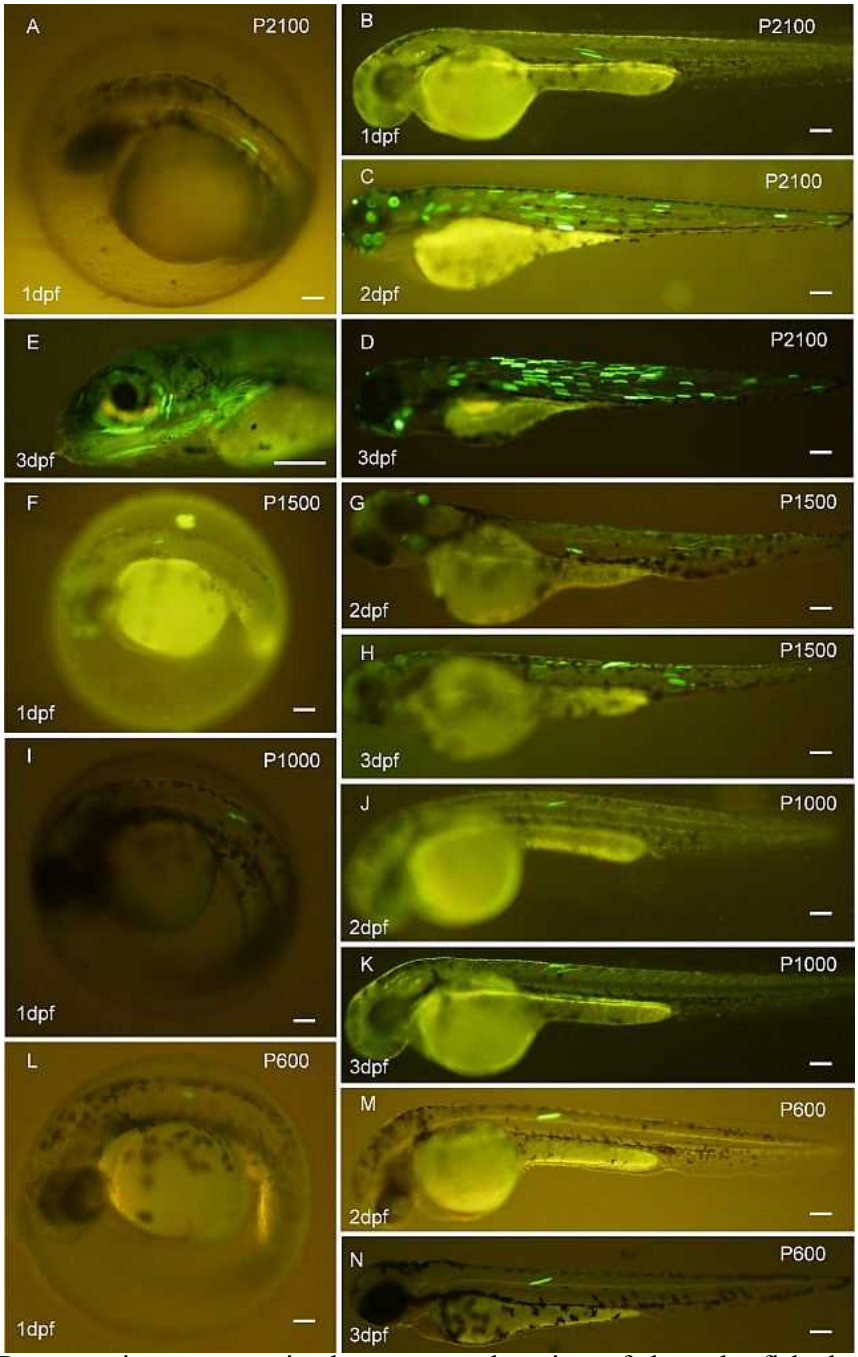

Fig. 2-5. EGFP expression patterns in the myotomal region of the zebrafish through reporter construct (A-N).A zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C; 3dpf, D) injected with the P2100 reporter construct. D-E, EGFP expression patterns in craniofacial muscles in zebrafish larvae at 3 dpf. As 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.

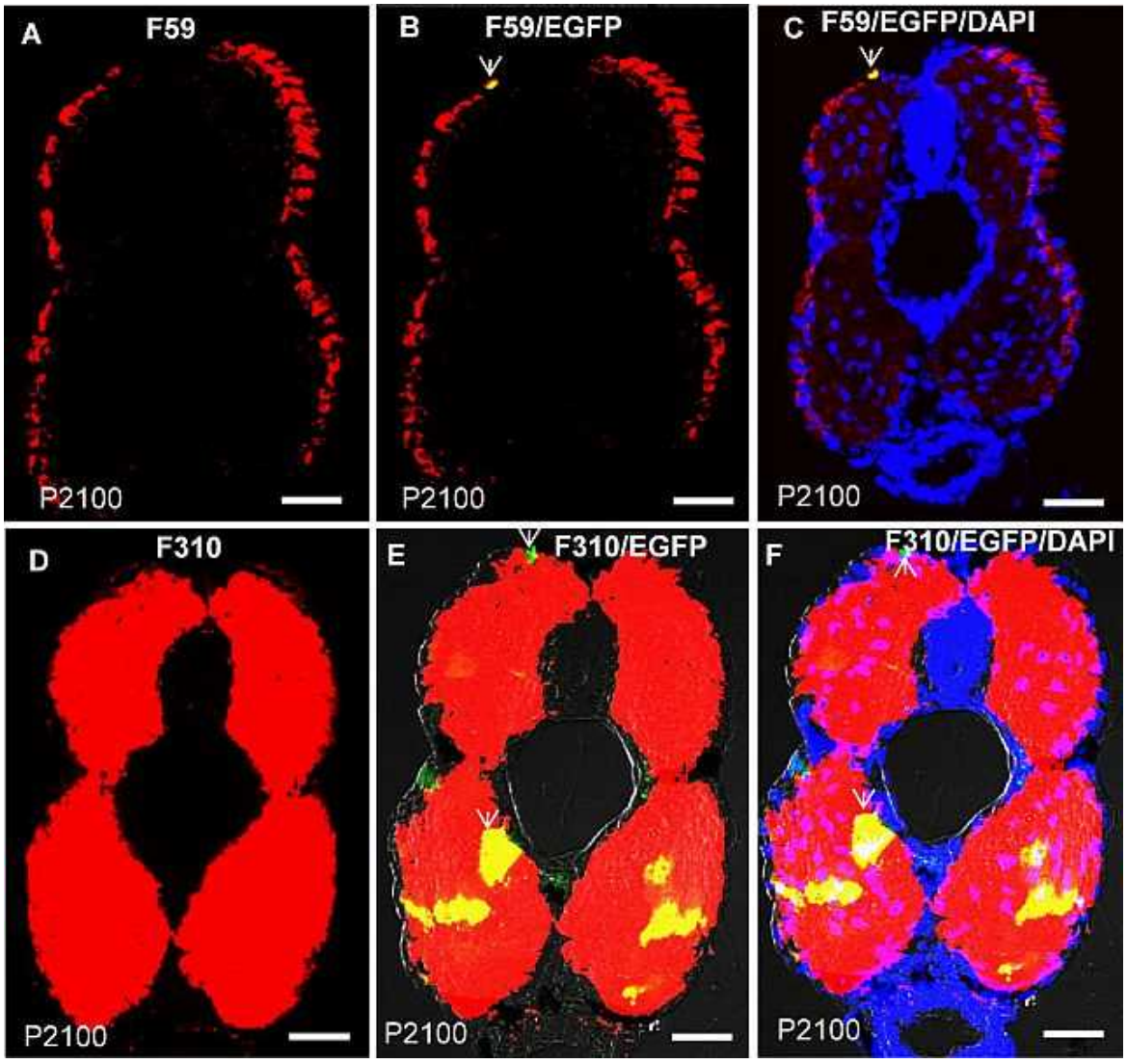

Fig. 2-6. Immunohistochemistry localizing EGFP expression in both fast and slow muscle fibers of P2100-injected zebrafish larvae. A-C, slow muscle fibers expressing EGFP as reacted with F59 antibody (A, F59 antibody view; B, F59 with EGFP; and C, F59 and EGFP with DAPI) in a P2100-injected larva at 3 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$_{M2528-1}$* promoter activity in zebrafish larvae

To confirm the specific activity of the *MYH$_{M2528-1}$* promoter in post-embryonic muscle hyperplasia, a stable transgenic zebrafish line, Tg:MYH$_{M2528-1}$:EGFP,was established using the P2100 construct and temporal and spatial EGFP expression was analyzed. Similar to the *in vivo* reporter assay (Fig. 2-5), Tg:MYH$_{M2528-1}$:EGFP displayed EGFP expression at 1 dpf (Fig. 2-7A-B). After hatching at 2 dpf, EGFP continued to be expressed in the whole myotomal region of the larva (Fig. 2-7C). Observation with a fluorescent microscope also confirmed that Tg:MYH$_{M2528-1}$:EGFP expressed EGFP in the whole myotomal region (Fig. 2-7D) and craniofacial muscles (Fig. 2-7E) at 3 dpf.

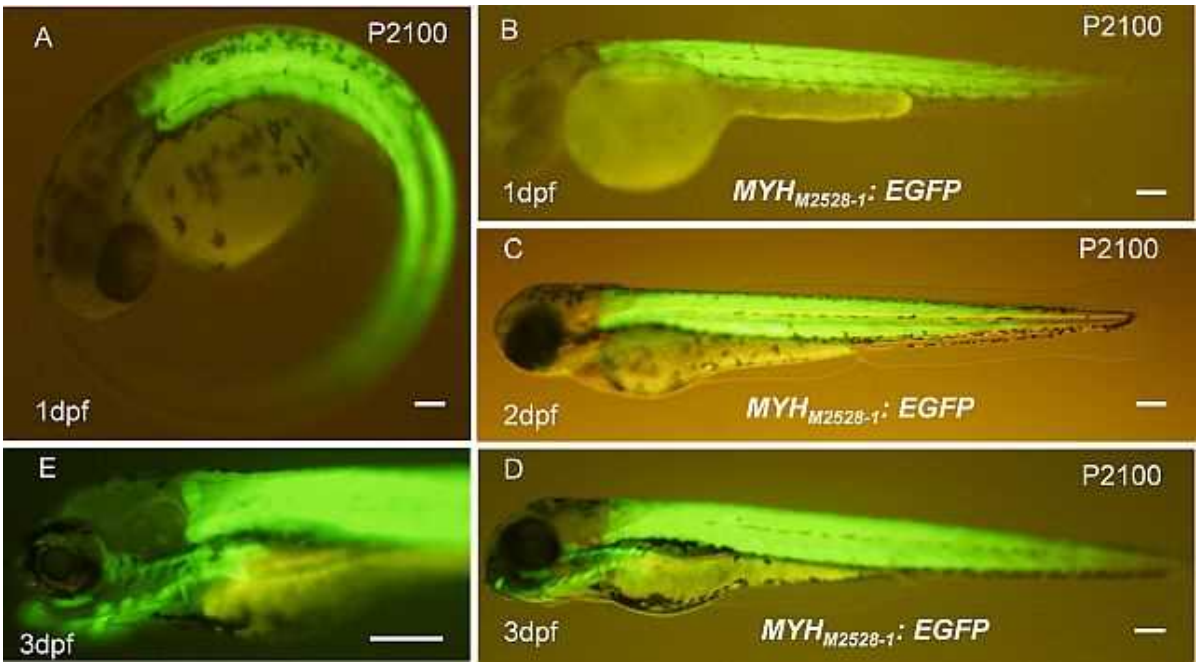

Fig. 2-7. Expression patterns of EGFP in theTg:MYH$_{M2528-1}$:EGFP stable transgenic line embryo and larva. A stable line was established by injecting the P2100 construct. EGFP expression was observed in the whole myotomal region at 1 dpf (A,B), 2 dpf (C), and 3 dpf (D). In addition to myotomal skeletal 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 makesup 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 2-8 shows the immunohistochemistry of Tg:$MYH_{M2528\text{-}1}$: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. 2-8A-C) as described above. Most slow muscle fibers were EGFP negative, but a fiber at the dorsal edge expressed EGFP (Fig. 2-8D-F, arrowhead). In fast muscle, many muscle fibers expressed EGFP, but their distribution was predominant in the apical surface region (Fig. 2-8G-I). In Fig. 2-8K and 2-8L, 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.

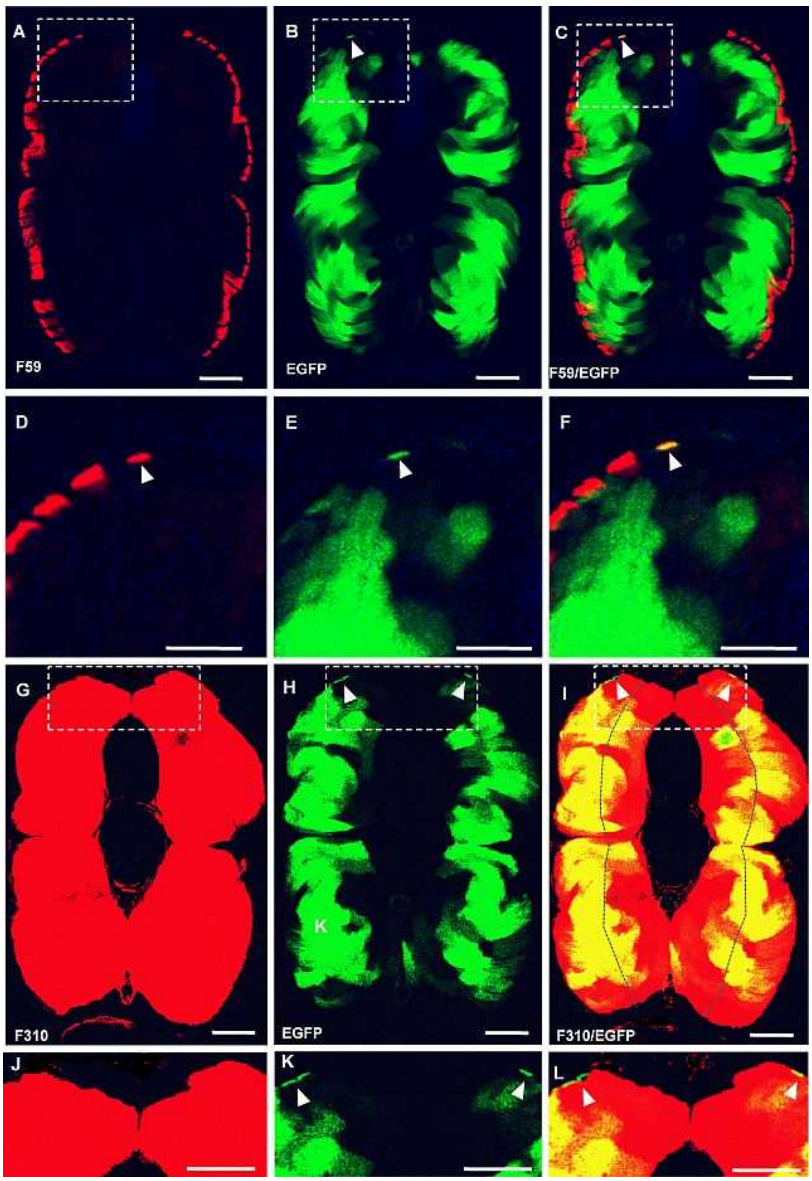

Fig. 2-8. Localization of EGFP-expressing myotomal muscle fibers in Tg:MYH$_{M2528-1}$:EGFP larvae (A-L). A-F, transverse section of myotomal region of Tg:MYH$_{M2528-1}$:EGFP at 3dpf. A-C, slow muscle fibers were stained red with a F59 antibody (A, F59 antibody; B, EGFP; C, merged view). D-F, the magnified 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. 2-9C,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. 2-9E-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).

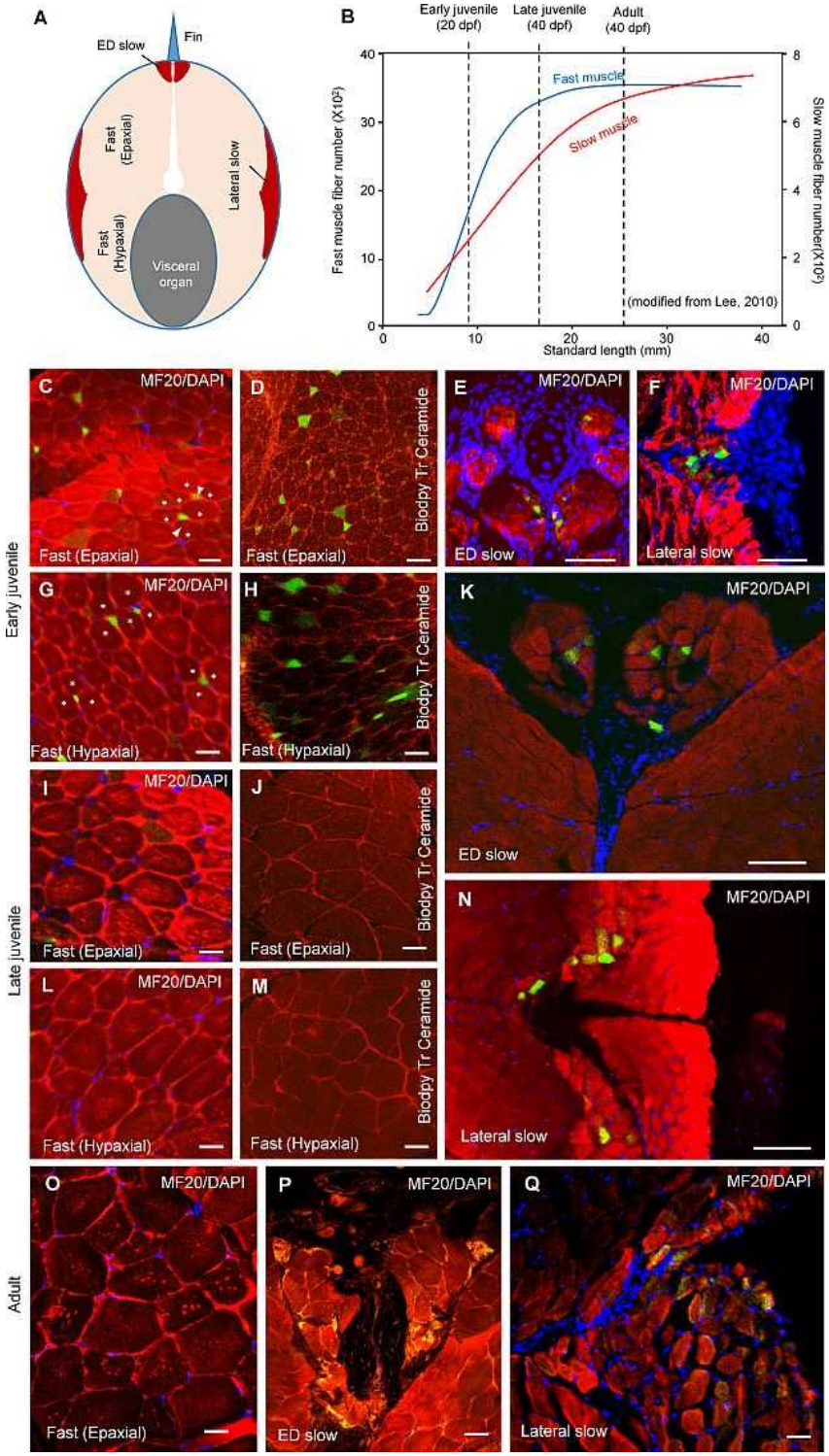

Fig. 2-9. Localization of EGFP-expressing myotomal muscle fibers in Tg:MYH$_{M2528\text{-}1}$:EGFP zebrafish (A-Q).(A)Cross-section of zebrafish. (B)body size-related increase in muscle fiber numbers in slow and fast muscles of zebrafish. Data cited from Lee (2010). (C-H) early, (I-N) late juvenile & (O-Q) adult stages. Scale bar: 50 μm

Consistent with the difference in the growth pattern between slow and fast muscles (Fig. 2-9B), promoter activity was not observed in fast muscle (Fig. 2-9I,J,L,M,O) but in slow muscle (Fig. 2-9K,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. 2-10).

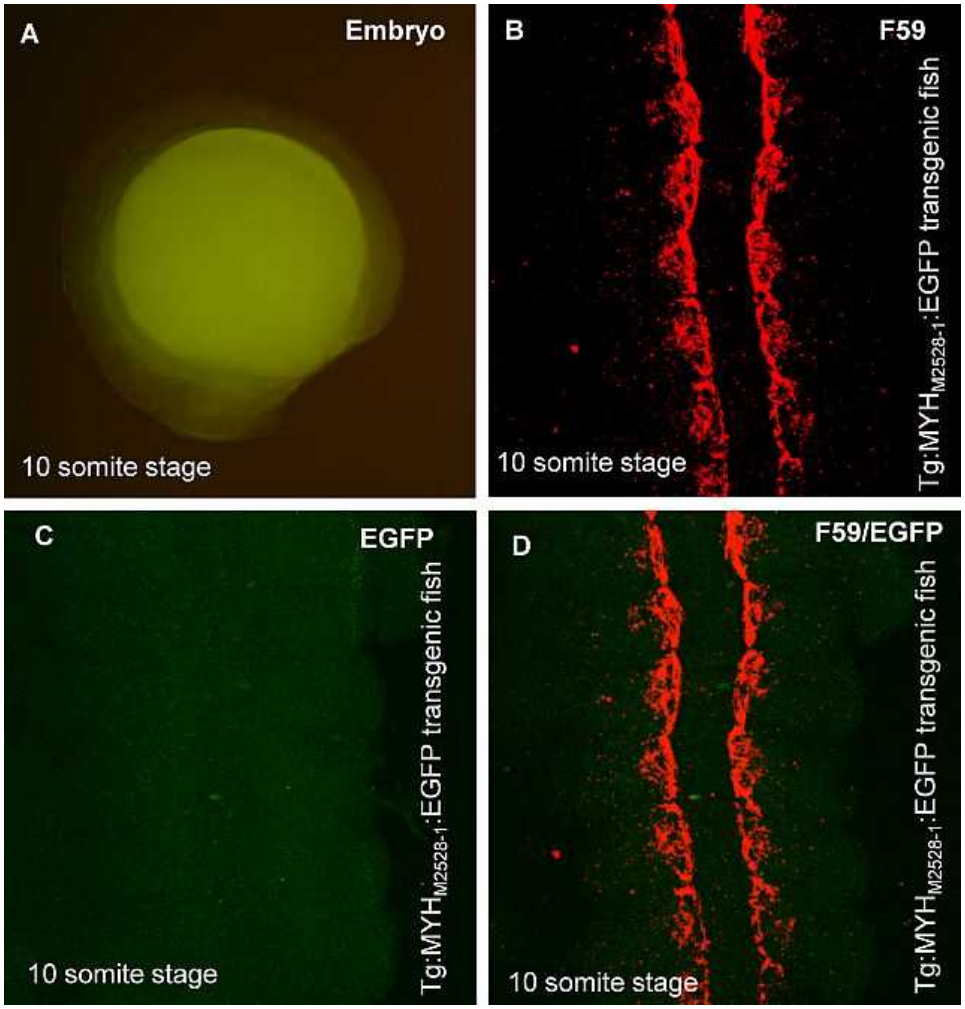

Fig. 2-10. Immunohistochemistry of Tg:MYH$_{M2528-1}$:EGFP transgenic embryo at 10 somite stage. A-D, adaxial cell does not expressed EGFP as not reacted with F59 antibody (A,10 somite stage Tg:MYH$_{M2528-1}$:EGFP transgenic embryo; B, F59 antibody view; C, EGFP; and D, F59 and EGFP) inaat 10 somite 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 cyclopamine at 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. 2-11D). Cyclopamine treated embryos showed very little change of EGFP expression compared to the control wild embryos treated with only ethanol (Fig. 2-11B,C,E,F). Depending on the dose, the rate of embryos showing EGFP expression in the muscle fibers varies from47.39% (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. 2-11G). 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. 2-11H,J). The EGFP expressing fiber was detected at only dorsal extreme of the myotome and is secondary slow muscle fiber (Fig. 2-11I,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.

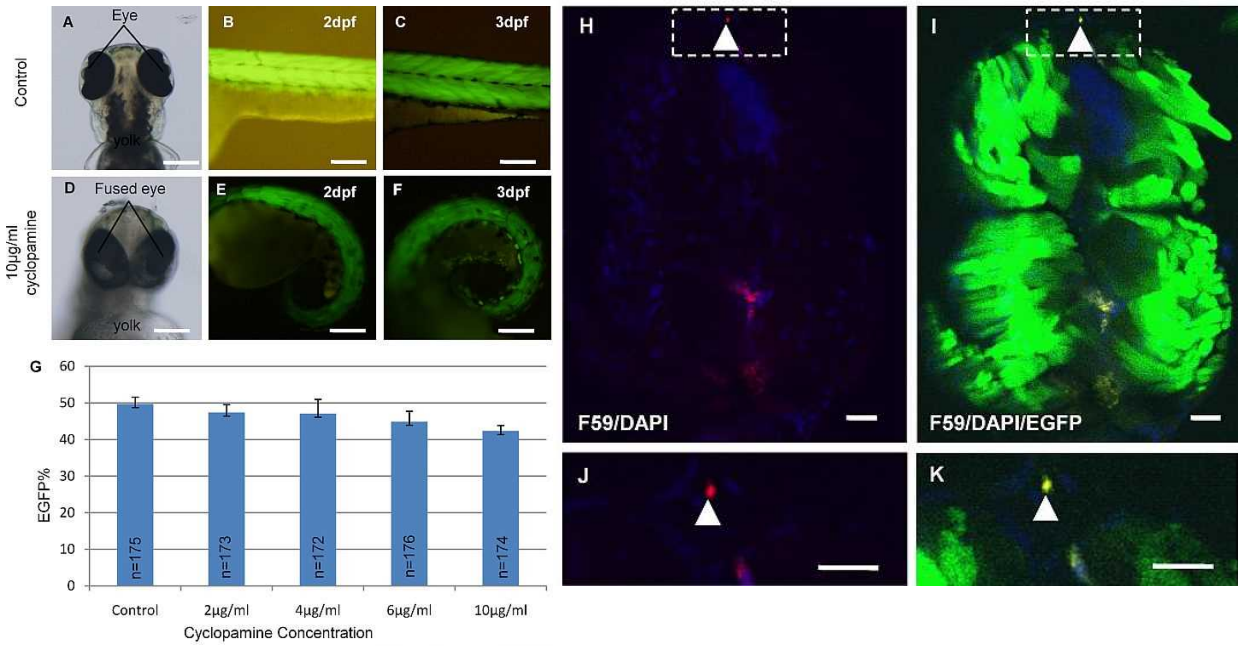

Fig. 2-11. Influence of Hedgehog (Hh) signaling pathway on the *MYH*$_{M2528\text{-}1}$ promoter and immunohistochemistry (A-K). Hh signaling was inhibited by cyclopamine treatment. Fertilized eggs of Tg:MYH$_{M2528\text{-}1}$:EGFPwere transferred into 2 – 10.0μg mL^{-1}cyclopamine solution. Control 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 disappear 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 that the mortality rate and pathogenesis of various age-related diseases are associated with the functional status and mass of skeletal muscle in humans. In mammals, however, the growth and regeneration capacity of skeletal muscle quickly decreases after birth (Rowe & Goldspink, 1969; Schultz, 1996), and skeletal muscle mass severely declines during aging. In this regard, indeterminate growth, continuous production of neonatal muscle fibers until death, is a rare characteristic of teleost skeletal muscle.

A 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 this unique process. In this regard, orthologous gene in respect to $MYH_{M2528\text{-}1}$ has not been found (www.ensembl.org). 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. rVISTA analysis has been tested between torafugu 2100 kb upstream sequence from startcodon of $MYH_{M2528\text{-}1}$ and zebrafish myhz1 promoters of three isoforms like myhz1.1, myhz1.2 and myhz1.3 and found no conserve region among them (Data not shown).

Here, 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 a region that had adequate promoter activity; 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. 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.

Notably, there is also a marked difference in adult body size between torafugu (over 6kg of adult body weight) and zebrafish (4-5cm of adult body length). Rowlerson et al. (1997) compared production of neonatal muscle fibers at the post-larval stage between sea bream *Sparus aurata* (large fish) and zebrafish. Based on the difference in MYH composition in neonatal muscle fibers at the adult stage, they suggested that large and small fish have different genetic requirement for the production of these fibers. Our results, however, clearly showed that the transcriptional network mediating MYH expression in neonatal muscle fibers at larval and post-larval stages is conserved between large (torafugu) and small (zebrafish) fish. However, an important difference is the duration of promoter activity in fast muscle. *MYH*$_{M2528\text{-}1}$ promoter activity in fast muscle ceased at 40dpf, 17mm SL(Fig. 2-9I, J,L,M) and 60dpf, 25mm SL (Fig. 2-9O) of zebrafish in this study, whereas the *MYH*$_{M2528\text{-}1}$ expression in torafugu fast muscle is still up-regulated at a far larger size (over 1kg body weight) (Akolkar et al., 2010; Asaduzamman et al., 2013). In addition, when compared with the number of *MYH*$_{M2528\text{-}1}$ expressing muscle fibers in the adult torafugu fast muscle (Akolkar et al., 2010; Asaduzamman et al., 2013), the number of EGFP-positive fast muscle fibers in Tg:MYH$_{M2528\text{-}1}$:EGFP zebrafish seemed to be reduced (Fig. 2-9C,D,G,H). Because fast muscle occupies a large portion of skeletal muscle in the teleost, the degree of growth in fast muscle is an important factor indetermination of adult body size.

As mentioned above, production of neonatal muscle fibers in teleost fast muscle continues at the post-larval stage but ceases at a definitive size (Johnston, 2001, Fig. 2-9B). Conversely, the growth of teleost slow muscle is 'indeterminate' in a true sense, in which muscle fiber production lasts until death (Johnston, 2001, Fig. 2-9B). Our transgenic line expresses EGFP in both slow and fast muscles at larval (Fig. 2-6) and early juvenile stages (Fig. 2-8C-H) but only in slow muscle at the late juvenile and adult stage (Fig. 2-9I-Q). This clear contrast between slow and fast muscles in growth patterns with advancing age is important because sarcopenia in mammals is often accompanied by a conversion of fast muscle fibers to slow muscle fibers (vonHaehling et al., 2010). What molecular mechanisms determine this difference in the promoter activity between slow and fast muscles is an interesting question with respect to muscle growth and aging.

Our goal was to elucidate the molecular mechanisms responsible for the difference in muscle growth between mammals and teleosts. In mammals, production of neonatal muscle fibers at post-larval stages occurs only during regeneration after injury and disease (Rowe & Goldspink, 1969). Although it is not clear whether the same signaling is shared between regeneration and growth, common MYHs, both neonatal and embryonic, are expressed in neonatal muscle fibers and produced during adult stage regeneration and embryonic hyperplastic muscle growth in mammals (Whalen et al., 1981; Lyons et al., 1990). Daou et al. (2013) reported that NFAT signaling is primarily important to induce neonatal and embryonic *MYHs* in neonatal muscle fibers in the adult mouse. We found that the $MYH_{M2528\text{-}1}$ promoter also contains three putative NFAT binding sites (Fig. 2-1) and deletion constructs showed an apparent reduction in the promoter activity in accordance with number of the NFAT binding sites (Figs. 2-3 and 2-4).

These results suggest NFAT signaling is also involved in the activation of the $MYH_{M2528\text{-}1}$ promoter in the teleost, presenting the possibility that indeterminate muscle growth is due to constitutive activation of NFAT signaling in skeletal muscle. We also found multiple sites for several representative myogenesis-related transcription factors in the promoter sequence (Fig. 2-1). MyoD and MEF2 are representative myogenic regulatory factors for muscle differentiation (Chanoine et al., 2004) and deletion constructs also demonstrated reduced promoter activity in harmony with number of the MyoD and MEF2 binding sites. Pax3 is expressed in myogenic precursor cells and may be involved in indeterminate muscle growth (Froehlich et al., 2013b). Therefore, it will be necessary to examine whether all of these factors, including NFAT, are actually involved in the promoter activity and also in the regulation of indeterminate muscle growth. This ambiguity is examined in the next chapter.

CHAPTER 3

Multiple *Cis*-acting Factors Function in the Regulation of *MYH*$_{M2528\text{-}1}$ Promoter Activity

Abstract

In Chapter 2, we successfully isolated torafugu $MYH_{M2528\text{-}1}$ promoter which induces gene expression specifically in newly formed muscle fibers at zebrafish larval and post-larval stages, indicating its functional roles for indeterminate muscle growth. However, the *cis*-elements involved in its promoter activity 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. We discovered that myoblast determining factor (MyoD) binding elements play a key role and participate in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression. We discovered that paired box protein (Pax3) are required for promoting $MYH_{M2528\text{-}1}$ expression and myocyte enhancer factor-2 (MEF2) binding sites participate in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression in slow/fast skeletal muscles. We further discovered that the nuclear factor of activated T-cell (NFAT) 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. 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.

3.1. Introduction

Myosin is a universal actin-based motor protein that forces a wide range of motile processes in eukaryotic cells. Muscle tissues contain the most classic sarcomeric myosin, called myosin II, which consists of 2 heavy chains (myosin heavy chains, MYHs) and 4 light chains (Sellers, 2000). MYH is the most important functional and structural domain of the myosin molecule and thus a key determinant factor of the skeletal muscle development (Ikeada et al., 2007). This skeletal muscle development in fish clearly differs from mammals. In mammals, postnatal muscle growth only involves the hypertrophy of pre-existing muscle fibers formed prior to birth (Rowe and Goldspink, 1969), whereas fish larvae show hyperplasic muscle growth, the increase in the number of muscle fibers which takes place in discrete germinal zones situated mainly at the dorsal and ventral extremes of the myotome via stratified hyperplasia (SH) (Rescan, 2005). Subsequently, the SH is followed by a mosaic hyperplasia (MH) process that leads to the formation of new fibers throughout the whole myotome (Rowlerson and Veggetti, 2001). Interestingly, our previous studies showed that torafugu *Takifugu rubripes* $MYH_{M2528\text{-}1}$ was expressed at the dorsal and ventral extremes of the myotome in torafugu larvae (Akolkar et al. 2010) and small diameter muscle fibers distributed between matured large muscle fibers in torafugu adult (Asaduzzaman et al., 2013), indicating its key role on fish characteristic indeterminate muscle growth. In the chapter 2, we successfully identified $MYH_{M2528\text{-}1}$ promoter which induced gene expression in neonatal muscle fibers produced by larval and post-larval hyperplasic muscle growth in zebrafish *Danio rerio*. However, *cis*-acting factors which bind and regulate the promoter activity remain unknown. Fish muscle growth is controlled by a variety of transcription factors such as myogenic regulatory factors (MRFs), paired-box transcription factor (Pax3) (Froehlich et al., 2013b) and MADS box transcription factor related to the myocyte

enhancer factor-2 (MEF2) (Chanoine et al., 2004). MRFs are well-known myogenic factors among vertebrates (Perry and Rudnick, 2000; Rescan, 2001; Bergstrom et al., 2002; Chanoine et al., 2004; Berkes and Tapscott, 2005). Further vital transcription factors in myogenesis are MEF2 family members, which has binding capacity to an A/T-rich sequence existing in many muscle-specific promoters and enhancers (Jordan et al., 2004; Berkes and Tapscott, 2005). These are supposed to inspire the expression of approximately all muscle-specific genes. Previous studies have reported that MEF2 instructed the fast fiber of skeletal *MYH* transcription *in vivo* (Allen et al., 2005). So far, the transcriptional activities of *MYH*s have been comprehensively examined 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, medaka, zebrafish, and torafugu) in a temperature condition (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008) and in fiber-type specification (Asaduzzaman et al., 2011, 2013). Nevertheless, it is still far from complete understanding of the transcription factors functioning in the regulation of gene expression specifically in neonatal muscle fibers produced by indeterminate muscle growth.

To reveal the scheme of indeterminate muscle growth is also related to understanding sarcopenia in mammals. Mammalian skeletal muscles undertake marked biological aging called sarcopenia, the degenerative loss of skeletal muscle mass, quality, and strength associated with aging. Especially in humans, sarcopenia is a critical problem globally, related with increasing of aged population (Clark & Manini, 2008, 2010). A variety of studies using mammal models like mice and rats have recognized several genes implicated in senescence, with appropriate genetic modifications resulting in a marked interruption in the senescence of various organs, including skeletal muscle (Froehlich et al., 2013a). On the other hand, these mammal models simply

showed interruption in senescence and ultimately still achieve a severe sarcopenia phenotype. In this regard, teleosts are an outstanding model because the organismal senescence of their skeletal muscles up to date an immense structure during a method to control sarcopenia which can be revealed (Froehlich et al., 2013a). However, the molecular mechanisms of transcriptional regulation accountable for the indeterminate muscle growth found in teleosts are utterly unknown. Therefore, our present study was to reveal the *cis*-regulatory elements contained in the torafugu $MYH_{M2528\text{-}1}$ promoter by *in vivo* reporter assay. The transient transgenic analyses by using reporter vector containing various deletion and mutation forms of the 5'-flanking region of a certain gene are efficient for identifying *cis*-acting elements accountable for its expression. Here, we used zebrafish to examine the regulatory mechanisms concerned in the expression of torafugu $MYH_{M2528\text{-}1}$, as it is a profitable animal model to generate transgenic lines and are easily accessible to transient reporter analysis for quantification. As well, transgenic zebrafish that articulate enhanced green fluorescent protein (EGFP) under the control of muscle-specific promoters are helpful for visualizing active gene expression outlines, and dissecting regulatory transcription network in live embryos (Jin et al., 2009; Park et al., 2009). In chapter 1, we reported the isolation of the upstream promoter region of torafugu $MYH_{M2528\text{-}1}$ and demonstrated that the 2.1 kb 5'-flanking region regulated developmental muscle-specific expression in zebrafish embryos.

In this chapter, we attempted to identify *cis*-elements responsible for the $MYH_{M2528\text{-}1}$ promoter activity. As shown in Chapter 1, several putative transcription factor binding sites for MyoD, Pax3, MEF2, and NFAT were retrieved from the $MYH_{M2528\text{-}1}$ promoter sequence. We constructed

deletion mutants lacking above transcription factor binding sites individually or in combination and revealed that these binding sites actually function in the promoter activity.

3.2. Materials and methods

3. 2.1 Experimental fish

Torafugu larvae at 10days post-fertilization (dpf) stage and the skeletal muscle of an adult torafugu *T. rubripes* (body mass ~ 1 kg) (Oshima Fishery Hatchery Co., Ltd, Nagasaki, Japan) were used for the RNA extraction. As well, from embryo to adult zebrafish *D. rerio* were raised at the zebrafish rearing facility at the Department of Aquatic Bioscience, The University of Tokyo which used for RNA collection. Therefore, 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).

3.2.2 Generation of deletion constructs of various cis-elements

Inverse PCR-based site-directed mutagenesis technique was used to generate the deletion and mutation constructs of MyoD, Pax3, MEF2 and NFAT binding sites. Specific forward and reverse primers were designed for the deletion of *cis*-elements (Table 3-1). 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 DNA Ligation Kit (Mighty Mix-Takara, Tokyo, Japan). The plasmid DNA for

microinjection was isolated from the positive colony of each of deletion and mutation constructs using GenElute^{TM} plasmid Mini-prep Kit (Sigma-Aldrich).

Table 3-1: Nucleotide sequences of oligonucleotide primers used in the generation of deletion constructs of various *cis*-elements from P2100 constructs of $MYH_{M2528\text{-}1}$

Experiment	Primer Name	Neolceotide Sequence (5′……..…3′)	Length (bp)
MyoD binding site deletion	P2100ΔMyoD-1F	AGGTAAAAATGTGACAGTTAACC	23
	P2100ΔMyoD-1R	ACACTTACTGTATACAAAGGCTA	23
	P2100ΔMyoD-2F	TTAGAGAATGCATAGCCCCCTT	22
	P2100ΔMyoD-2R	GGCTAACAGCTCTTCTTCCATT	22
Pax3 binding site deletion	P2100ΔPax3-1F	GCCTGTAACTAAAGACTTGAC	21
	P2100ΔPax3-1R	GGTTTCTCGTATTTTCTCTACGT	23
	P2100ΔPax3-2F	ATAACTATGGCTGCACAAGCG	21
	P2100ΔPax3-2R	GTAGACATAGTTTATGAAGCATCTTT	26
MEF2 binding site deletion	P2100ΔMEF2-1F	CAACACAAAGAGAACAATGCACA	23
	P2100ΔMEF2-1R	GACAAGTTTATAATGACCACAATTAGT	27
	P2100ΔMEF2-2F	GCCTCTAGAGCATAAATTTGAGG	23
	P2100ΔMEF2-2R	TATCTGTCTGTCCTCTGTCTGT	22
	P2100ΔMEF2-3F	ACAGCTACAATTAATAACTATGGCT	25
	P2100ΔMEF2-3R	TATGAAGCATCTTTCATGCAATATCA	26
	P2100ΔMEF2-4F	TTTCTTTTGAATATTTAATGTG	22
	P2100ΔMEF2-4R	TAGTTAAATCCCTTAGTTTTT	21
NFAT binding site deletion	P2100ΔNFAT-1F	GATGAATTGCAGGTGCTGAAAG	22
	P2100ΔNFAT-1R	CATATGTCATTCACACTAATTCTGCA	26
	P2100ΔNFAT-2F	TCCCTCCTTTTCTTTAAAGCTC	22
	P2100ΔNFAT-2R	AGGGGATTATTTCTCTCAGCA	21
	P2100ΔNFAT-3F	GAAAGATGCTTCATAAACTATGTC	24
	P2100ΔNFAT-3R	ATTTCTTCATTTTCTTTCTTTCTTTCT	27

3. 2.3 *In vivo* reporter assay

Each EGFP reporter construct was diluted to 100 ng/µl 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.

3.2.4 Immunohistochemical analysis

For immunohistochemistry, larvae were fixed with 4% PFA in Tris-buffered saline (25 mM Tris-HCl[pH 7.4], 137 mM NaCl, 2.7 mM KCl) 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) 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).

3.2.5 Real-time PCR analysis

For relative quantification, the reaction was performed in a total volume of 20 μl, containing 10 μl 2×SYBR premix Ex TaqII 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 in 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* gene were listed in Table 3-2.

Table 3-2. Nucleotide sequences of oligonucleotide primers used in real-time PCR analysis

Experiment	Primer Name	Nucleotide Sequence (5′……..…3′)	Length (bp)
Real-time PCR analysis	*EGFP* -Forward	AGC AAA GAC CCC AAC GAG AA	20
	EGFP-Reverse	GCG GCG GTC ACG AAC TC	17
	ß-actin-Forward	TGC TGT TTT CCC CTC CAT TG	20
	ß-actin-Reverse	TCT GTC CCA TGC CAA CCA T	19

3.2.6 Validation of transcription factor using RT-PCR through gene specific primer

For partial amplification of MyoD, Pax3, MEF2 and NFAT, cDNA sequences of both zebrafish and torafugu were collected from ensemble genome browser (http://www.ensembl.org), National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Fugu Genome Project (http://www.fugu-sg.org/). Gene specific primer pairs were designed using Primer 3 software-web version 4.0.0 (http://primer3.ut.ee/) based on sequences of zebrafish and torafugu collected sequences. The primer sequences are given in Table 2. Total RNA from embryo, larvae and adult of zebrafish and torafugu was extracted with ISOGEN solution (Qiagen, Germany) and then

single-strand cDNA was synthesized using PrimeScriptfirst strand cDNA synthesis kit (Takara, Tokyo, Japan). PCR was performed following the procedure as per the manufacturer's instruction for 35 cycles. All test samples were amplified simultaneously from equal volume of first strand cDNA with the particular primer pair using a master PCR mix. For each reaction, master PCR mix contained PCR buffer, 2 mM of dNTPs, 0.2mM of each primer, template cDNA, and 1.0 unit of Ex-taq DNA polymerase (Takara, Tokyo, Japan). Subsequently, PCR reactions were run in a programmable thermal cycler (Applied Biosystems). PCR products were run in 1.5 % agarose gel and visualized by gel documentation system (Bioinstrument, ATTO, Tokyo, Japan) after staining with Syber safe.

Table 3-3: Nucleotide sequences of oligonucleotide primers used in the validation of transcription factor in zebrafish and torafugu

Experiment	Primer Name	Neolceotide Sequence (5′……..…3′)	Length (bp)
Zebrafish Pax3	Pax3aF	TGCAATTAGCCACCTTGTTCA	21
	Pax3aR	AGGTTCCAATGAGCCCAAA	19
	Pax3bF	GCCCATCACTCTCTCCAAACTATCC	25
	Pax3bR	TCGTCAGCTCGAGTGCCA	18
Zebrafish NFAT	NFATC1F	TCTCTTGACTGGAATACACGCTTA	24
	NFATC1R	AGGAAGGTTGGCTGATGGAT	20
	NFATC2F	AACCAAACATCCAGTAACAGCA	22
	NFATC2R	TTCATCCACTTTCCCTCCAC	20
	NFATC3F	TCACCCTGAACACACCAATC	20
	NFATC3R	CCACCCATCACACCTTGAG	19
Zebrafish MyoD	MyoDF	TTTCTTTCTTTATGCGGTGGA	21
	MyoDR	TGTTCGTTTTCGTCGCTTT	19
Zebrafish MEF2	MEF2AF	AGCTCCTCCTGCTCCTCCT	19
	MEF2AR	TCTTTCCCCGTCCACCTAC	19
Torafugu NFAT	NFATC1	ATGAGGTGGAGGAGAGCAGC	20
	NFATC1	ATCTGAGGGGAGGTGGATG	19
Torafugu Pax3	Pax3-1F	TCGGGCTACTCGGACAGC	18
	Pax3-1R	ACTTTGTCCGTACTGGCCGT	20
	Pax3-2F	AGGTGATGGGTTTGCTGAACCC	22
	Pax3-2R	ACTTTGTCCGTACTGGCCGT	20
Torafugu MyoD	MyoDF	TGCTGCTCTTCATCCTCACTC	21
	MyoDR	TTCGGGTTGGTGTTTGTGC	19
Torafugu MEF2	MEF2F	TGCGTAGGAAGGGTGTGG	18
	MEF2R	TCCAGCGACCGATTCAGG	18

3.2.8 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. Data were represented as the mean ± SD and the differences were considered significant at $P<0.05$.

3.3. Results

3.3.1 MyoD binding sites are involved in the transcriptional control of *MYH*$_{M2528\text{-}1}$ expression in skeletal muscle

Promoter deletion analyses proved that EGFP expression in myotomal region usually linked with a smaller *MYH*$_{M2528\text{-}1}$ promoter region (Chapter 1). These results designated that the key *cis*-acting element(s) that control the transcription of *MYH*$_{M2528\text{-}1}$ is located within the 2,100 bp 5'-flanking of *MYH*$_{M2528\text{-}1}$. Analysis of the 2,100 bp 5'-flanking region by Genomatix Matinspector and TFsearch programs identified two putative MyoD binding sites, designated as MyoD_1 and MyoD_2. MyoD is a representative myogenic regulatory factor for muscle differentiation (Chanoine et al., 2004) and account to be involved in the transcriptional regulation of muscle-specific genes during development in fish (Liang et al., 2008). To determine the role of these MyoD binding sites, we deleted MyoD_1 and MyoD_2 sites. All deletion mutant constructs were microinjected into zebrafish embryos for transient expression analysis. Single deletion of MyoD_1 or MyoD_2 site significantly reduced EGFP expression in muscle fibers compared to the embryos injected with the P2100, and double deletion of both MyoD_1 and MyoD_2 binding

sites showed more pronounced reduction of EGFP expression (Fig. 3-11B-E). On the other hand, fluorescent optics also demonstrated that EGFP expression levels in myotomal compartments were different from embryo to embryo. Therefore, EGFP-expressing embryos were grouped into very high (>200), high (200-100), medium (50-100), low (10-50) and very low (<10) categories. The deletion of MyoD binding sites significantly reduced the number of muscle fibers expressing EGFP per embryos (Fig. 3-1C-F). As well, reduction of such EGFP expression in MyoD binding sites deletion constructs were also confirmed by using real-time PCR analysis (Fig. 3-2).

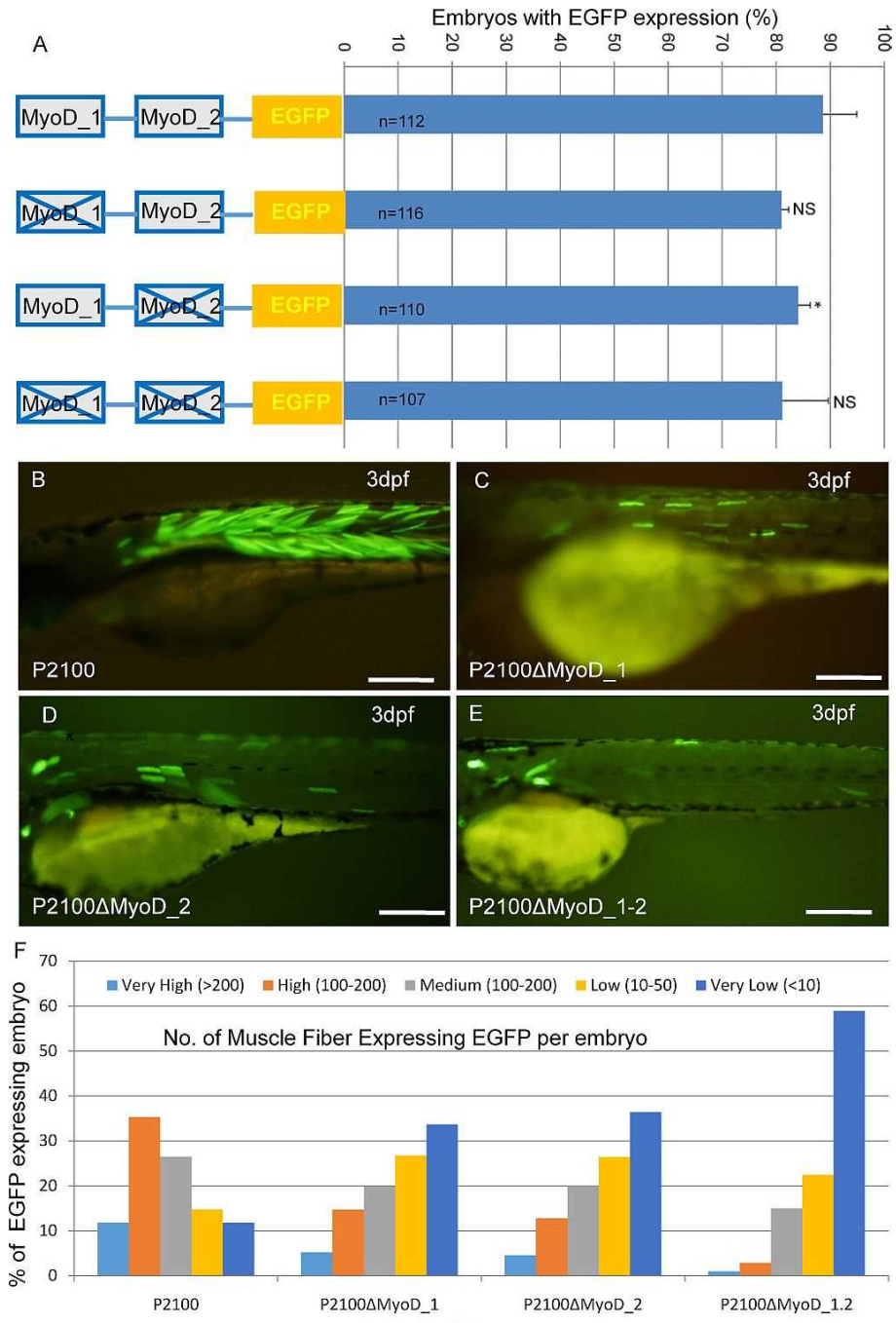

Fig. 3-1: MyoD binding *cis*-elements play a key role in the transcriptional regulation of$MYH_{M2528\text{-}1}$ expression. A) Bar graph showing percentages of embryos that expressed EGFP in hyperplastic muscle fibers in microinjection with different MyoD binding elements deletion constructs. B-E) all panels are side views showing EGFP expression in myotomal muscles fibers of zebrafish larvae at 3dpf in different MyoD binding sites deletion constructs. J) Bar graph showing the number of EGFP expressing myotomal muscle fibers per embryo in different MyoD binding sites deletion constructs. Scale bars 50 µm. * P<0.05.

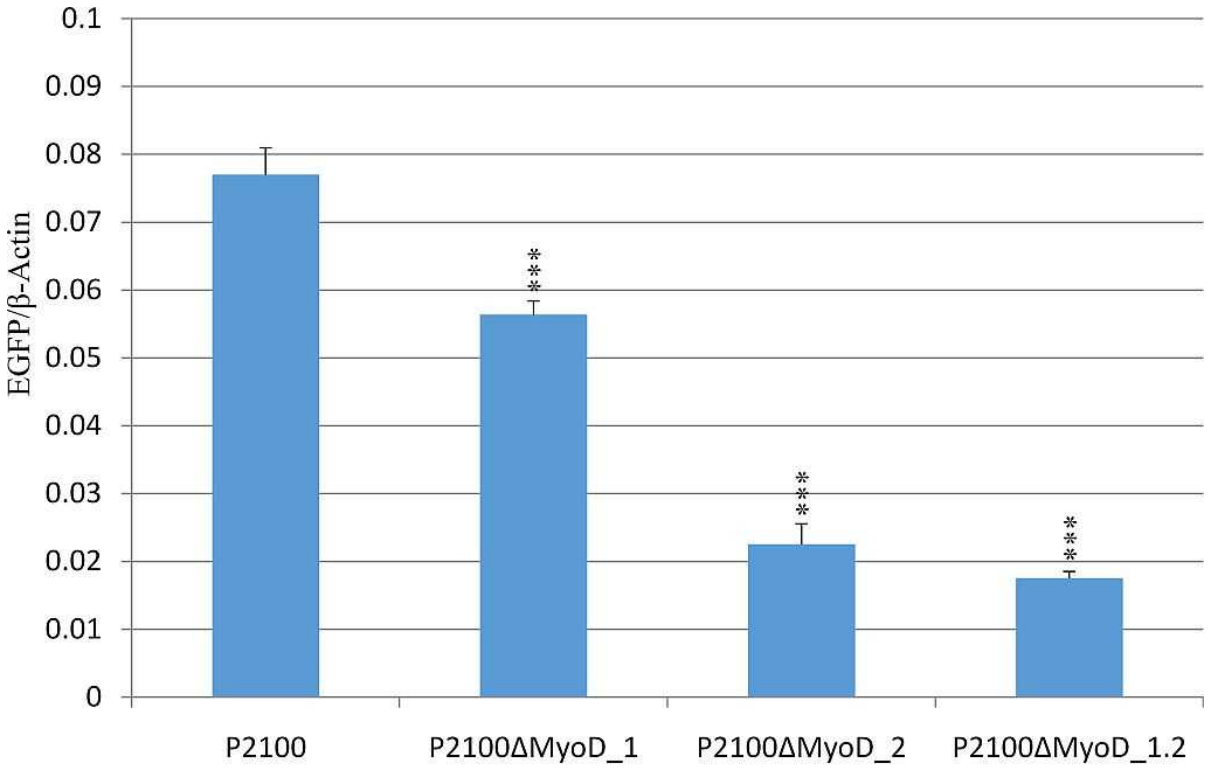

Fig. 3-3: Bar graph showing reduced relative EGFP expression in the P2100ΔMyoD_1, P2100ΔMyoD_2 and P2100ΔMyoD_1.2 constructs using real-time PCR analysis. Difference are significant in ANOVA followed by Tukey test at ***P<0.001.

Furthermore, immunohistochemistry of MyoD binding sites deleted larvae at 3dpf stage was performed. Reduction of EGFP expression by single and double deletion of MyoD binding site, was confirmed in both fast and slow muscle region (Fig. 3-3A-R).

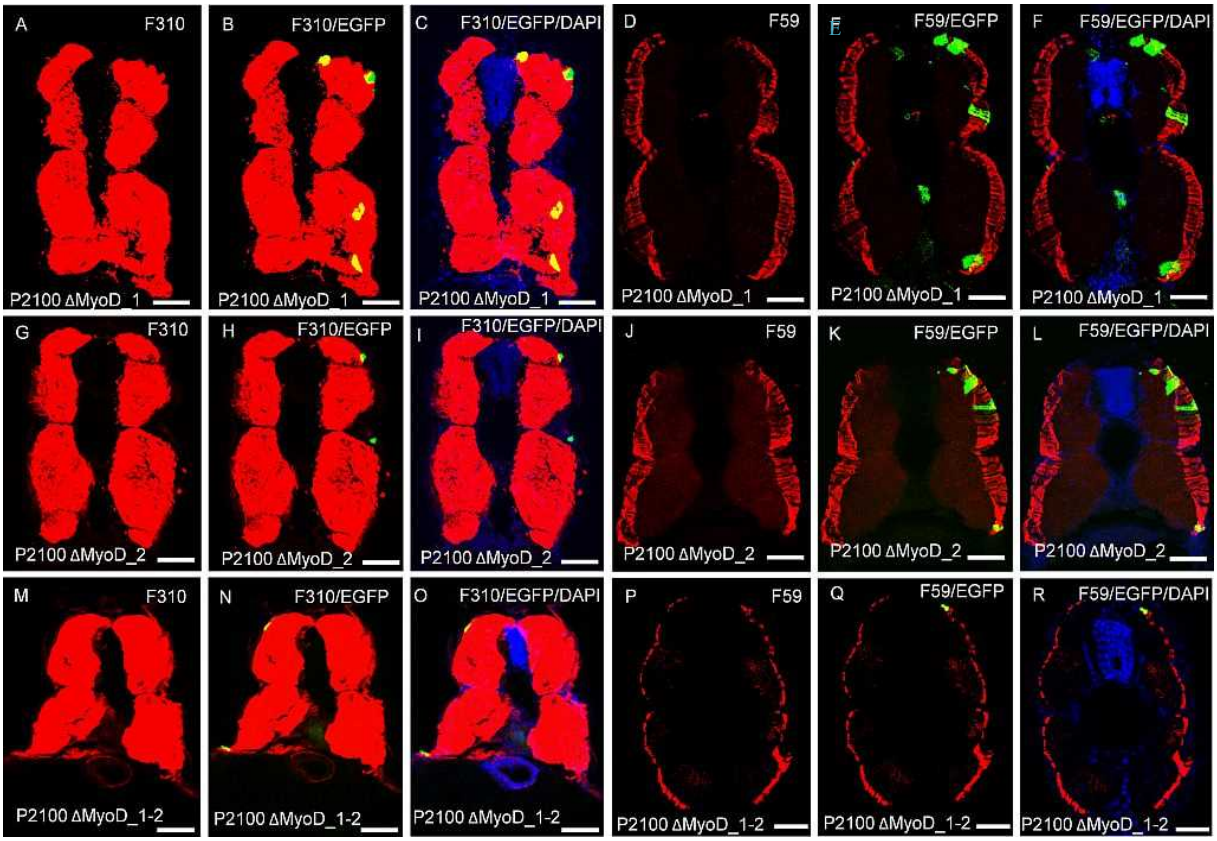

Fig. 3-3: Immunohistochemistry localizing EGFP expression in zebrafish muscle fibers due to the deletion of MyoD binding sites. A-F) EGFP expression was localized in both slow and fast muscle as reacted with F59 and F310 antibodies in larvae microinjected with the individualMyoD_1deletion construct;. G-L) Enhanced EGFP expression was also localized in slow muscle fibers in larvae microinjected with MyoD_2 binding site deletion construct. M-R) Embryos microinjected with the deletion construct of P2100ΔMyoD_1.2also did not show EGFP expression in fast muscle fibers. Scale bars: 20µm.

These data indicate that MyoD binding sites actually participate in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression. However, it should be noted that EGFP expression was not completely inhibited in skeletal muscles in all MyoD binding sites deletion constructs.

3.3.2 Pax3 binding sites are implicated in the transcriptional control of $MYH_{M2528\text{-}1}$ expression in zebrafish skeletal muscle

As like MyoD, Genomatix Matinspector and TFsearch programs also recognized two putative Pax3 binding sites in 2,100 bp 5'-flanking region of $MYH_{M2528\text{-}1}$. These Pax3 binding sites were designated as Pax3_1 and Pax3_2. Pax3 is a marker of myogenic precursor cells and appears to be highly expressed throughout myoblast proliferation and into differentiation in teleost (Froehlich et al., 2013a). Recently, Rescan et al. (2013) reported that Pax3 is upregulated during hyperplasia in rainbow trout. Fish species like salmonids and giant danio are able to accomplish continued growth well into adulthood through self-renewal of muscle pioneer cells via a Pax3 dependent mechanism (Froehlich et al., 2013b).

Therefore in this study, we assumed that thus putative Pax3 binding sites may be involved in transcriptional regulation of $MYH_{M2528\text{-}1}$. To conclude the role of these Pax3 binding sites, we deleted these sites individually or in various combinations (Fig. 3-4). Here, all mutant constructs were microinjected into zebrafish 1 or 2 cell stages. As a result, deletion of Pax3 binding sites significantly reduced EGFP expression compare with wild type-construct (P2100) (Fig. 3-4A).

Deletion of both two Pax3 binding sites showed in a mark reduction in the number of EGFP expressing embryos (Fig. 3-4B-E). However, fluorescent microscope observation demonstrated that EGFP expression levels in myotomal compartments were vary from embryo to embryo. Therefore, EGFP-expressing embryos were categorized into very high (>200), high (200-100), medium (50-100), low (10-50) and very low (<10) groups. Among embryos injected with Pax3 binding sites deletion construct, highest number of embryo were classified in low expression groups (Fig. 3-4F). Reduction of such EGFP expression were also confirmed by using real-time PCR analysis (Fig. 3-5).

Furthermore, immunohistochemistry was performed to detect what muscle type expresses EGFP after microinjection. Pax3_1 deletion construct showed EGFP expression only in slow muscle but not in fast muscle fibers (Fig. 3-6A-F). Pax3_2 deleted construct showed EGFP expression only in fast muscle (Fig. 3-6G-L). Correspondingly, double deletion of Pax3_1 and Pax3_2 sites showed EGFP expression only in fast muscle (Fig. 3-6M-R). As a whole, these data proposed that Pax3 binding sites take part in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression.

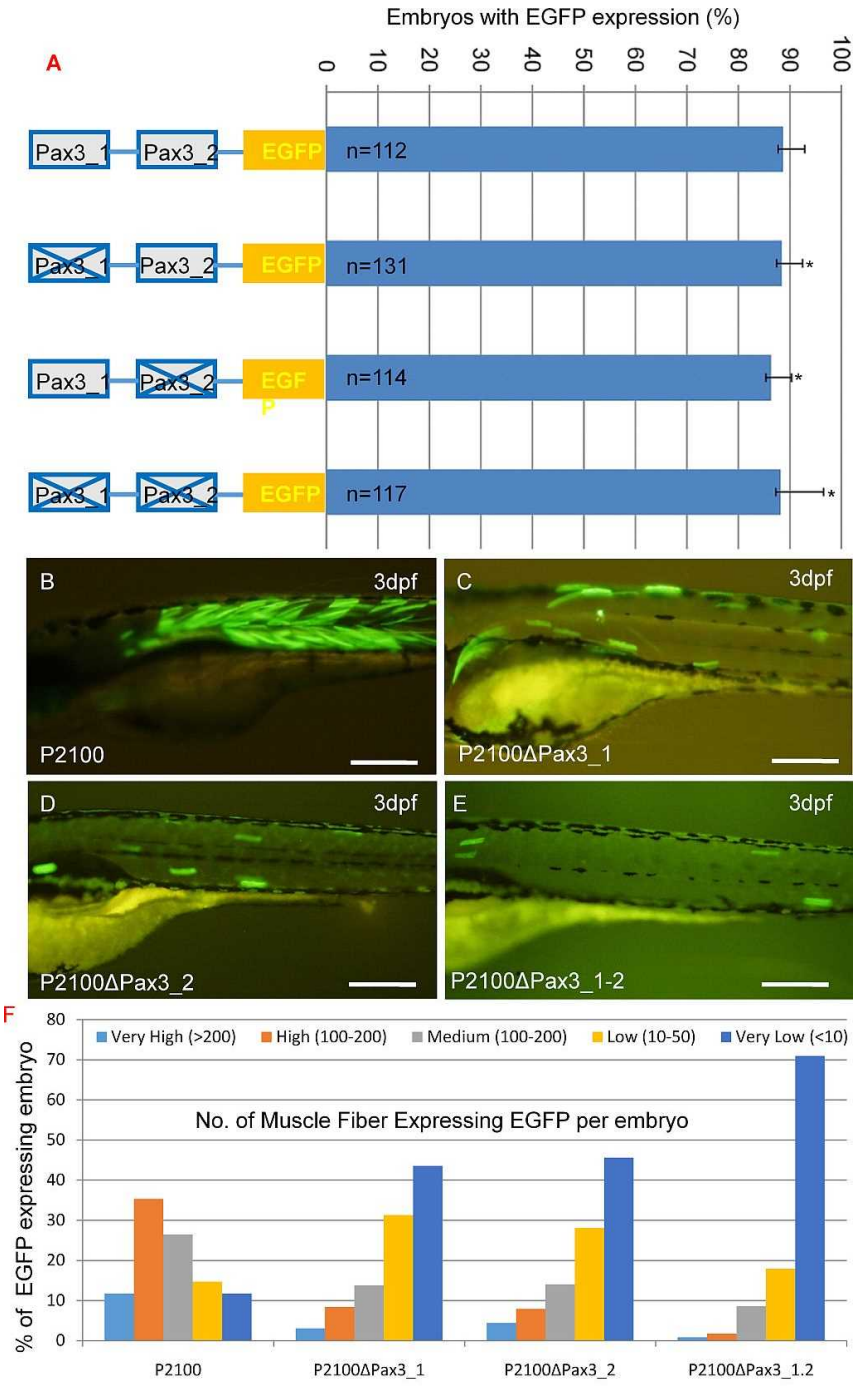

Fig. 3-4: Pax3 binding *cis*-elements play a key role in the transcriptional regulation of$MYH_{M2528\text{-}1}$ expression. A) Bar graph showing percentages of embryos that expressed EGFP in hyperplasic muscle fibers in microinjection with different Pax3 binding elements deletion constructs. B-E) all panels are side views showing EGFP expression in myotomal muscles fibers of zebrafish larvae at 3dpf in different Pax3 binding sites deletion constructs. J) Bar graph showing the number of EGFP expressing myotomal muscle fibers per embryo in different Pax3 binding sitesdeletion constructs. Scale bars 50 µm. * P<0.05.

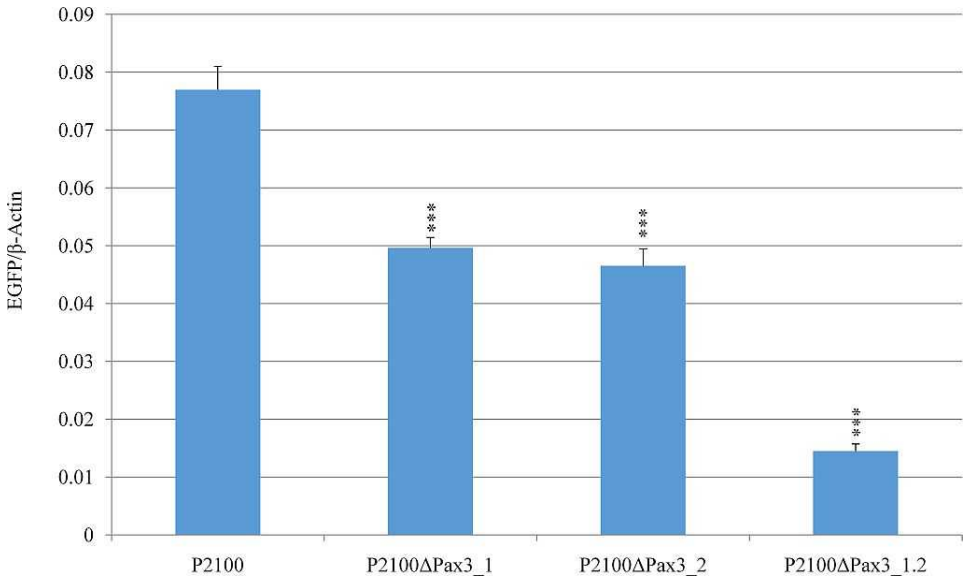

Fig. 3-5: Bar graph showing reduced relative EGFP expression in the P2100ΔPax3_1, P2100ΔPax3_2 and P2100ΔPax3_1.2 constructs using real-time PCR analysis. Difference are significant in ANOVA followed by Tukey test at ***<0.001.

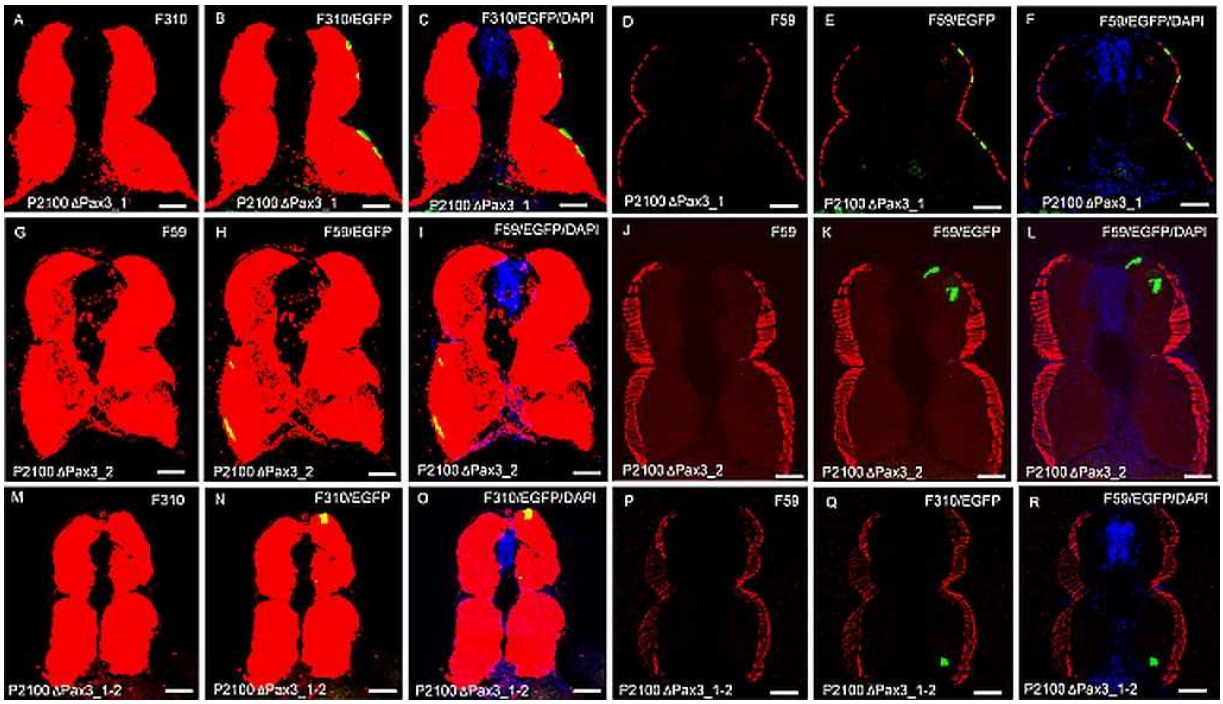

Fig. 3-6: Immunohistochemistry of all Pax3 mutated zebrafish at 3dpf stage. A-F) EGFP expression was localized in slow muscle due to deletion of Pax3_1 binding site. G-L) EGFP expression was also detected in slow muscle fibers due to deletion of Pax3_2 binding site. M-R) Embryos microinjected with the deletion construct of P2100ΔPax3_1.2 also did not show EGFP expression in slow muscle fibers. Scale bars: 20µm.

3. 3.3 MEF2 elements participate in the transcriptional regulation of *MYH*$_{M2528-1}$expression

Analysis of the 2,100 bp 5′-flanking region of *MYH*$_{M2528-1}$ by the Genomatix Matinspector program identified four putative MEF2 binding sites spanning the region at -1827/-1804 (MEF2_1), 1643-/-1619 (MEF2_2), -651/-633 (MEF2_3) and 84/-61 (MEF2_4) (Fig.2-2). MEF2 family members have been reported to be involved in muscle differentiation (Chanoine et al., 2004). Therefore, we deleted these MEF2 binding sites individually and in various combinations from the P2100 construct to determine their role in the promoter activity (Fig. 3-7). Deletion mutant constructs were microinjected into zebrafish embryos for transient expression analysis. Single deleting of any one of these four MEF2 binding sites reduced the *MYH*$_{M2528-1}$ promoter activity (Fig. 3-7B). Therefore, we decided to generate the multiple MEF2 binding site-deletion mutant constructs (Fig. 3-7). Deleting multiple MEF2 binding sites significantly reduced EGFP expression percentages in muscle fibers (Fig. 3-7A). Moreover, fluorescent optics showed that deleting all four MEF2 binding sites resulted in significant reduction of EGFP expression in skeletal muscle (Fig 3-7C-I). EGFP-expressing embryos were also categorized by number of EGFP positive fibers into very high (>200), high (200-100), medium (50-100), low (10-50) and very low (<10) groups. Here, each deleted MEF2 binding sites construct microinjected into zebrafish embryo demonstrated highest number of embryo which included in low groups (Fig. 3-7J).

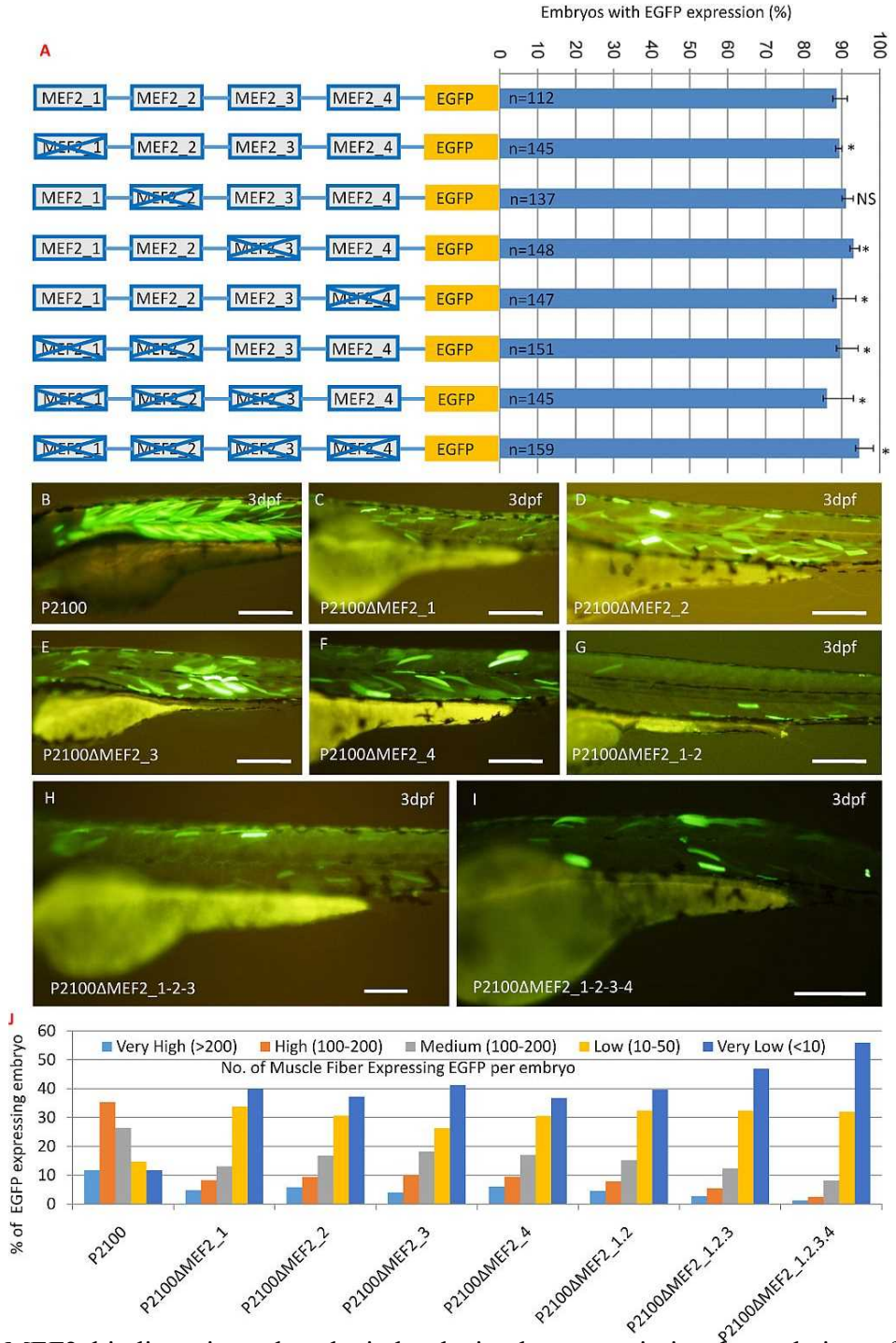

Fig. 3-7:MEF2 binding sites played vital role in the transcriptional regulation of*MYH*$_{M2528\text{-}1}$ expression. A) Bar graph showing percentages of embryos that expressed EGFP in zebrafish with MEF2 deletion constructs. C-I) all panels are side views showing EGFP expression in zebrafish larvae at 3dpf in different MEF2 binding sites deletion constructs. J) Bar graph showing the number of EGFP expressing myotomal muscle fibers per embryo in different MEF2 binding sites deletion constructs. Scale bars 50 µm. * P<0.05.

As well, these results have also been validated by RT-PCR experiment showing reduced EGFP expression (Fig. 3-8). Immunohistochemistry showed that EGFP was detected only in slow muscle fibers by MEF2 binding site deletion in zebrafish larvae at 3dpf stages (Fig. 3-9A-P). Therefore, these data indicated that these MEF2 binding sites in the $MYH_{M2528\text{-}1}$ promoter were involved in $MYH_{M2528\text{-}1}$ promoter expression in zebrafish. However, it should be noted that EGFP expression was not completely inhibited in the skeletal muscle, suggesting additional regulatory sequences might also be involved in regulating $MYH_{M2528\text{-}1}$ gene expression in skeletal muscles.

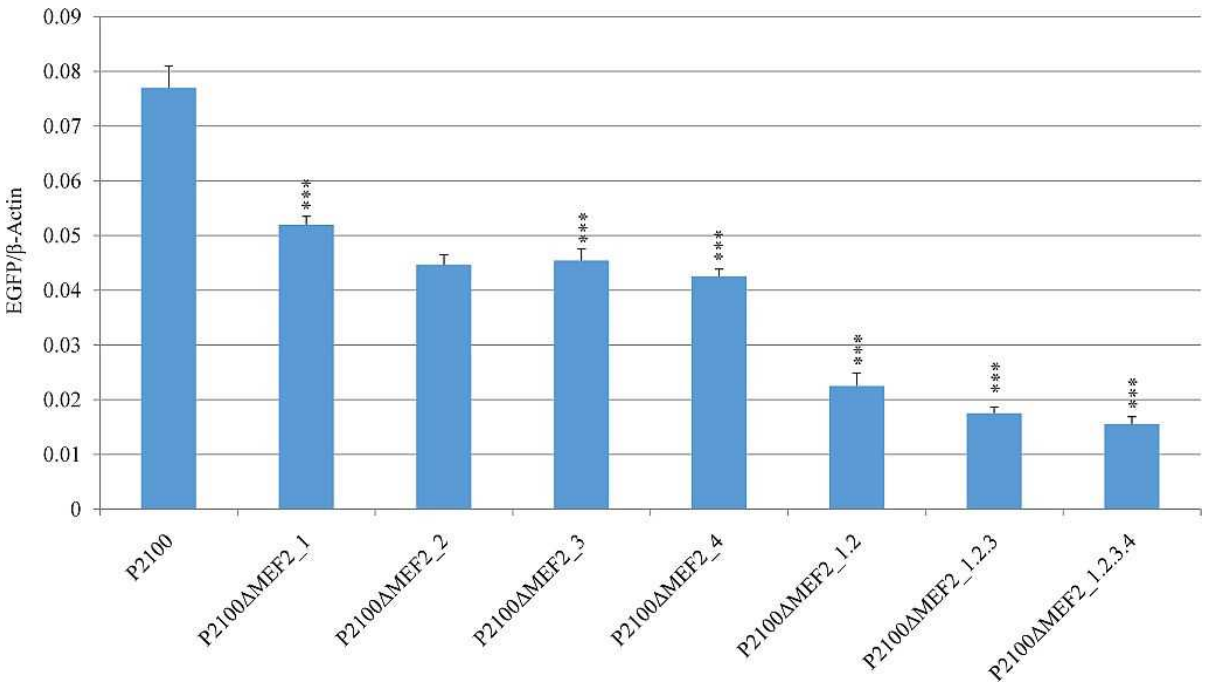

Fig. 3-8: Bar graph showing reduced relative EGFP expression in the multiple deletion construct of MEF2 using real-time PCR analysis. Difference are significant in ANOVA followed by Tukey test at ***<0.001.

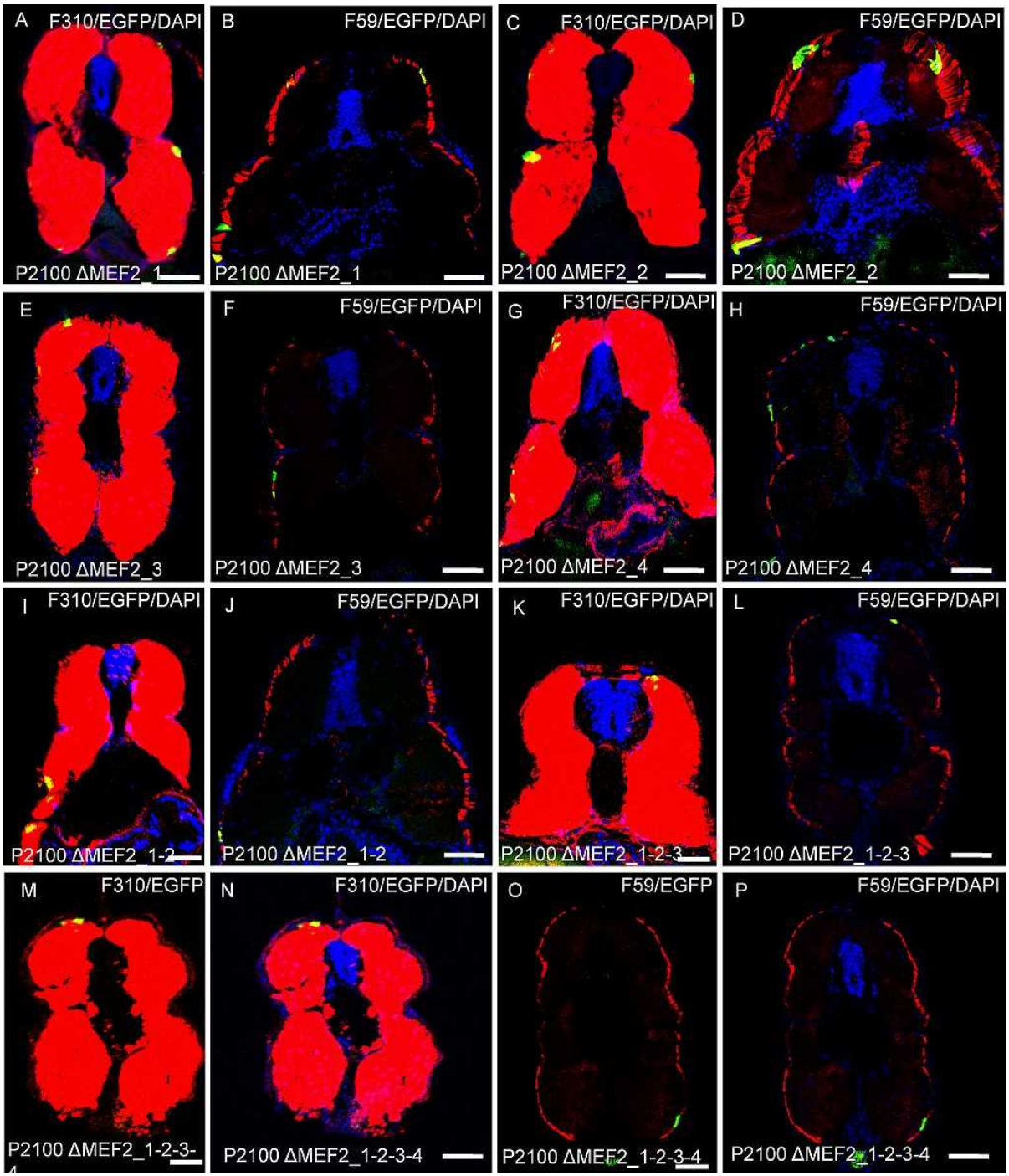

Fig. 3-9: Immunohistochemistry of all MEF2 mutated zebrafish larvae at 3dpf stage. A-P) EGFP expression was localized in slow muscle of zebrafish larvae due to all deletion of MEF2 binding sites where majority did not show EGFP expression in fast muscle fibers. Scale bars: 20µm.

3.3.4 NFAT elements play a significant role in the transcriptional regulation of *MYH$_{M2528\text{-}1}$* expression in zebrafish larvae

Genomatics software found three putative NFAT binding sites spanning the region at 2076/-2072 (NFAT_1), -1151/-1147 (NFAT_2), and -684/-666 (NFAT_3) within this 2100-bp promoter region of *MYH$_{M2528\text{-}1}$* (Fig. 2-2), focusing to be involved to induce neonatal and embryonic *MYHs* in neonatal muscle fibers produced by regeneration after injury in the adult mouse (Daou et al., 2013). Therefore, we hypothesized that these NFAT binding sites might have a significant role in the transcriptional regulation of *MYH$_{M2528\text{-}1}$* in relation to indeterminate muscle growth. To examine whether NFAT binding sites is functional in the transcriptional regulation of *MYH$_{M2528\text{-}1}$*, we carried out an experiments using deleted promoter constructs in which one site, multiple sites in combination, or all of the NFAT binding sites were deleted from P2100 constructs. Zebrafish embryos microinjected with the deletion constructs of NFAT_1, NFAT_2, NFAT_1.2 and NFAT_1.3 showed significantly reduced EGFP expression in muscle fibers compared to the P2100 injected embryos, and deletion of multiple and all the NFAT binding sites have more distinct effects (Fig. 3-10A). The deletion of NFAT binding sites also reduced the number of EGFP expressing muscle fibers per each embryo in compared to control (Fig. 3-10B-G). Immunohistochemistry showed that deletion of NFAT_1 binding sites larvae showed EGFP expression in slow muscles (Fig. 3-11A-D). NFAT_2 and NFAT_3 deleted larvae showed low number of EGFP expression in both slow and fast muscles (Fig. 3-11E-L) whereas in combined deletion showed only in slow muscle (Fig. 3-11M-T). These results clearly demonstrated that NFAT binding sites play a significant role in the transcription regulation of *MYH$_{M2528\text{-}1}$* expression. However, the deletion of all NFAT binding sites could not completely abolish the EGFP expression. These data suggest that NFAT binding sites, together with other *cis*-elements

are necessary for directing the $MYH_{M2528\text{-}1}$ expression in relation with muscle hyperplasia.

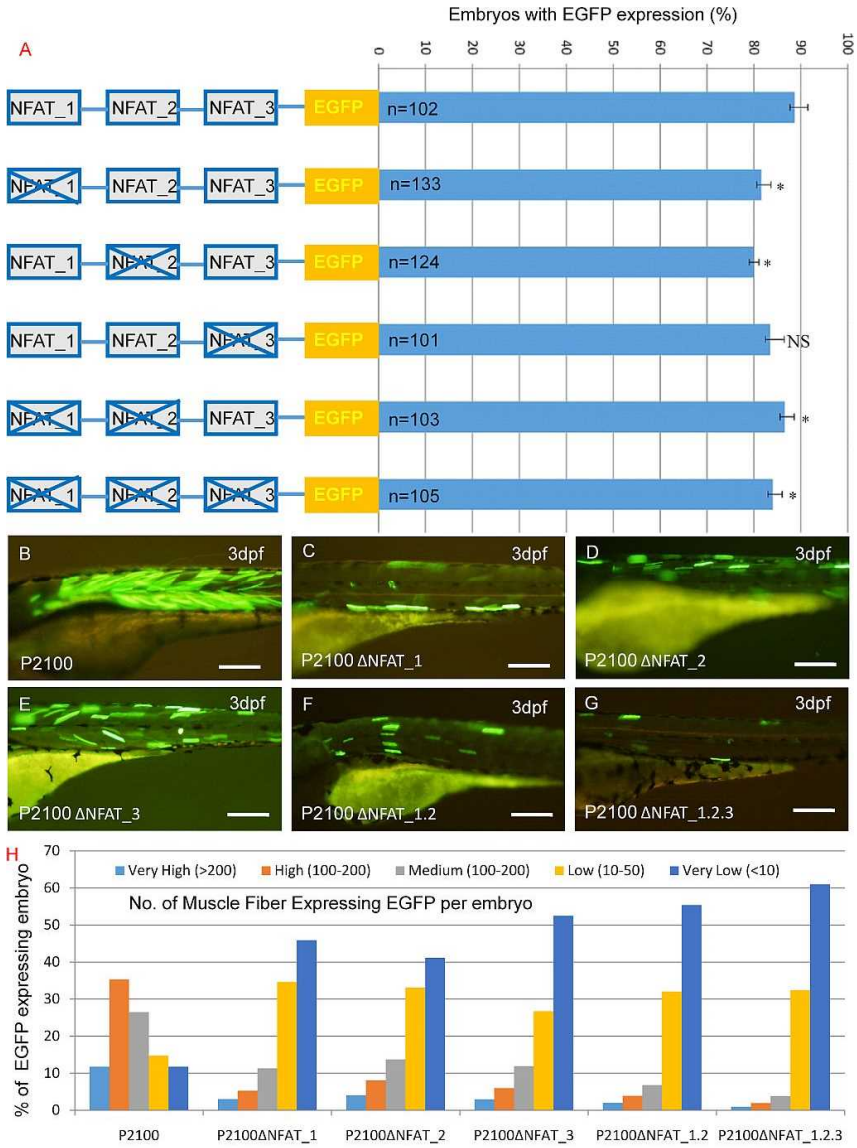

Fig. 3-10: NFAT binding *cis*-elements participate a key role in the transcriptional regulation of$MYH_{M2528\text{-}1}$ expression. A) Bar graph showing percentages of embryos that expressed EGFP in zebrafish with NFAT deletion constructs. B-G) all panels are side views showing EGFP expression in zebrafish larvae at 3dpf in different MEF2 binding sites deletion constructs in compared to control. J) Bar graph showing the number of EGFP expressing in myotomal muscle per embryo in different NFAT binding sites deletion constructs. Scale bars 50 µm. * P<0.05.

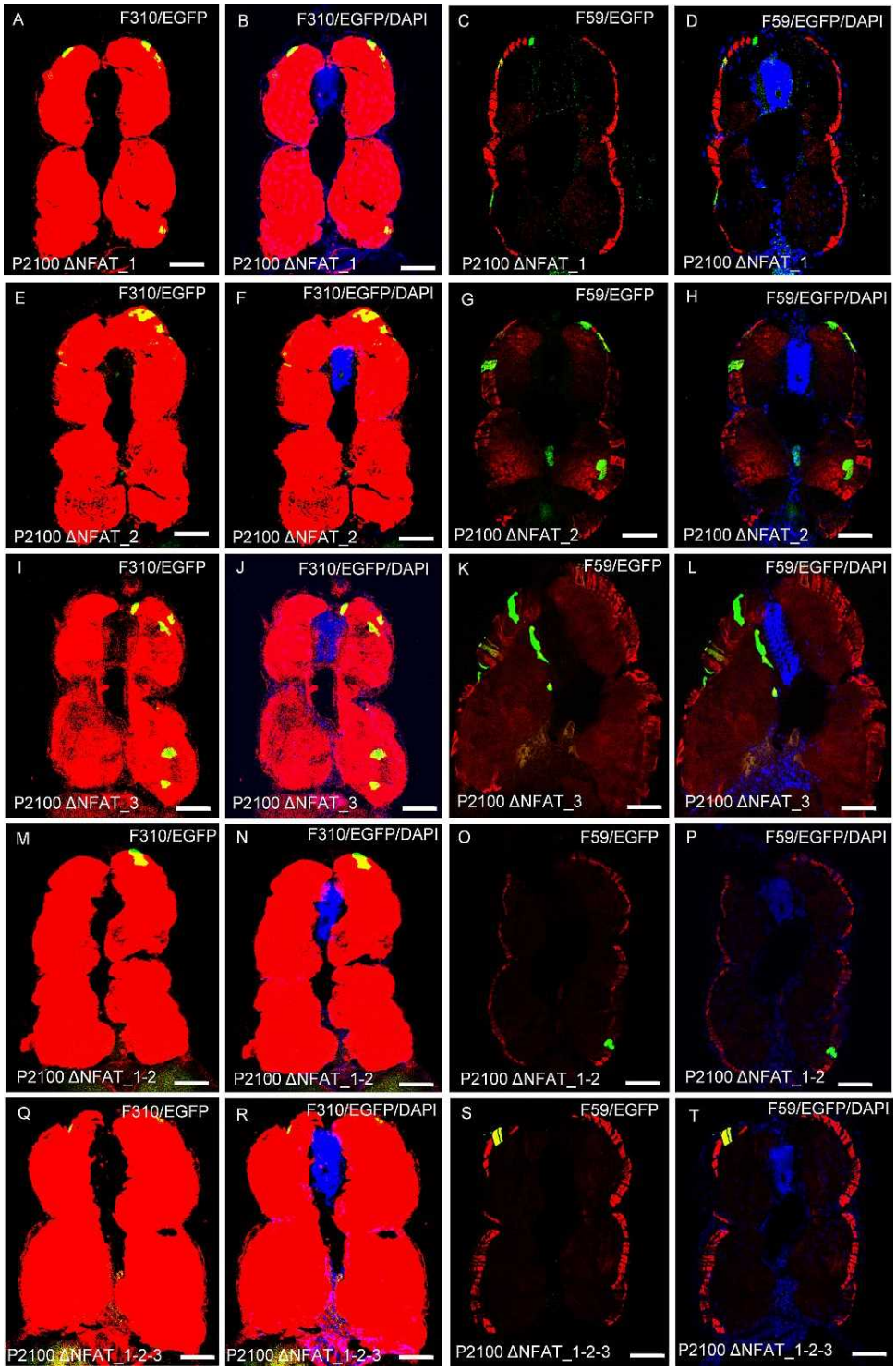

Fig. 3-11: Immunohistochemistry of all NFAT mutated zebrafish larvae at 3dpf stage. A-D) EGFP expression was detected in slow muscle of zebrafish larvae due to NFAT_1 binding site. E-T) Low EGFP expression was found in both slow &fast muscle fibers after deletion of NFAT_2 and NFAT_3 whereas deletion of their combination showed in slow muscle . Scale bars: 20µm.

3.3.5Validation of expression of transcription factors

We have validated the expression of four transcription factor family members, myoD, Pax3a, Pax3b, Mef2a, and NFATc1, NFATc2, and NFATc3 in zebrafish and myoD, Pax3a Mef2b, and NFATc1 in torafugu during their developmental stages. Among them, MyoD expression was found to be in the after post-natal and in adult stages of zebrafish (Fig. 3-13A-C) but not in embryonic stage.

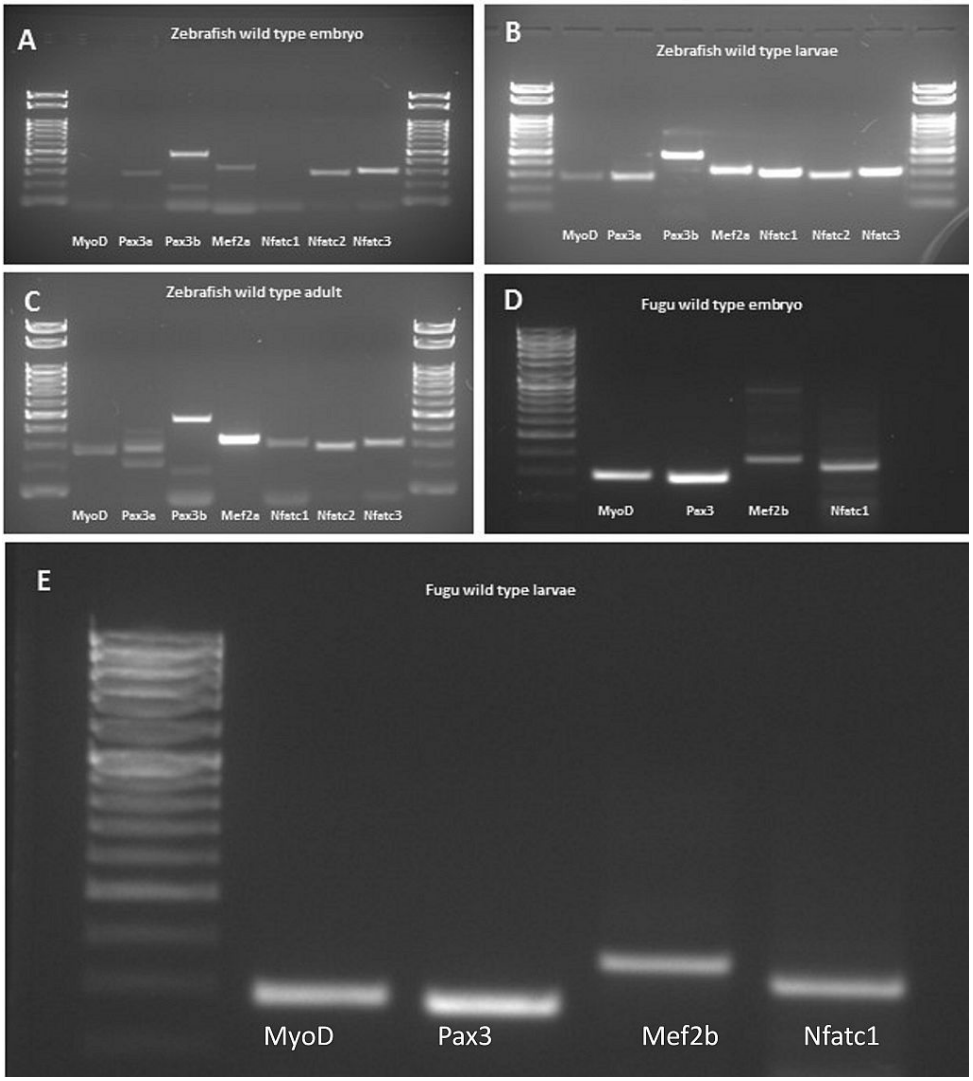

Fig. 3-12: Expression of MyoD, Pax3, MEF2 and NFAT transcription factor in zebrafish (A-C) and torafugu (D-E) as revealed by gene specific primer through RT-PCR.

Pax3 (Pax3a and Pax3b), MEF2 (MEF2b) and NFAT (NFATc1/2/3) were expressed throughout zebrafish life cycles (Fig. 3-12B). As well, these four transcription factors were also detected in torafugu embryo and larval stages (Fig. 3-12D-E). Although all family members were not examined, these results supports our speculation that MyoD, Pax3, MEF2, and NFAT have important function on the $MYH_{M2528\text{-}1}$ promoter activity during teleost hyperplastic muscle growth.

3.4. Discussion

Skeletal muscles in mammals has important role in maintaining posture, locomotion, ventilate the lungs but it is to be documented as an eminent part in the systemic regulation of aging and age-related diseases. It has been reported that age concerning disease like sarcopenia is associated with skeletal muscle mass in human (Ruiz et al., 2008; Demontis et al., 2013). Therefore clear contrast in skeletal muscle growth and aging between mammals and teleost fish is quite attractive. In mammal, growth of skeletal muscle is limited and the mass is reduced by aging, whereas teleost fish showed continuous production of neonatal muscle fibers throughout their lifespan. In this regard, molecular network regulating the expression of $MYH_{M2528\text{-}1}$ which shows characteristic expression correlated with larval and post-larval hyperplasic muscle growth would provide useful approach into how determinate and indeterminate growth are dictated in mammals and teleost fish. 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 growth stage-related *MYH* expression. We reported that the 2.1 kb 5'-flanking region of torafugu $MYH_{M2528\text{-}1}$

found to be involved in indeterminate muscle growth in zebrafish (Chapter 1, Ahammad et al., 2015). This chapter we tried to identify several regulatory elements involved in the hyperplastic expression of $MYH_{M2528\text{-}1}$ in skeletal muscles using zebrafish.

Myogenic regulatory factors like MyoD, myogenin, myf5, and MRF4 have been shown to regulate the transcription of numerous muscle-specific genes. The transcriptional regulation of these factors is often associated with myogenesis during developmental condition. As well, MyoD transcription factors has found to be a milestone in understanding the molecular network controlling the differentiation of the muscle lineage (Chanoine et al., 2004). In the present study, deleting multiple MyoD binding sites significantly reduced $MYH_{M2528\text{-}1}$ promoter activity in myotomal compartments of fish. It has been revealed that the expression of common carp fast skeletal *MYH*, named *FG2*, required the upstream sequence in the 5'-flanking region, which contained MyoD binding site (Gauvry et al., 1996). It was also reported that MyoD and Myf5 binding sites are crucial for myogenesis in individual somites in zebrafish (Hinits et al., 2009). These lines of studies designated that MyoD binding sites are involved in the transcriptional regulation of *MYH*s expression in fish. However, it should be noted that deleting all MyoD binding sites in the torafugu $MYH_{M2528\text{-}1}$ 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.

Paired box transcription factor like Pax3 expression appears to be highly expressed in myoblast differentiation (Froehlich et al., 2013a). It has also been reported that Pax3 may also play a key role in inducing differentiation (Young and Wagers, 2010). In zebrafish, several isoforms of

Pax3 have been identified (Seo et al. 1998). Furthermore, Pax3 may be more applicable to adult growth and myofiber maintenance (Kirkpatrick et al. 2010). Pax3 appears to be most important during primary myogenesis where it does appear to be expressed during postnatal life (Froehlich et al., 2013b). Deleting multiple Pax3 binding sites from 2.1kb 5'-flanking of $MYH_{M2525\text{-}1}$ has significantly reduced the promoter activity in zebrafish muscle fiber. Taken together with previous studies, our results implicated that Pax3 binding sites have significant role to activate the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression in zebrafish during indeterminate muscle growth through muscle hyperplasia.

Previous studies reported that MEF2 binding sites are vital for the transcriptional regulation and muscle differentiation (Chanoine et al., 2004). Also in teleost fish, it has been reported that MEF2 binding sites are essential for temperature-dependent expression of *MYH*s in medaka and common carp (Kobiyama et al., 2006; Liang et al., 2008), transcriptional regulation of fast muscle-specific torafugu $MYH_{M743\text{-}2}$ (Asaduzzaman et al., 2011) and slow muscle-specific torafugu $MYH_{M86\text{-}2}$ (Asaduzzaman et al., 2013). Therefore, it was tremendous interest to understand whether or not the MEF2 binding sites similarly participate in the regulation of torafugu $MYH_{M2528\text{-}1}$ in relation to muscle hyperplasia. Sequence analysis recognized four MEF2 binding elements in the 2100 bp 5'-flanking region of $MYH_{M2528\text{-}1}$ (Fig. 2-2). We revealed that MEF2 participated in the transcriptional regulation of $MYH_{M2528\text{-}1}$ expression of whole myotomal part (Fig. 3-9). In mammals, MEF2 was found to be functional factor in both slow and fast muscle fiber. The involvement of MEF2 in the transcriptional regulation of muscle-specific *MYH*s represents a pathway from calcineurin to DNA (Wu et al., 2000; Mckinsey et al., 2002; Asaduzzaman et al. 2013). However, medaka (*Oryzias latipes*) fast myotomal muscle expressed

different myosin heavy chain genes at certain acclimation temperatures which likely controlled by MEF2 family members (Liang et al., 2008). As well, MEF2 transcription factor participated in some slow fiber-specific genes by the calcium-dependent phosphatase (Wu et al., 2000).Thus, MEF2 appear to be participated in the transcriptional regulation of $MYH_{M2528\text{-}1}$, however, and additional regulatory factors might also be involved in regulating $MYH_{M2528\text{-}1}$ gene expression in skeletal muscles.

TFsearch analysis by genomatics software identified three putative NFAT binding sites in 2100 bp 5'-flanking region of$MYH_{M2528\text{-}1}$ (see Fig. 2-2). Previous studies demonstrated that a novel transcriptional paradigm where NFAT proteins cooperated with MRF during somitogenesis (Armand et al., 2008). As well, NFAT proteins involved in the regulation of the different *MYHs* isoform expression which promotes the growth of myotubes (Rao et al., 1997; Kegley et al., 2001; Horsley et al., 2001;Horsley et al., 2003). Daou et al. (2013) reported that the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway is involved in the modulation of the adult muscle fiber type which played a key role in establishing the early muscle fiber type during neonatal period. Therefore, it was of great interest to examine the role of the NFAT binding sites in the regulation of hyperplastic muscle-specific $MYH_{M25228\text{-}1}$ promoter in zebrafish. Our study revealed that NFAT binding sites play a significant role in the transcriptional regulation of $MYH_{M25228\text{-}1}$ expression (see Fig. 3-10). During adult skeletal muscle myogenesis, the role of NFAT binding sites in the transcriptional regulation of *MYH*s promoters has been documented in mammals (Calabria et al., 2009; Schiaffino, 2010). Rao et al. (1997) identified five NFAT genes and all most of all NFAT proteins are expressed in adult skeletal muscles (Calabria et al., 2009). However, among the NFAT protein group, NFATc2 played a

fundamental role in skeletal muscle growth during secondary myogenesis (Horsley et al., 2001). Correspondingly, NFATc2 has been shown to promote the fusion of myoblasts with neighboring myotubes (Horsley et al., 2003). Subsequently, NFATc2 not only played an important role in muscle development during secondary myogenesis, but also in primary myogenesis by controlling the expression of the neo-*MYHs* (Daou et al., 2013). In consistent with the other studies on mammalian muscles especially mice, our study revealed an important role of NFAT binding sites in the transcriptional regulation of hyperplastic muscle-specific $MYH_{M2528\text{-}1}$ expression in zebrafish. However, it should be noted that deleting all four NFAT binding sites from the 2100 bp regulatory region did not completely abolish its activity in directing EGFP expression in whole muscle fibers, suggesting that other regulatory element(s) might be also involved in its expression. Regulation of $MYH_{M2528\text{-}1}$ gene expression by various *cis*-elements emerges 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 $MYH_{M2528\text{-}1}$. In summary, our study on the torafugu $MYH_{M2528\text{-}1}$ promoter implied that hyperplasic muscle-specific expression is achieved through an up-regulatory mechanism that activate the expression in the zebrafish muscle fibers. In addition, we conclude that together with MEF2, MyoD, Pax3 and NFAT binding elements play a key role in the transcriptional regulation of the torafugu $MYH_{M2528\text{-}1}$ gene.

CHAPTER 4

General Discussion

Fish is an attractive model vertebrate to study the mechanisms involved in muscle development and growth due to its specific features. Foremost, fast and slow muscle fibers generally separated into anatomically divergent regions in contrast to other vertebrate muscles in which muscle fibers of different fiber-types form mosaic pattern (Ennion et al., 1995; Johnston, 2006). Skeletal muscle growth in fish also distinctly differs from that of mammals. The new muscle fibers (hyperplasic muscle growth) continue to be produced by 'indeterminate' manner in fish, whereas in mammals, postnatal muscle growth is caused only by hypertrophy of pre-existing muscle fibers that are formed during embryonic development, bestowing upon mammals 'determinate' growth (Rowe and Goldspink, 1969). Morphological, functional and metabolic characteristics of skeletal muscles in vertebrate are primarily interrelated to the cellular expression of different MYHs (Cobos et al., 2001). Therefore, understanding transcriptionl regulation of *MYHs* is crucial to revealing molecular networks responsible for vertebrate muscle formation and growth. In this regard, it is noted that fish are documented to have highly diverged MYH multigene family rather their higher vertebrate counterparts (Kikuchi et al., 1999; Watabe, 2002; Watabe and Ikeda, 2006; Ikeda et al., 2007; Ikeda et al., 2010). Fish *MYHs* expression patterns has been detected where a specific *MYH* demonstrated the recruitment of new muscle fibers i.e. neonatal muscle fibers produced by post-embryonic hyperplasia in common carp (Ennion et al., 1995), sea bream (Rowlerson et al., 1997), zebrafish (Rowlerson et al., 1997), providing greater insight and fundamental approaching into indeterminate muscle growth in relation with *MYH* expression. Our previous study also showed torafugu *Takifugu rubripes* fast-type *MYH*, $MYH_{M2528\text{-}1}$ is specifically expressed in neonatal muscle fibers produced by larval and post-larval muscle hyperplasia. The present study was mostly focused on the identification of sufficient promoter

region that allow *MYH*$_{M2528\text{-}1}$ expression (Chapter 2) and various *cis*-acting elements responsible for the expression (Chapter 3).

Torafugu is an useful genome model because of its smallest genome size among vertebrates. By using torafugu genome, we speculated that molecular networks involved in the *MYH* expression in relation to the indeterminate muscle growth are retrieved effectively. On the other hand, functional analysis on molecules for transcriptional regulatory network in torafugu showed difficulties of routinely rearing and breeding them in the laboratory compared to other experimental teleost model such as zebrafish (Alestrom et al., 2006). Transgenic approach using zebrafish is one of promising and valuable tool for the gene expression analysis *in vivo* (Long et al., 1997; Motoike et al., 2000; Zhang and Rodaway, 2007).Therefore, to identify spatio-temporary regulated promoter of torafugu *MYH*$_{M2528\text{-}1}$, we employed *in vivo* reporter assay technique using zebrafish. As a result, we successfully retrieved the 2,100 bp 5'-flanking region of *MYHM*$_{2528\text{-}1}$ as an enough promoter to govern the spatial and temporal gene expression during larval and post-larval hyperplasic muscle growth (Chapter 2). 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 throughout teleosts. In other words, our reporter P2100 construct of MYH$_{M2528\text{-}1}$:EGFP can be applied on various fish species to visualize neonatal muscle fiber production. We also established novel stable transgenic zebrafish line named Tg:MYH$_{M2528\text{-}1}$:EGFP, which showed consistent expression of EGFP in neonatal muscle fibers produced by larval and post-larval hyperplastic muscle growth (Chapter 2). These are powerful tool for future analysis on teleost skeletal muscle growth by *in situ* imaging of the neonatal muscle fiber production.

Teleost skeletal muscle has unique characters that make them an attractive model for studies on vertebrate muscle formation. One of representative characteristics of teleost is that biochemically distinct muscles are separated in contrast with higher vertebrates including mammals. As the distribution of slow and fast muscles are clearly segregated in trunk musculature, fast muscle comprises the majority of trunk muscle whereas slow muscle is found in a narrow lateral layer just under the skin called lateralis superfacial region (LS). In addition to LS, another type of slow muscle is located at the median fins in fish belonging to the order Tetradontiformes, termed erector and depressor muscle (ED) (Winterbottom, 1974). The erectors lie superficially to the depressors and connect the anterior faces of fin ray bases to the front of the pterygiophore, the bone between vertebral spines that provides support to dorsal and anal fins. These muscle fibers contain distinctive MYH distinguished by immunohistochemistry with MYH specific antibodies (Rowlerson et al., 1985; Scapolo and Rowlerson, 1987). Interestingly, slow and fast muscle region showed distinct *MYH*$_{M2528\text{-}1}$ promoter activity. In Tg:MYH$_{M2528\text{-}1}$:EGFP zebrafish, EGFP expression in fast muscle ceased at definitive body size whereas the expression in slow muscle was constitutive activated regardless of the body size (Chapter 2). Lee (2010) reported recruitment of newly formed muscle in fast muscle ceased at 17mm SL of zebrafish. In consistent with Lee (2010), Tg:MYH$_{M2528\text{-}1}$:EGFP showed EGFP expression in both slow and fast muscle fiber at larval stage but it ceased in fast muscle region from late juvenile to adult stage (Chapter 2). On the other hand, EGFP expression in slow muscle was constitutively activated even at adult stage. Promoter activity in fast muscle is important to understand difference in adult body size among fish species. Torafugu is large fish showing over 6kg of adult body weight whereas zebrafish is small fish, 4-5cm of adult body length. *MYH*$_{M2528\text{-}1}$ promoter activity in fast muscle ceased at 40dpf, 17mm SL of zebrafish in this study, whereas

the *MYH*$_{M2528\text{-}1}$ expression in torafugu fast muscle is still up-regulated at a far larger size (over 1kg body weight) (Akolkar et al., 2010; Asaduzamman et al., 2013). In addition, when compared with the number of *MYH*$_{M2528\text{-}1}$ expressing muscle fibers in the adult torafugu fast muscle (Akolkar et al., 2010; Asaduzamman et al., 2013), the number of EGFP-positive fast muscle fibers in Tg:MYH$_{M2528\text{-}1}$:EGFP zebrafish seemed to be reduced (Chapter 2). Because fast muscle occupies a large portion of skeletal muscle in the teleost, the degree of growth in fast muscle is an important factor in determination of adult body size of teleost fish.

Skeletal muscle gene expression is regulated by a complex group of *cis*-elements that derive from one of two major families of transcription factors. Among them, the most widely studied ones are the myogenic regulatory factor (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). The MRFs are necessary for the determination, specification and differentiation of skeletal muscle lineage (Perry and Rudnick, 2000; Chanoine et al., 2004). These MRFs also play a significant role in regulating muscle development (Tapscott, 2005). During the embryonic development of several teleost species, MyoD is first appeared in adaxial cells of the presomitic mesoderm (Weinberg et al., 1996; Delalande and Rescan, 1999; Temple et al., 2001; Xie et al., 2001;Tan and Du, 2002; Hall et al., 2003; Cole et al., 2004; Zhang et al., 2006;Galloway et al., 2006; Kim et al., 2009) showed that mesodermal cells are intended to become muscle cells in myogenesis (Galloway et al., 2006). In zebrafish, role of MyoD is characterized as tiny triangular patches and expressed in the segmentation period during embryonic development (Weinberg et al., 1996). Its motif like bHLH (basic-helix-loop helix) act sequentially in myogenic differentiation. MyoD is also an important effector for the fast muscle

fibers (Ehlers et al., 2014). In zebrafish, it has also been reported that MyoD are sufficient to promote slow muscle formation from adaxial cells as wel as it required for fast muscle differentiation (Hammond et al., 2007; Groves et al., 2005). Genomatix MatInspector program identified two MyoD binding sites in 2100 bp 5'-flanking region of $MYH_{M2528\text{-}1}$ (Chapter 2). Deletion mutation analysis through inverse PCR of these putative binding sites showed that MyoD binding sites participates in the transcriptional regulation of $MYH_{M2528\text{-}1}$ (Chapter 3).

Pax3 is a gene that includes to the paired box (Pax) family of transcription factors. Paired-box (Pax) family has been extensively played a great role in concerned with skeletal myogenesis (Williams and Ordahl, 1994; Seale et al., 2000; Lepper et al., 2009). Recently, Pax3 was recognized as a key regulator of normal skeletal muscle differentiation where it has a vital role to initiates the myogenic program (Maroto et al., 1997; Rawls and Olson, 1997; Tajbakhsh et al., 1997). Particularly, Pax3 is expressed during developmental and growth stages of myogenesis (Buckingham and Relaix, 2007; Lagha et al., 2008). However, Pax3 is likely to express in the lateral fast muscle before they differentiate into new myofibers (Steinbacher et al., 2008). Regarding embryonic (primary) stages of myogenesis, Pax3 appears to be most important (Froehlich et al., 2013b) and a key myogenic factor that involved in the regulation of indeterminate teleost myogenesis program (Froehlich et al., 2013a). Rescan et al. (2013) revealed that up-regulation of Pax3 genes have a role in muscle hyperplasia. Although Pax3 gene is appear to be well conserved in zebrafish (Seo et al., 1998; Hammond et al., 2007; Kirkpatrick et al., 2010; Seger et al., 2011), zebrafish has two Pax3 isoforms that differ from each other by glutamine residue (Vogan et al., 1996). As for $MYH_{M2528\text{-}1}$ promoter, Genomatix MatInspector program and manual screening identified two putative Pax3 binding sites (Chapter 2). Deletion

mutation analysis through inverse PCR, RT-PCR and immunohistochemistry suggested that Pax3 has great role in the transcriptional regulation of *MYH*$_{M2528\text{-}1}$ (Chapter 3).

MEF2 transcription factors are believed to function in the pathway for skeletal muscle development (Martin et al., 1993; Molekentin and Olson, 1996; Wang et al., 2001; Dodou et al., 2003). During embryogenesis, MEF2 genes are crucial for activation of muscle gene expression (Olson et al., 1995). Therefore, expression of MEF2 genes has also been characterized in zebrafish (Ticho et al., 1996). Additionally, this gene cooperates with the MyoD to drive skeletal muscle development during embryonic and postnatal period (Molekentinet al., 1995; Black and Olson. 1998; Chanoine et al., 2004; Potthoff et al., 2007). MatInspector program identified 4 putative MEF2 binding site in the 2100 bp 5'-flanking region of *MYH*$_{M2528\text{-}1}$ (Chapter 3). Deletion analysis through inverse PCR, immunostaining and RT-PCR results confirmed that MEF2 binding sites took part in the transcriptional regulation of *MYH*$_{M2528\text{-}1}$ expression (Chapter 3).

NFAT gene family involved in muscle precursor cell and skeletal muscle differentiation (Sakuma et al., 2003). Rather than regulating muscle differentiation, NFAT pathway initiates and regulates embryonic muscle differentiation (Armand et al., 2008; Daou et al., 2013). While at least three NFAT isoforms are expressed in skeletal muscle but each isoforms have a unique role in skeletal muscle development (Bourajjaj et al., 2008). The 2100 bp 5'-flanking sequence of torafugu *MYH*$_{M2528\text{-}1}$ contains 3 putative NFAT binding sites (Chapter 3). Therefore, it was of great attention to analyze the role of these NFAT binding sites in the transcriptional regulation of torafugu *MYH*$_{M2528\text{-}1}$ promoter expression in zebrafish. Our present experiment also showed an

important role of NFAT binding sites in the transcriptional activation of *MYH*$_{M2528\text{-}1}$ in zebrafish (Chapter 3).

It is noted that NFAT function in regeneration of mammalian skeletal muscle. In mammals, production of neonatal muscle fibers at post-larval stages occurs only during regeneration after injury and disease (Rowe & Goldspink, 1969). During skeletal muscle regeneration, common *MYHs*, named neonatal and embryonic ones are expressed in regenerated new fibers (Whalen et al., 1981; Lyons et al., 1990). Daou et al. (2013) reported that NFAT signaling is primarily important to induce neonatal and embryonic *MYHs* in neonatal muscle fibers in the adult mouse. We found that the *MYH*$_{M2528\text{-}1}$ promoter also contains three putative NFAT binding sites (Chapter 2) and deletion of these sites showed an apparent reduction in the promoter activity in accordance with number of the NFAT binding sites (Chapter 3). These results indicate the possibility that indeterminate muscle growth is due to constitutive activation of regeneration-related signaling in skeletal muscle.

At last, the present study provides greater insight into regulation of gene expression mediating indeterminate muscle growth in teleost and the transcriptional regulatory mechanism of *MYH*$_{M2528\text{-}1}$ that are specifically expressed in neonatal muscle fibers during larval and post-larval hyperplastic growth in fish. It is clear that *MYH*$_{M2528\text{-}1}$ is transcriptionally regulated by its 5'-flanking regions, although we cannot exclude the contribution of other regions. Various *cis*-elements in the 5'-flanking regions of *MYH*$_{M2528\text{-}1}$ are participated in the transcriptional regulation of slow and fast muscle-specific expression in relation with muscle hyperplasia. Even though some of the previous studies revealed that transcriptional regulatory mechanisms

essential for the slow and fast muscle-specific *MYH*s in fish, to our knowledge this study is the first reports about the regulation of gene expression mediating indeterminate muscle growth in teleost. A better consideration of the transcription factors responsible for the $MYH_{M2528\text{-}1}$ expression would provide a greater insight into how neonatal muscle fibers are formed and maintained in fish growth. In this study, most of the *cis*-regulatory regions are recognized by deletions and mutations and immunohistochemical analysis in zebrafish larvae. However, deletions of complete putative *cis*-regulatory regions may affect the other transcription factors binding sites. As well, the estimated transcription factor may not inevitably bind a putative binding site. Therefore, further studies are required to examine whether and how the predicted transcription factors bind to the promoters and whether the predicted transcription factors work together with other cofactors to activate $MYH_{M2528\text{-}1}$ expression in fish.

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Summary and Conclusion

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 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, 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. Conversely, in teleost skeletal muscles, both hyperplasia and hypertrophy occur throughout the organism's lifespan. This 'indeterminate' muscle growth is a quite 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. 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. 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. Our previous studies identified a MYH

gene (*MYH*), $MYH_{M2528\text{-}1}$, in the torafugu (*Takifugu rubripes*) genome with specific expression in neonatal muscle fibers produced by larval and post-larval muscle hyperplasia. Therefore, understanding the mechanisms of $MYH_{M2528\text{-}1}$ transcription regulation will provide a basis to dissect the molecular network involved in the production of neonatal muscle fibers by indeterminate muscle growth.

Here, the present study examined the torafugu $MYH_{M2528\text{-}1}$ promoter via an *in vivo* reporter assay using zebrafish. We successfully isolated $MYH_{M2528\text{-}1}$ promoter 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. We also identified several transcription factor binding sites plaining essential roles in the activation of the $MYH_{M2528\text{-}1}$ promoter.

1. **Identification of the promoter which activates gene expression mediating indeterminate muscle growth in teleost**

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. Exons 1 and 2 are transcribed as an untranslated region, and the start codon is located in exon3. 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 zebrafish as an *in vivo* reporter assay. For P5000, ~ 97% of the injected embryos displayed strong EGFP expression along skeletal muscle fibers. Although almost the same

expression pattern was observed in zebrafish larvae injected with P5000 throughP600, 5' flanking regions shorter than 2,100bp resulted in a significant reduction in EGFP expression in the myotomal muscle fibers. This finding indicates that *cis*-acting element(s) in between -2,100 to -600 bp in 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 myoblast determining factor (MyoD), four myocyte enhancer element 2 (MEF2), two paired box 3 (Pax3), and three nuclear factor of activated T-cell (NFAT) binding sites.

To confirm the specific activity of the $MYH_{M2528\text{-}1}$ promoter in larval and post-larval 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. Tg:$MYH_{M2528\text{-}1}$:EGFPdisplayed EGFP expression at 1 dpf. After hatching at 2dpf, EGFP continued to be expressed in the whole myotomal region of the larva. In teleosts, slow and fast muscle fibers occupy distinct regions of the myotomal skeletal muscle. Fast muscle fibers comprise the deep portion of the myotome whereas slow muscle fibers are segregated into a wedge-shaped region of the myotome surface. In fast muscles of teleost larva, the apical surface region actively produces neonatal muscle fibers via hyperplasia. 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. The immunohistochemistry of Tg:$MYH_{M2528\text{-}1}$:EGFP at the larval stage (3dpf) showed that the distribution of EGFP-expressing muscle fibers was consistent with the above-mentioned myogenic zone via hyperplasia atthe teleost larval stage.After the larval stage, myogenic cells in fast muscle scattered amongst existing muscle

fibers produce neonatal muscle fibers. 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. 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. In 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. Thus the distribution of EGFP-positive muscle fibers well 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.

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, whereas the number of slow muscle fibers continually increases throughout lifespan. Consistent with the difference in the growth pattern between slow and fast muscles, promoter activity was not observed in fast muscle but in slow muscle at the late juvenile (17mm SL) and adult stage (25mm SL), respectively. Specific expression of EGFP in relation to larval and post-larval hyperplastic muscle growth was also confirmed by adaxial cell observation and hedgehog signal inhibition experiment. Taken together, 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.

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. Our results also showed that the transcriptional network is conserved between large (torafugu) and small (zebrafish) fish. However, an important difference is the duration of promoter activity in fast muscle. $MYH_{M2528\text{-}1}$ promoter activity in fast muscle ceased at 17mm SL of zebrafish in this study, whereas the $MYH_{M2528\text{-}1}$ expression in torafugu fast muscle is still up-regulated at a far larger size (1kg body weight). Because fast muscle occupies a large portion of skeletal muscle in the teleost, the degree of growth in fast muscle is an important factor in determination of adult body size.

2. Multiple *cis*-acting factors function in the regulation of $MYH_{M2528\text{-}1}$ promoter activity

To identify the key regulatory *cis*-element for the gene expression in indeterminate muscle growth in teleost, a deletion analysis was performed within the P2100 construct. As described in the previous section, the 2100-bp 5'-flanking region from the start codon of $MYH_{M2528\text{-}1}$ contains putative transcription factor-binding sites for MyoD, Pax3, MEF2, and NFAT, all of which have been implicated in the expressional regulation of muscle-specific genes during development and growth of vertebrate including fish. To determine the role of these transcription factor binding sites, we deleted these sites individually or in various combinations and microinjected them into zebrafish eggs to observe change in the EGFP expression pattern.

MyoD is a representative myogenic regulatory factor (MRF) and account to be involved in the transcriptional regulation of muscle-specific genes during development in fish. The 2100-bp 5'-

flanking region of *MYH*$_{M2528\text{-}1}$ contains two putative MyoD binding sites. Deletion of each or both sites significantly reduced EGFP expression compared with the non-deletion construct. The reduction of EGFP expression was also confirmed by real-time PCR analysis and immunohistochemistry.

Pax3 is a marker of myogenic precursor cells and appears to be highly expressed throughout myoblast proliferation and into differentiation in teleost. Recently, Rescan et al. (2013) reported that Pax3 is upregulated during hyperplastic muscle growth in rainbow trout. *MYH*$_{M2528\text{-}1}$ promoter contains two putative Pax3 binding sites. Deleting all two Pax3 binding sites showed a mark reduction in number of EGFP expressing embryos and number of EGFP positive muscle fibers per embryo. Reduction of EGFP expression was also confirmed by using real-time PCR and immunohistochemistry.

MEF2 family members are vital for the transcriptional regulation in the muscle differentiation. Additionally, MEF2 cooperates with MyoD to drive skeletal muscle development during embryonic and postnatal period. In fish, it has been reported that MEF2 binding sites are essential for temperature-dependent expression of *MYH*s in medaka and common carp, transcriptional regulation of muscle fiber type-specific torafugu *MYHs*. *MYH*$_{M2528\text{-}1}$ promoter contains four putative MEF2 binding sites. We deleted these binding sites individually or in various combinations. Deleting these binding sites individually or in various combinations showed a significant reduction in number of EGFP positive muscle fibers per embryo. Reduction of EGFP expression was also confirmed by using real-time PCR and immunohistochemistry.

NFAT pathway initiates and regulates embryonic muscle differentiation in vertebrates. The 2100-bp 5'-flanking region of $MYH_{M2528\text{-}1}$ contains three putative NFAT binding sites. As well as in above transcription factor binding sites, deletion of NFAT binding sites individually or in various combinations showed significant reduction in the EGFP expression in zebrafish myotomal region. Reduction of EGFP expression was also confirmed by real-time PCR and immunohistochemistry.

Deletion mutation analysis suggests that MyoD, Pax3, MEF2 and NFAT binding sites are crucial for the $MYH_{M2528\text{-}1}$ promoter activity in indeterminate muscle growth in teleost. Expression of all these transcription factors in zebrafish and torafugu was confirmed by RT-PCR in this study. It is noted that NFAT function in regeneration of mammalian skeletal muscle. In mammals, production of neonatal muscle fibers at post-larval stages occurs only during regeneration after injury and disease. During skeletal muscle regeneration, common *MYHs*, named neonatal and embryonic ones are expressed in regenerated new fibers. Daou et al. (2013) reported that NFAT signaling is primarily important to induce neonatal and embryonic *MYHs* in the adult mouse. Our results suggest the possibility that indeterminate muscle growth is due to constitutive activation of regeneration-related signaling cascade in teleost skeletal muscle. However, the deletion of MyoD-, Pax3-, MEF3- or NFAT-binding sites failed to eliminate $MYH_{M2528\text{-}1}$ expression completely, suggesting participation of other transcription factor(s) on the promoter activity.

Conclusion

This study presents a piece of information about the transcriptional regulatory mechanisms involved in gene expression mediating indeterminate muscle growth in teleost. This study is the first to report the specific promoter involved in teleost indeterminate muscle growth. Although there are intriguing characteristics of teleost indeterminate muscle growth, little is known about the underlying causative molecular mechanisms. A better understanding of the *cis*-elements responsible for the transcriptional regulation of hyperplastic growth-specificexpression of $MYH_{M2528\text{-}1}$would provide a basic insight into how myotomal muscle mass are increased and maintained during indeterminate muscle growth in teleost. In this study, the *cis* regulatory elements are determined by deletion mutant analyses using zebrafish *in vivo* reporter assay. Further studies onthe $MYH_{M2528\text{-}1}$ promoter such as interactome analysis on the promoter will provide new insights not only into skeletal muscle biology but also into vertebrate growth and aging.