Myogenic regulatory factors Myf5 and Myod function distinctly during craniofacial myogenesis of zebrafish

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Abstract

The functions of Myf5 and Myod are well known in trunk myogenesis. However, the roles that Myf5 and Myod play during craniofacial myogenesis are far from well known. We observed that zebrafish myf5 was detected in the primordia of the obliques, lateral rectus, sternohyoideus, and pharyngeal mesoderm cores. In contrast, myod transcripts were expressed in all head muscle precursors at later stages. Knockdown of myf5 revealed that Myf5 was required for the development of the obliques, lateral rectus, sternohyoideus, and all pharyngeal muscles, whereas knockdown of myod proved that Myod was required for the development of superior rectus, medial rectus, inferior rectus, lateral rectus, and the ventral pharyngeal muscles. myod mRNA did not rescue the loss of the cranial muscle caused by injecting myf5-morpholino, or vice versa, suggesting that the functions of Myf5 and Myod were not redundant in head paraxial mesoderm, a finding different from their functions in trunk myogenesis. Myf5, but not Myod, was required for the forward migration of myf5-positive oblique precursors. All evidences reveal that Myf5 and Myod function independently during cranial myogenesis. On the basis of the expression patterns of myf5 and myod, we propose a model to present how Myf5 and Myod are involved in head myogenesis of zebrafish.

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Introduction

The paraxial mesoderm comprises the anterior (head or cephalic) mesoderm and the posterior (trunk somites and tail) mesoderm of vertebrates (Pownall et al., 2002). Unlike trunk muscle, which originates from somites, head muscle is derived from the unsegmented, nonepithelial paraxial mesoderm flanking the hindbrain and midbrain and from the prechordal mesoderm regions. Head muscle develops in two regions: branchiomeric and nonbranchiomeric (Noden, 1983; Couly et al., 1992; Trainor et al., 1994; Hacker and Guthrie, 1998; Mackenzie et al., 1998). Branchiomeric muscle includes the muscles of mastication, derived from the first or mandibular arch; the muscles of facial expression, derived from the second or hyoid arch; and the muscles of the pharynx and larynx, derived from more caudal arches (Kaufman and Bard, 1999). Non-branchiomeric head muscle includes extraocular muscles, derived from the anterior-most paraxial and prechordal mesoderm; and tongue muscles, derived from the hypoglossal cord and originating in the anterior somites (Mackenzie et al., 1998; Kaufman and Bard, 1999).

The basic helix–loop–helix myogenic regulatory factors (MRFs) play crucial functions that trigger the expression of muscle structural proteins and permit the assembly of functional myofibers (Molkentin and Olson, 1996). Myf5 and Myod direct the myogenic lineage, evidenced by the finding that double-mutant mice do not form skeletal muscle, a result of the absence of precursor myoblasts (Rudnicki et al., 1993; Kaul et al., 2000; Kablar et al., 2003). In the absence of these factors, progenitor cells remain multipotent and their cell fates can change (Tajbakhsh et al., 1996; Kablar and Rudnicki, 1999). However,
in Myf5−/− mutants, muscles occur normally in limb and branchial arch progenitors (Kablar et al., 1997); in Myod−/− embryos. Skeletal muscles in trunk develop normally and Myf5 is significantly up-regulated (Rudnicki et al., 1992). Although Myod mutant embryos exhibit the delayed development of limb musculature, the limb myogenesis still keeps on processing (Kablar et al., 1997), indicating that Myod and Myf5, have functional redundancy during somitogenesis (Pownall et al., 2002). In zebrafish, embryos that received myf5- or myod-morpholino oligonucleotide (MO) alone developed somites normally (Lee et al., 2006), suggesting that myf5 and myod perform complementary functions during somitogenesis. However, Kassar-Duchossoy et al. (2004) state an epistatic relationship among MRFs, that is, Mrf4 acts upstream of myod and directs embryonic multipotent cells into the myogenic lineage. This finding contradicts the theory that myogenic identity is conferred only by Myf5 and Myod.

Several transcription factors and signaling modulators play important roles in mediating the response of signals from surrounding tissues to induce expression of MRFs during skeletal myogenesis (Borycki and Emerson, 2000; Sabourin and Rudnicki, 2000; Tajbakhsh and Buckingham, 2000). However, the finding of distinct regulatory networks of MRFs in head and in trunk myogenesis has been reported by many investigators (Patapoutian et al., 1993; Tajbakhsh et al., 1997; Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). Mice lacking Myf5 and the paired homeodomain transcription factor Pax3 do not develop skeletal muscle in the trunk or limb, yet head muscle forms normally (Tajbakhsh et al., 1997). Furthermore, mice lacking Capsulin and MyoR fail to express Myf5 in the branchial arch progenitors (Kablar et al., 1997); in Myod mutant embryos exhibit the delayed development of limb muscle. This observation and whole-mount in situ hybridization have been described previously (Lee et al., 2006), except that myf5 and myod knockdown of Myf5 abolished the migration of cranial muscle primordia. Therefore, on the basis of the expression pattern of myf5 and myod, we propose three putative pathways of how Myf5 and Myod regulate the development of craniofacial muscles.

Materials and methods

Zebrafish transgenic lines

Two transgenic lines of zebrafish, Tg(α-actin:RFP) and Tg(myf5:EGFP), were used in this study. The enhanced green fluorescent protein (EGFP) reporter cDNA was isolated from the plasmid pZa-EGFP-ITR (Hsiao et al., 2001), which contains an upstream 4-kb segment of zebrafish fast muscle α-actin fused with the EGFP reporter and flanked by an internal repeat sequence of an adenovirus-associated virus, was replaced by red fluorescent protein (RFP) cDNA from carp (DrsRed, Clontech). The pDsRed 2-1 (Clontech) was cut first with NotI then with BamHI after the NotI site was blunted. It was ligated into the pZa-EGFP-ITR vector that was cut with XhoI, blunted, then cut with BamHI. The resultant plasmid, namely pZa-DsRed-ITR, was linearized by NotI and resuspended at a concentration of 25 ng/μl in double-distilled water mixed with 0.1% (v/v) phenol red prior for microinjection to generate the transgenic line Tg(α-actin:RFP). Parental pairs that produced RFP-positive embryos were separated and mated with wild-type individuals to confirm the putative germ-line transmitting parent. After screening, RFP-positive F1 embryos were raised to adulthood and crossed with wild-type zebrafish to generate a heterozygotic F2 generation. RFP-positive F2 individuals were then crossed with each other to generate homozygotic F3 fish, which were used to produce 100% RFP-positive F4 offspring. A similar strategy was used to generate the transgenic line Tg(myf5:EGFP), except that the microinjected plasmid was pZMYP-BAC80E, which contained an upstream region of zebrafish myf5 was fused with the EGFP reporter.

Knockdown of Myf5 and Myod in zebrafish embryos

Antisense MOs were designed specifically for translation inhibition of myf5-MO, TACGTCCATGATGGTTGTTGTTGT, which was complementary to nucleotides (nt) 28-52, respectively, of zebrafish myf5 cDNA (GenBank accession no. NM131576). The myod-MO, GTTTTTTCTACCTCAACAGCC-ATA, was complementary to nt 180–204 of zebrafish myod cDNA (GenBank accession no. NM131262). All oligonucleotides were prepared at a stock concentration of 1 mM and were diluted to the desired concentrations, that is, either 4, 2, or 1 ng, for microinjection into each embryo.

Fish embryos and whole-mount in situ hybridization

The procedures for zebrafish culture, embryo collection, fluorescent observation, and whole-mount in situ hybridization have been described previously (Lee et al., 2006), except that myf5 (GenBank accession no. NM131576), myod (GenBank accession no. NM131262), myogenin (GenBank accession no. NM131006), myf4 (GenBank accession no. NM001003982), α-actin (GenBank accession no. AF180887), and mrf4 (GenBank accession no. NM001007124) were used as probes. They were digoxigenin-labeled, after we cloned their partial DNA fragments. The design of head muscle and the developmental stage of zebrafish was following those of Schilling and Kimmel (1997).

mRNA preparation for the rescue experiment

Capped mRNAs of myf5 and myod were synthesized according to the protocol of the manufacturer (Epicentre). The resultant mRNAs were diluted to 44 ng/μl and 22 ng/μl for myf5 mRNA and myod mRNA, respectively, with distilled water. Approximately 2.3 nl was used in injection into one-cell stage embryos.
**Time-lapse and imaging analyses**

Embryos derived from the transgenic line Tg(myf5:EGFP) were dechorionized and anesthetized with buffered ethyl m-aminoobenzoate (Tricaine; Sigma). Then, embryos were transferred to 0.5% agar containing an embryo medium with 10 mM HEPES and Tricaine. Axiovert 200 M Inverted Microscope (Zeiss) was used to capture the image approximately every 20 min from 40- to 48-hpf period. Embryos were always kept at 28.5°C on the heated microscope stage during time-lapse analysis. Image analysis was processed by using MetaMorph 7.0 software (Molecular Derice).

**Results**

**All cranial muscles are tagged with RFP in the transgenic zebrafish line Tg(α-actin:RFP)**

To characterize the functions of Myf5 and Myod during head development, we generated a zebrafish transgenic line Tg(α-actin: RFP) that carried a DNA construct in which the RFP reporter was driven by a zebrafish fast-muscle α-actin promoter. Whole-mount in situ hybridization revealed that the RFP reporter gene started to transcribe at 14 h postfertilization (hpf) in the somites of embryos derived from Tg(α-actin:RFP), indicating that the transcription of transgenic and endogenous α-actin genes was initiated at the same stage (Figs. 1A, B). Red fluorescent signal was observed first in somites at 20 hpf (data not shown), and this signal appeared weaker in the newly formed somites than in the old ones (Figs. 1C, D). Similarly, α-actin transcripts were detected first in the head region at 36 hpf, as were RFP transcripts. However, RFP signals were observed first in all cranial muscles at 55 hpf (data not shown). After 55 hpf, all cranial skeletal muscles of this transgenic Tg(α-actin:RFP) fish were tagged clearly by RFP (Figs. 1E, F, G, H). We also noticed that the expression of α-actin in the head was similar to that of myod.

**Expression patterns of myf5 and myod in zebrafish head muscle development**

We detected the spatiotemporal expression patterns of myf5 and myod from 24 through 48 hpf. At 24 hpf, myf5 was detected in the posterior region near the eye (Fig. 2A). At 30 hpf, these myf5-positive cells distributed at the first branchial arch mesoderm core (Figs. 2A, B, C). In addition, myf5 started to express in the inferior oblique (io) and lateral rectus (lr) muscle primordial cells (Fig. 2B). At 32 hpf, myf5 was expressed not only in io and lr, but also in the muscle primordia of superior oblique (so) muscle and of the first, the second, and the third branchial arches (Fig. 2C). After 32 hpf, myf5 transcripts rapidly were absent in the first arch and lr, but expression increased in the third branchial arch mesoderm core (Figs. 2C, E). At 36 hpf, myf5 was expressed predominately in sternohyoides (sh), which migrates from the anterior somites (Fig. 2E). At 36 through 48 hpf, myf5 continued to be expressed in the muscle primordia of so, io, sh, and the mesoderm cores of the second and the third branchial arches (Figs. 2G, I). After 48 hpf, myf5 transcripts gradually decreased in the head region.

Unlike the expression pattern of myf5, myod transcripts were detected first in the head muscle primordia of the superior rectus (sr), medial rectus (mr), and inferior rectus (ir), lr, and in the first branchial arch mesoderm core at 32 hpf (Fig. 2D). At 36 hpf, myod was detected in the first (masticatory plate, MP; intermandibularis, IM) and the second arch mesoderm cores (constrictor hyoideus dorsalis, CHD; constrictor hyoideus ventralis, CHV). Thereafter, these mesoderm cores were cleared individually into dorsal (MP in the first arch; CHD in the second arch) and ventral (IM in the first arch; CHV in the second arch) areas (Figs. 2F, H, J (Schilling and Kimmel, 1994). At 42 to 48 hpf, all the cranial muscle were myod-positive (Figs. 2H, J).

By comparing the expression patterns of myf5 and myod in head, we found that almost all the cranial muscle expressed myf5 in the early stages and then expressed myod afterward. At 32 hpf, the first arch mesoderm core was myf5-positive. However, when the first arch subdivided into MP and IM, the myf5 transcripts started to decrease at 36 hpf, as in the second and third arch. The expression of myf5 was decreased greatly after 42 hpf. Instead, myod became positive in these muscles at 36 hpf. A group of myf5-negative muscle primordia, such as mr/ir/sr, became myod-positive after 32 hpf (Figs. 2F, H). In addition, after we compared the spatiotemporal expressions of myf5, myod, myogenin, and α-actin in the wild-type embryos and the RFP reporter signaling in the transgenic line Tg(α-actin: RFP) (please see Supplemental data 1), we hypothesize that Myf5 and Myod are involved in cranial muscle development but that they play roles differently.

**Functions of Myf5 and Myod in zebrafish cranial muscle development**

To determine whether myf5 and myod play roles during craniofacial muscle development, we microinjected MOs to knock down Myf5 or Myod specifically. When myf5-MO was injected into zygotes from Tg(α-actin:RFP) fish, only the muscle primordia of sr, mr, and ir were RFP-positive at 72 hpf (Fig. 3A vs. B), even until 7 days postfertilization (dpf; data not show); the remainder of the cranial muscle was lost in myf5 morphants, even at 7 dpf. The transcripts of myod and myogenin were only detected in the primordia of sr, mr, and ir at 36 hpf (Fig. 3D) and 58 hpf (Figs. 3H, J) in myf5 morphants. The MO-induced phenotypes were dose-dependent (Table 1). Next, we detected etl expression in myf5 morphants to reveal whether the loss of ventral cranial muscle in myf5 morphants was due to the abnormal development of pharyngeal ventral mesoderm cores. Results showed that the pharyngeal ventral mesoderm cores of myf5 morphants developed normally (Fig. 3E vs. F). These results indicate that Myf5 knockdown did not affect cranial mesoderm cores development. Myf5 expression in the arch 1 and 2 mesoderm cores is necessary for initiating the further myogenesis of ventral mesoderm cores. In addition, although myod and myogenin were expressed in all cranial muscle (Figs. 3G, I), Myf5 knockdown resulted in restricting myod and myogenin expression in the primordia of sr, mr, and ir (Figs. 3H, J). Thus, we propose that Myf5 is necessary for the development of the extraocular muscles so, io, and lr, and all pharyngeal muscles.

When myod-MO was microinjected to specifically inhibit the translation of myod in the embryos derived from both wild-type
and Tg(α-actin:RFP) fish, the RFP signals were present in the extraocular muscles so and io, and in the dorsal pharyngeal muscles, such as lap, do, ah, ao, and sh at 72 hpf (Figs. 4Avs. B, C vs. D), although the RFP signals were reduced slightly. Nevertheless, the RFP signals were lost in the extraocular muscles sr, mr, ir, and lr and in the ventral pharyngeal muscles ima, imp, ih, and hh. Moreover, the myf5 transcripts appeared normal in myod morphants both at 30 hpf (Fig. 4F) and at 36 hpf (Fig. 4H), whereas the myogenin transcripts were expressed slightly in so, io, lap/do, ah, ao, and sh (Fig. 4I vs. J). Like RFP signals, neither myf5 nor myogenin was expressed in the extraocular muscles sr, mr, ir, and lr and in the ventral pharyngeal muscles ima, imp, ih, and hh in myod morphants. The red fluorescent signal was too weak to be observed in the primordia of am in myod morphants before 72 hpf, but myogenin was detected by using whole-mount in situ hybridization, supporting the theory that myogenin was expressed in the promordia of am. However, neither the RFP signal nor in situ hybridization was detected in sr, mr, ir, ima, imp, ih, and hh, even until 7 dpf (data not shown). The phenotypes induced by MO treatment were dose-dependent (Table 2). Again, these results suggest that the absence of cranial muscle in the myod morphants was myod-specific, not due to the delay of development in the MO-treated embryos. Although myod was expressed in all cranial muscles, myod knockdown did not affect the expression of myf5 in so, io, dorsal arch, and sh (Figs. 4F, H), but myogenin transcript and the RFP-labeled muscle

Fig. 1. Tagging all head muscles by using the transgenic zebrafish line Tg(α-actin:RFP). Endogenous α-actin transcripts (A) and red fluorescent protein (RFP) reporter transcripts (B) were detected by whole-mount in situ hybridization of zebrafish embryos at 14 hpf (lateral view: A, B). RFP expressions in the embryos derived from the transgenic line (α-actin:RFP) at 22.5 hpf (C, D), 72 hpf (E, F), and 120 hpf (G, H) were observed from a lateral view (E, G) and from a ventral view (F, H). Panel D is a magnification of panel C. RFP appeared in the formed somites (D, arrowhead) but was absent in the newly forming somite (D, arrow) at 22.5 hpf. Meanwhile, all cranial muscles were labeled by red fluorescent signal in the embryos both at 72 and at 120 hpf. The muscles are designated following the scheme of Schilling and Kimmel (1997): ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; dpw1–5, dorsal pharyngeal wall 1–5; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; lr, lateral rectus; mr, medial rectus; sh, sternohyoideus; so, superior oblique; sr, superior rectus; and tv 1–5, transvs. ventralis 1–5.
fibers were reduced, indicating that Myod helped to enhance the myogenesis of the head muscles so, io, do, am, ah, ao, and sh. Myod is required for the development of the extraocular muscles sr, mr, ir, and lr and the ventral pharyngeal muscles ima, imp, ih, and hh. Myf5 and Myod play their own distinct roles during cranial myogenesis of zebrafish.

Embryos that received either myf5- or myod-MO did not lose all the cranial muscle. However, when both myf5- and myod-MOs were injected into embryos derived from the transgenic line Tg(α-actin:RFP), all cranial muscle labeled with RFP was absent in the head region (Figs. 5A, B). We also detected the expression of myogenin and myf4 in this myf5/myod morphant.
and found that myogenin and myf4 were not expressed in any cranial muscle (Figs. 5C vs. D, E vs. F). Furthermore, Myogenin and MRF4 did not initiate cranial myogenesis of zebrafish when both Myf5 and Myod lost their function.

To determine whether there is a redundant function between Myf5 and Myod during cranial muscle development, we co-injected myf5-MO with myod mRNAs in embryos. Interestingly, myod mRNA did not rescue the loss of the cranial muscle caused by myf5-MO: only the mr, ir, and sr were observed (Fig. 5G). When myf3 mRNA and myf5-MO were co-injected, all the cranial muscles displayed a normal phenotype (Table 1). Similarly, the number of embryos displaying the normal
myf5 and Myod play their own distinct roles during cranial muscle development of zebrafish. Because the primordium of sh has been described as migratory muscle (Schilling and Kimmel, 1997), we studied migratory cranial muscle and found that not only sh but also so and io migrated from the posterior eye field toward the anterior region as the developmental stage progressed (Figs. 2C, I). The myf5 transcripts were detected in so and io from 32 to 48 hpf, and these signals migrated from the posterior eye field toward the anterior region (Figs. 2C, E, G, I). To confirm the migration of these myf5-positive so and io muscles, we used the transgenic line Tg(myf5:EGFP), in which an upstream region of zebrafish myf5 was fused with EGFP, to trace the migration of cranial muscle that expresses myf5. To analyze the spatiotemporal migration of so and io, we found that the GFP signal could be detected in so and io clearly at 36 hpf (Fig. 7A). As a later developmental stage, the GFP signal moved from the posterior eye field toward the anterior region (Figs. 7A, E, I and Supplementary Movie 1). Meanwhile, myod transcripts were detected first in so and io at 42 hpf, when so and io migrated at the top and bottom of the eye field, respectively (Figs. 2G, H). The spatiotemporal movement of the GFP signals in so and io was similar to the myf5 and myod transcripts detected by in situ hybridization.

To understand whether myf5 plays a role in cranial muscle migration, we detected the transcript of met, a migration cell marker (Haines et al., 2004). Results showed that the met transcripts were observed in both so and io of wild-type embryos at 36, 42, and 48 hpf (Figs. 7B, F, J). But the met signal was not detected in the embryos injected with myf5-MO (Figs. 7C, G, K). However, met was transcribed in myod morphants (Figs. 7D, H, L). This evidence supports the finding that myf5 but not myod is required for the migration of primordial so and io.

Similar effects were also observed in other head muscles, such as sh primordium (Schilling and Kimmel, 1997). Compared with myf5 and myod staining, met transcripts were detected in migratory cells that originated from the anterior somites, such as sh, lb, and phm primordia at 36 hpf (Figs. 8A, B, C). Knocking down the Myod level resulted in up-regulating the myf5 expression in the primordia of sh, lb, and phm (Fig. 6B). Compared to the control embryos, the met transcripts were normally expressed in the sh, lb, and phm (Figs. 8C, E). However, in myf5 morphants, the met signal was detected only in the phm primordia (Fig. 8D). Moreover, myogenin transcripts were lost in myf5 morphants, but myogenin was expressed normally in myod morphants in the migratory muscle cells at 48 hpf (Figs. 8F, G, H). Embryos derived from the transgenic line Tg(α-actin:RFP) were also used to support this finding. RFP signal was lost in sh, fb, and phm in myf5 morphants (Figs. 8I vs. J), but RFP signal was observed in myod morphants (Figs. 8I vs. K). Taken together, this evidence leads us to believe that myf5 has an important role in cranial muscle migration.

**Discussion**

In vertebrates, presomitic and somitic multipotential mesodermal cells give rise to myogenic progenitor cells (also known as premyogenic cells), which commit to form the skeletal...
muscles of the trunk, limbs, and head. Meanwhile, the more anterior nonsomitic paraxial and prechordal head mesoderm is the source of some head muscles (reviewed by Wachtler and Jacob, 1986; Christ and Ordahl, 1995). The migration of paraxial mesoderm to the branchial arches contributes precursors that develop into facial muscles in chicks (Hacker and Guthrie, 1998; Noden et al., 1999). Like other vertebrates, zebrafish cranial muscles originate from the paraxial mesoderm (Kimmel et al., 1990; Noden, 1983). Muscles that originate from the paraxial mesoderm of the first and second pharyngeal arches develop the dorsal (lap, do, am, ah, ao) and the ventral (ima, imp, ih, hh) portions of cranial muscles.

Fig. 4. Myod is required for the development of posterior extraocular recti and ventral branchial muscles. Embryos derived from the transgenic line Tg(α-actin:RFP) (A–D) and from the wild-type strain (E–J) were used. Embryos injected with 4 ng of myod-morpholino oligonucleotide (MO) to inhibit specifically myod translation were followed to observe the development of cranial muscle (B, D) and the expression of myf5 (F, H) and myogenin (J; lateral views: A, B, E–J; ventral views: C, D) at the stages indicated. The anterior extraocular recti (io and so), dorsal branchial muscle (ah, ao, do, and lap), and sh developed normally in the myod transgenic morphant (A vs. B; C vs. D). The posterior extraocular (sr, mr, ir, and lr) and the ventral branchial muscle (ima, imp, ih, and hh) were totally lost. When wild-type embryos were injected with myod-MO, myf5 was expressed normally in the myod morphants at 30 hpf (E vs. F) and at 36 hpf (G vs. H). Whereas, the expression of myogenin was decreased in myod morphants at 58 hpf (I vs. J), indicating a reduction in myogenin-positive muscle fibers.
Myf5 and Myod play crucial functions to trigger the expression of muscle structural proteins and finally to permit the assembly of myofibers (Molkentin and Olson, 1996; Buckingham, 2001). Tajbakhsh et al. (1997) reported that Pax3 and Myf5 of mice follow two distinct myogenic pathways and that Myod acts genetically downstream of these genes for myogenesis in trunk muscle development; Myf5 and Myod regulate the head muscle formation independently. The regulation of myf5 is markedly different from that of myod. Obviously, more detailed knowledge about the functions of Myf5 and Myod on cranial muscle development is needed.

In this study, we provide strong evidence to show the distinct roles that Myf5 and Myod play during craniofacial muscle development of zebrafish. In the myf5-knockdown morphants, the development of all the cranial muscles, except sr, mr, and ir, was impeded severely. The primordia of sr, mr, and ir developed normally in the myf5 morphants. Furthermore, in situ hybridization also proved that myf5 was not transcribed in sr, ir.

<table>
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**Fig. 5.** Myf5 and Myod function independently to activate progenitor lineages in muscles of the head region. Embryos co-injected with 4 ng of myf5-morpholino oligonucleotide (MO) with 4 ng of myod-MO to inhibit specifically both myf5 and myod translation, respectively, were used to observe the development of cranial muscle (A, B) and the expression of myogenin and mrf4 (D, F) at 72 hpf. Panel B was magnified from the head area of panel A. No red fluorescent protein (RFP) signal was detected in muscle primordia in the myf5 and myod double-knockdown morphants derived from the transgenic line Tg(α-actin:RFP; A, B). Similarly, the transcripts of myogenin (C vs. D) and mrf4 (E vs. F) were not detected in the myf5 and myod double-knockdown morphants (I vs. J). The myod mRNA did not rescue the formation of RFP-labeled primordia muscles in myf5 morphants (G). Similarly, the myf5 mRNA did not rescue the formation of RFP-labeled primordia muscle in myod morphants (H). For abbreviations, see the legend of Fig. 1.
mr, and ir. On contrast, few cranial muscles, such as sr, mr, and ir, and the ventral set of muscles (ima, imp, ih, and hh) were totally lost in the myod-knockdown morphants, suggesting that Myf5 and Myod may function independently and distinctly in some cranial muscles during zebrafish embryogenesis. The embryos injected with either myf5- or myod-MO in this study could survive more than 8 days. Thus, we conclude that the MO-induced phenotypes are specific.

Regulatory networks of Myf5 and Myod during cranial myogenesis are intricate

Myf5 and Myod are indispensable for cranial muscle development of zebrafish, because Myf5 or Myod have its own role without being redundant. Based on the expression patterns of myf5 and myod, there are three different regulatory mechanisms of all craniofacial myogenesis (Fig. 9).

During cranial muscle development, the arch I and II mesoderm cores are subdivided into dorsal and ventral mesoderm cores. The dorsal mesoderm cores are the precursor of lap, do, am, ah, and ao, whereas the ventral mesoderm cores are the precursors of ima, imp, ih, and hh. We find that both myf5 and myod are detected in the dorsal mesoderm cores, but only myod is expressed in the ventral groups. On the basis of data shown in this study, we propose two regulatory pathways (Fig. 9C): in pathway I, Myf5 per se is capable of initiating myogenesis. Once Myf5 is expressed, the expression of myod starts to increase, and then myogenesis proceeds further. This finding is illustrated by the expression of the muscle differentiation marker myogenin: myod and downstream
Myf5 is required for the forward migration of the superior oblique (so) and inferior oblique (io) primordia toward the anterior eye region. Embryos derived from the transgenic line Tg(myf5:EGFP), in which an upstream region of zebrafish myf5 was fused with enhanced green fluorescent protein (EGFP), were used to trace the migration of cranial muscle that express myf5. The primordia of io and so labeled with EGFP were clearly visible in the embryos derived from the transgenic line Tg(myf5:EGFP) at 36–48 hpf under fluorescent microscopy (A, E, I; arrows). Whole-mount *in situ* hybridization of wild-type zebrafish embryos showed that the expression of *met*, a cell marker of migration, was positive in the io and so at 36–48 hpf (B, F, J; arrows). Embryos injected with *myf5*-morpholino oligonucleotide (MO) and *myod*-MO to inhibit *myf5* and *myod* translations, respectively, expressed *met* at the stages indicated. The *met* transcript was not expressed in the io and so of *myf5* morphants (C, G, K), but *met* was expressed normally in the io and so of *myod* morphants (D, H, L; arrows). For abbreviations, see the legend of Fig. 1.
myogenin were not expressed in the dorsal mesoderm cores of the myf5 morphants (Fig. 3). In contrast, myogenin transcripts are detected in the primordia of myod morphants (Fig. 4), suggesting that dorsal arch muscle development of cranial myogenesis does not initiate in the absence of Myf5. However, knockdown of myod does not impair myogenesis, because the downstream differentiation marker myogenin is still expressed in these muscles, although myogenesis proceeds less efficiently.

The alternative pathway for regulating the arch mesoderm core is Pathway II: muscle primordia subdivided from the myf5-positive core are initiated to undergo myogenesis by Myod. We find that ima, imp, ih, and hh are lost in both myf5 morphants and myod morphants. These results indicate that both myf5 and myod are necessary for ventral core mesoderm to undergo myogenesis.

Unlike in the arch mesoderm core, the development of extraocular muscles is regulated by three different pathways (Fig. 9C). Although both myf5 and myod transcripts were detected in io and so, the primordia of io and so are lost in the myf5 morphants but not in the myod morphants, indicating that Myf5 and Myod modulate the development of the extraocular muscles io and so through Pathway I. On the other hand, lr is lost in the myf5 and myod morphants, suggesting that Myf5 and Myod modulate the development of the extraocular muscles lr through Pathway II. In the development of sr, mr, and ir; however, myod transcript is expressed but not myf5 transcript.
This result is also supported by tracing the GFP signal from the transgenic line that carries the upstream region of zebrafish myf5 fused with GFP reporter (Fig. 7). Thus, it is Myod but not Myf5 that modulates the development of the muscle primordia of sr, mr, and ir through Pathway III. On the other hand, the primordia of sh, which originates from the anterior somites, was lost in the myf5 morphants but not in myod morphants, suggesting that sh is modulated through Pathway I. Thus, it is worthwhile to study whether other factors are involved for controlling the development of all cranial muscles.

Myf5 and Myod function differently for craniofacial and for trunk myogenesis

Myf5 and Myod function distinctly during development of the dorsal and ventral cranial muscles

![Fig. 9. A plausible model to present the distinct modulation of Myf5 and Myod during craniofacial myogenesis of zebrafish. (A) Schematic illustration of the dynamic expression of myf5 and myod in the cranial muscle of zebrafish embryos at 32 and 42 hpf. The myf5- or myod-positive muscle fibers are labeled in red; myf5- and myod-negative ones are labeled in gray. (B) Schematic illustration of the presence (red) or absence (gray) of cranial muscle fibers in the embryos injected with either myf5-morpholino oligonucleotide (MO; left) or myod-MO (right) at 72 hpf. (C) Schematic diagram of all cranial muscles that are categorized into three groups (represented in red, blue, and green) on the basis of three regulatory pathways of myf5 and myod during development: (I) for so, io, sh, and dorsal arch, not only does myf5 modulate myogenesis directly to generate myogenesis at the basal level but myf5 also triggers myod expression to enhance myogenesis at a high level; (II) for lr and ventral arch, myf5 defines the cell fate of muscle and myod is the major factor of myogenesis; and (III) for sr, mr, and ir, myod modulates myogenesis directly.

Myf5−/− and Myod+/− mutant mice are viable and fertile (Kaul et al., 2000, Rudnicki et al., 1992). However, Myf5/Myod null embryos do not form skeletal muscles and die at birth because of respiratory failure (Rudnicki et al., 1993). Thus, Myf5 and Myod function redundantly for skeletal myogenesis. However, unexpectedly, we found that this redundancy present for trunk myogenesis is not the case for cranial myogenesis in zebrafish, as evidenced by co-injection of myf5-MO with myod mRNA not rescuing the myf5-MO-induced phenotype (Fig. 5G). Similarly, co-injected myod-MO and myf5 mRNA did not rescue the defects caused by myod-MO (Fig. 5H). Therefore, we reason that the functions of Myf5 and Myod are not redundant in cranial myogenesis. Nevertheless, the cranial muscle sh, which originates from trunk paraxial mesoderm, is an exception to this rule. The expression of myf5 in sh is up-regulated in the myod morphants (Fig. 6B), suggesting that the primordium of sh progresses as does trunk myogenesis, even when they migrate into the head region. This finding also indicates that Myf5 and Myod indeed have different regulatory mechanisms between head and trunk paraxial mesoderm, suggesting that cranial myogenesis is governed by a head-specific regulatory cascade, which is fundamentally distinct from the regulatory cascade in the trunk.
necessary for the specification of the dorsal cranial muscles but not for the ventral ones. Thus, we propose that Myf5 may be a key modulator to control the development of major cranial muscles, whereas Myod and Tpf2a may be key modulators to control the development of the ventral and dorsal cranial muscles, respectively.

**Epistatic relationship of the zebrafish MRFs in cranial myogenesis**

Recently, the epistatic relationship of mouse MRFs has been proposed that both Myf5 and Mrf4 act upstream of Myod to direct embryonic multipotent cells into the myogenic lineage (Kassar-Duchossoy et al., 2004). However, the epistatic relationship of the MRFs in cranial myogenesis is still unclear. Our study shows that embryos coinjected with myf5- and myod-MOs lost all the cranial muscles in the head region (Figs. 5A, B). In addition, in situ hybridization revealed that myogenin and mrf4 were not expressed in myf5/myod morphants (Figs. 5D, F). This evidence indicates that Myogenin and Mrf4 are not capable of initiating cranial myogenesis. We speculate that myogenin and mrf4 may serve as differentiation genes.

**Myf5 is required for the migration of myogenic precursor cells**

In rodent, met null mice lost the hypaxial mesoderm derived from the migratory muscles, such as the diaphragm, tongue, limb, and associated shoulder musculature. Also, met served as a migratory marker (Bladt et al., 1995; Dietrich et al., 1999). In zebrafish, the primordium of the cranial muscle sh, which originates from the anterior somites, has been defined (Schilling and Kimmel, 1997). In this report, we show that the primordia of io and so migrate during cranial myogenesis (Fig. 7). Whole-mount in situ hybridization showed that met is expressed in so, io, and sh primordia (Figs. 7, 8). Interestingly, met transcript is lost in the myf5 morphants, but met displays normally in the myod morphants, suggesting that myf5 is necessary for cranial muscle primordia migration.

In the development of mouse limb, myf5 and myod transcripts are not detected in the migratory muscle primordia until they migrate into the limb buds (Tajbakhsh and Buckingham, 1994; Birchmeier and Brohmann, 2000). Unlike the mouse limb, in zebrafish cranial muscle development, the migratory cells are myf5- and myod-positive, even when they undergo migration. In Xenopus, p38 mitogen-activated protein kinase (MAPK) regulates the expression of Xmyf5 and affects distinct myogenic programs (Keren et al., 2005). Inhibition of p38 MAPK prevents the expression of Xmyf5 but not of Xmyod. The ventral body wall muscles, whose migratory precursors originate in the ventral part of somites, are reduced greatly when p38 MAPK and Xmyf5 are knocked down. Together, the early activation of Myf5 and Myod in frog and fish may suggest the rapidity of their early developments to produce functional muscles for swimming.

In summary, we address the functions of Myf5 and Myod during cranial muscle development in zebrafish. Myf5 and Myod play distinct roles in cranial myogenesis. A putative model that demonstrates three pathways for Myf5 and Myod function in craniofacial muscle development is proposed. In addition, the expression of met in morphants supports the finding that Myf5, a well-known MRF that is involved in trunk myogenesis, also controls the cranial cell migration. This article is the first report to reveal the functions of Myf5 and Myod during cranial myogenesis in zebrafish.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.042.

**References**


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