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Regulation of gene expression mediating indeterminate muscle growth in teleosts

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ABSTRACT

Teleosts are unique among 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 2100 bp 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 to –600 bp 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.

Abbreviations: ANOVA, analysis of variance; DAPI, diamidine-20-phenylindole dihydrochloride; EGFP, enhanced green fluorescent protein; MEF2, myocyte enhancer element 2; MYH, myosin heavy chain; NFAT, nuclear factor of activated T-cell; Pax3, paired box 3; PFA, paraformaldehyde; RACE, rapid amplification of cDNA ends; SPSS, statistical package for social science; TBSTw, Tris-buffered saline with 0.1% tween 20; TEEA, transient embryonic excision assay

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1. Introduction

Skeletal muscle comprises a large portion of the mass of vertebrates. The bulk of vertebrate growth, therefore, depends on an increase in skeletal muscle mass during a species's lifespan. Skeletal muscles display two types of growth patterns, hypertrophy and hyperplasia. The former is characterized by an increase in the size of existing muscle fibers (myocytes) while the latter results in an increase in the number of muscle fibers. In mammals, however, the contribution of hyperplasia to muscle growth is quite small in the postnatal period and further growth primarily depends on hypertrophy (Rowe and 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 and Rando, 2005). Conversely, in teleost skeletal muscles, both hyperplasia and hypertrophy occur throughout the organism's lifespan (Johnston et al., 2001; Mommsen, 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 among 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 and Manini, 2008, 2010). Various studies using mammalian models such as mice and rats have identified several genes involved in senescence, with relevant genetic modifications resulting in a marked delay in the senescence of various organs, including skeletal muscle (Froehlich et al., 2013a). However, these modified mammalian models merely display a delay in senescence and eventually still achieve a severe sarcopenia phenotype. In this regard, teleosts are an attractive model because the naturally negligible senescence of their skeletal muscles presents a potentially powerful system through which a method to inhibit sarcopenia can be discovered (Froehlich et al., 2013a). However, the molecular mechanisms responsible for the indeterminate muscle growth found in teleosts are completely unknown.

Myosin heavy chain (MYH) is a subunit of myosin, the most abundant protein in skeletal muscle. Many isoforms of MYH exist, and their variation in expression is the primary determinant of the differential physiological properties of muscle fibers, such as slow *vs.* fast twitch (Weiss et al., 1999). The expression patterns of MYH isoforms also change along with the progression of growth stages such as embryonic, neonatal, and adult (Berg et al., 2001). Interestingly, several studies have reported that new muscle fibers (neonatal muscle fibers) produced by post-embryonic hyperplasia express specific MYH isoforms in common carp (Ennion et al., 1995), sea bream (Rowlerson et al., 1997), and zebrafish (Rowlerson et al., 1997). Our previous studies also identified a MYH gene (*MYH*), $MYH_{M2528\text{-}1}$, in the torafugu (*Takifugu rubripes*) genome with specific expression in neonatal muscle fibers produced by muscle hyperplasia at the larval, juvenile and adult stages (Akolkar et al., 2010; Asaduzzaman et al., 2013). $MYH_{M2528\text{-}1}$ was expressed in dorsal and ventral extreme, a region producing neonatal muscle fibers by stratified hyperplasia at larval stage and subsequently in small diameter muscle fibers generated by mosaic hyperplasia at the juvenile stages (Asaduzzaman et al., 2013). In adult torafugu, both fast and slow muscles expressed $MYH_{M2528\text{-}1}$ but the expression was restricted to small diameter neonatal fibers, implying that this gene is associated with muscle hyperplasia from larval to adult stages (Akolkar et al., 2010). 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 through post-embryonic hyperplasia.

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 among different fish species.

2. Results

2.1. *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. S1). Exons 1 and 2 are transcribed as an untranslated region, and the start codon is located in exon3 (Fig. S1 and Fig. 1).

2.2. *The 2100 bp 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 (Fig. 1), 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 (Figs. 2A,B and 3A). The EGFP expression was detected at 1 dpf and continued to be expressed in the whole myotomal region of larva at 2 dpf (Fig. S2A–E). At 3 dpf, EGFP was found to be expressed in the craniofacial and myotomal muscles (Figs. 2K and S2E). In the myotomal region, both slow and fast muscle fibers expressed EGFP (Fig. S3). Although almost the same expression pattern was observed in zebrafish larvae injected with P5000 through P600 (Fig. 2), 5′ flanking regions shorter than 2100 bp resulted in a significant reduction in EGFP expression in the myotomal muscle fibers (Figs. 2M–R and S2). As shown in Fig. 3A, the ratio of EGFP-expressing larvae per injected larvae of P1500–P600 was significantly reduced compare with those of P5000–P2100 injected larvae. In addition, the number of