**dickkopf-3-related Gene Regulates the Expression of Zebrafish myf5 Gene through Phosphorylated p38a-dependent Smad4 Activity**

Received for publication, July 6, 2010, and in revised form, December 13, 2010 Published, JBC Papers in Press, December 15, 2010, DOI 10.1074/jbc.M110.161638

Ren-Jun Hsu, Chiu-Chun Lin, Ying-Fang Su, and Huai-Jen Tsai

From the Institute of Molecular and Cellular Biology, National Taiwan University, No. 1, Section 4, Roosevelt Road, Taipei 106, Taiwan

Myf5 is a myogenic regulatory factor that functions in myogenesis. An intrinsic microRNA, miR-In300, located within zebrafish myf5 intron I, has been reported to silence myf5 through the targeting of *dickkopf-3*-related gene (*dkk3r*). However, the molecular mechanism underlying the control of myf5 expression by *dkk3r* is unknown. By injecting *dkk3r*-specific morpholino-oligonucleotide (*dkk3r*-MO) to knock down Dkk3r, we found that the phosphorylated p38a protein was reduced. Knockdown of p38a resulted in malformed somites and reduced myf5 transcripts, which photocopied the defects induced by injection of *dkk3r*-MO. To block the MAPK pathway, phosphorylation of p38 was inhibited by introduction of SB203580, which caused the down-regulation of myf5 expression. The GFP signal was dramatically decreased in somites when we injected *p38a*-MO into embryos derived from transgenic line Tg(myf5(80K):GFP), in which the GFP was driven by the myf5 promoter. Although these *p38a*-MO-induced defects were rescued by co-injection with *p38a* mRNA, they were not rescued with *p38a* mRNA containing a mutation at the phosphorylation domain. Moreover, overexpression of Smad2 or Smad3a enhanced myf5 expression, but the defects induced by the dominant negative form of either Smad2 or Smad3a equaled those of embryos injected with either *dkk3r*-MO or *p38a*-MO. These results support the involvement of Smad2-Smad3a in p38a mediation. Overexpression of Smad4 enabled the rescue of myf5 defects in the *dkk3r*-MO-injected embryos, but knockdown of either *dkk3r* or *p38a* caused Smad4 protein to lose stability. Therefore, we concluded that Dkk3r regulates p38a phosphorylation to maintain Smad4 stability, in turn enabling the Smad2-Smad3a-Smad4 complex to form and activate the myf5 promoter.

In vertebrates, the determination and differentiation of trunk skeletal muscle is controlled by the basic helix-loop-helix family of transcription factors, such as Myf5, Myod, myogenin, and MRF4 (1). Myf5 is the first myogenic regulatory factor expressed in mammals (2), birds (3, 4), and fish (5) during early embryogenesis in a somite- and stage-specific manner. In zebrafish, myf5 is primarily detectable in the somites and segmental plates (5, 6). The transcription level of myf5 elevates substantially until 16 hpf, gradually declines to undetectable levels by 33 hpf, and is strictly repressed after somitogenesis (5). Lin et al. (7) had previously reported a strong, negative regulatory motif located at +502/+835 (1300) within the first intron of zebrafish myf5, having an orientation-dependent function. However, after further investigation, Hsu et al. (8) discovered a novel microRNA, named miR-In300, located at +609/+622, which binds to the 3' UTR of the mRNA of the long isoform *dickkopf-3* (*dkk3*), also named *dickkopf-3*-related gene (*dkk3r*). Knockdown of *dkk3r* with *dkk3r*-specific morpholino-oligonucleotide (*dkk3r*-MO) resulted in the down-regulation of myf5 expression, suggesting that Dkk3r is involved in the upstream positive regulation of myf5. Therefore, miR-In300 inhibits the translation of *dkk3r* mRNA, which, in turn, suppresses myf5 expression.

To advance these findings in this study, we asked what molecular mechanism(s) might underlie the modulation of myf5 expression by *dkk3r*. Dkk is a secretory protein encompassing two conserved cysteine-rich regions, Cys-1 and Cys-2, located at the C and N terminus, respectively, but gapped by non-conserved linkers of various lengths (9, 10). In zebrafish, the Dkk family is categorized as Dkk1 (AF116852), Dkk2, Dkk3 (NM_001089545.1), and Dkk3r (also named long isoform Dkk3; NM_001159283.1). The activation of myf5 gene expression by a secretory protein, such as Dkk3r, depends on intermediate molecules to transduce the signals. It has been reported that p38 mitogen-activated protein kinase (MAPK) is the most likely regulatory pathway involved in muscular development (11). There are two forms of p38 MAPK (p38) in zebrafish: p38a and p38b (12). Analysis of amino acid sequences showed that zebrafish p38a and p38b share identity with human and mouse/rat p38a of 86 and 84%, respectively, compared with identity of 69 and 66% in the sequences of p38β. This fact indicates that zebrafish p38a and p38b are two isoforms of p38a. Knock-out of p38α, -β, -γ, and -δ in mouse studies showed that only p38α knock-out resulted in myogenesis defects (13). The results indicate that p38α may play its most important role in the regulation of myogenesis. This is important in the context of the present study because zebrafish p38a and p38b may conserve the biological function of mouse p38α. Therefore, to understand how *dkk3r* controls...
myf5 expression in zebrafish, the p38α pathway was first examined in this study because the temporospatial expression of the p38α isoform, not the p38β isoform, totally corresponds with muscle development.

Members of the TGF-β superfamily play a conserved function from Drosophila to higher mammals during embryogenesis. Mothers against Decapentaplegic (Mad)-related proteins (Smads) have been reported to play a role as cytoplasmic transducers in TGF-β signaling (14–17). The activated receptor-regulated Smads (R-Smads), such as Smad1, -2, -3, -5, and -8, enter into nuclei to initiate the transcription of downstream genes (19, 20). R-Smads are required to form a complex with co-mediator Smads (Co-Smads), such as Smad4, which carries the activated R-Smads to nuclei. The binding of Co-Smad-R-Smads to promoters activates the expression of downstream genes. Smad complexes enter the nucleus to mediate TGF-β signal transduction, and the interaction between p38α and Smads has been reported in many cell lines (21, 22).

In addition, because Dkk3 is required for TGF-β signaling in Xenopus mesoderm induction (23) and because TGF-β family members are known to regulate myogenesis (24), we examined the role of Smads in Dkk3 regulation of Myf5, taking the position that Dkk3 may activate the myf5 promoter through the signal transduction of p38α and the cytoplasmic transducer Smads.

EXPERIMENTAL PROCEDURES

**In Vitro Transcription, Whole Mount in Situ Hybridization (WISH), Fluorescent Microscopy Observation, Western Blot Analysis, and Luciferase Activity Detection**

The following procedures were performed according to Lin et al. (25): the synthesis of capped mRNAs, the labeling of probes for WISH, and the observation of embryos under fluorescence microscopy. The following procedures were performed according to Hsu et al. (8): the analysis of total proteins from embryos, Western blotting, and the quantitative measurement of luciferase activity.

**Heat Induction of pHSP70/4-dn-p38α to Produce Recombinant DN-P38α**

Embryos were placed in a 50-ml tube for 4.5 h prior to the subject stages. The tubes were incubated at 37 °C for 30 min for heat induction. After embryos were removed from Embryo Medium (Zebrafish Book protocol) kept at room temperature, we placed the embryos at 28 °C and incubated them for 4 h. Finally, embryos were fixed with 4% paraformaldehyde at 4 °C overnight, and chorions were removed.

**Chromatin Immunoprecipitation (ChIP) Assay**

The ChIP assay was performed in vivo according to the protocols described by Lindeman et al. (26), using the ExactaCruz™ IP/WB Reagents kit (Santa Cruz), except that (a) zebrafish embryos were injected with His-tagged expression plasmid, such as pHis-smad2, pHis-smad3a, or pHis-smad4; (b) A-agarose beads blocked with sonicated salmon sperm DNA (Upstate Biotechnology) were used; (c) the chromatin solution was immunoprecipitated with polyclonal anti-His antibody; and (d) PCR primers specific for detection of myf5 promoter were used.

**Plasmid Constructs**

pCS2-wp38α—Wobble p38α (wp38α) forward primer (CATGGTACCAAAAGGAGGCTCCTCCTCTCTCA) and p38α reverse primer (GTGGATGAAATGGAGTCTTGA) were used to amplify a wp38α, which is not bound by p38α-MO, from zebrafish 16 hpf cDNA. The fragment of wp38α was ligated to the pGEM-T (Promega) and then ligated to EcoRI-cut pCS2 to generate pCS2-wp38α. The wp38α mRNA was synthesized with the SP6 promoter.

pCS2-DN-p38α—To mutate the p38α phosphorylation site from Thr-Gly-Tyr (TGY) to Asn-Gly-Phe (NGF), we used a set of forward primer (GGATCAGTGTGAGAAGAAGAA) and reverse mutated primer (GTGAGCACCAG-ACCCAT-CTCATCCTCATC) as well as a set of forward mutated primer (GATGAGAT-GAATGGTTTGTGGCACC) and reverse primer (GTGGATGAAATGGAGTCTTGA) to amplify the mutated phosphorylation site of p38α (DN-p38α). The amplified product was ligated to pGEM-T and then ligated to EcoRI-cut pCS2.

pCS2-smad2a, -smad3a, -smad3b, and -smad4—Primer sets of Xhol-smad2a forward primer (CTCGAGATGTGCTTCCATCCTTGGCTTTT) and Smad2 reverse primer (TTAGGACATACCTGGAGCCAGCG); Xhol-Smad3a forward primer (CTCGAGATGTCAATTTACCTTCTCCTAC) and Smad3a reverse primer (CTATGACACACTGGAGCGGCG); Xhol-Smad3b forward primer (CTCGAGATGTCTATATTGGCCTTCA) and Smad3b reverse primer (CTATGACACACTGGAGCGGCG); and Xhol-Smad4 forward primer (CTCGAGATGTCCATCACAAACACTCCCA) and Smad4 reverse primer (TCAGTCTAAGGTGTGGGCCT) to amplify the PCR products of Smad2, Smad3a, Smad3b, and Smad4, respectively, from zebrafish 16 hpf cDNA.

pRSETa-smad2 or -3a—With Xhol-smad2 or Xhol-smad3a forward primer and reverse primer, smad2 or -3a was amplified with PCR and ligated into pGEM-T. With Xhol and EcoRI, smad2 or smad3a was cut and ligated to pRSETa. pRSETa-smad2 or -3a was transformed to BL-21 for the expression of His tag-Smad2 or His tag-Smad3 recombinant protein.

pHSP70/4-dnp38α—From constructed pCS2-DN-p38α, the kdnp38α sequence was amplified with Sall-Kozak p38 forward primer (CTCGAGCGCCACCATGTGCGCAGAAAGAA) and p38α reverse primer using PCR. The amplified products were ligated to pGEM-T. The kdnp38α sequence, which was cut out with Sall and NolI, was ligated to Sall/NolI-treated pHSP70/4-EGFP to generate plasmid pHSP70/4-dn-p38α.

pHSP70/4-dnsmad2a/3a/3b—With pX17-dnsmad2, -dnsmad3a, and -dnsmad3b (32) as templates, respectively, Sall-Kozak smad2a forward primer (CTCGAGCGCCACCATGTCCTCCTTGGC) and NolI-dnsmad2a reverse primer (CGCCGGCGTTAGGCGCATGCGGAGCAGCG); Sall-Kozak smad3a forward primer (CTCGAGCGCCACCATGTCCTCCTTGGC) and NolI-dnsmad3a reverse primer (CGCCGGCGTTAGGCGCATGCGGAGCAGCG); and Sall-
DKK3r Regulates Phosphorylated p38a-dependent Smad4 Activity

Inhibition of Dkk3r Resulted in the Decrease of p38a Phosphorylation—dkk3r-MO was microinjected into one-cell stage zebrafish embryos, which were developed to 16 hpf and collected. Total protein of these embryos was extracted to perform Western blotting, and the amount of p38a protein level was detected. Compared with the control protein level extracted from the control MO-injected embryos (Fig. 1C), the amount of p38a protein remained unchanged in the dkk3r-MO-injected embryos, but the amount of the phosphorylated p38a was reduced (Fig. 1C). Meanwhile, both the amount of p38a and the amount of phosphorylated p38a were reduced in the p38a-MO-injected embryos (Fig. 1C). To investigate whether the other ERK MAPK is involved, we injected dkk3r-MO to observe the amount of phosphorylated ERK protein. The results showed that inhibition of Dkk3r did not affect the phosphorylation of ERK (Fig. 1D). This ruled out the involvement of Dkk3r in the ERK MAPK regulatory pathway. However, because the Dkk family is known to be the downstream regulator in Wnt signal transduction, we further tested for the involvement of zebrafish development through Wnt signal transduction. After injection of dkk3r-MO, the protein amounts of Wnt downstream phospho-ERK, β-catenin, and c-Myc were subjected to examination, and results showed that Dkk3r knockdown had no effect on either p-ERK, β-catenin, or Myc proteins (Fig. 1D), indicating that Dkk3r did not regulate muscle development through Wnt signal transduction. Thus, we suggested that Dkk3r might activate the phosphorylation of p38a to regulate myf5 gene expression, which, in turn, might affect muscle development.

Temporospatial Expression of p38a mRNA Corresponded with the Process of Somatic Muscular Development—RT-PCR was performed to observe the temporal expression of p38a mRNA. At various developmental stages, p38a mRNA was expressed consistently from one-cell stage to the completion of muscle development at 30 hpf (supplemental Fig. S1A). WISH was performed to observe the temporospatial expression of p38a mRNA. At 7 hpf, p38a transcript was uniformly expressed in the whole embryonic body (supplemental Fig. S1B). At 18 hpf, p38a mRNA was expressed in eye, brain, and somites (supplemental Fig. S1C), and p38a was continuously expressed in somites at 24 hpf (supplemental Fig. S1D). At 36 hpf, when trunk muscle development was completed, the expression of p38a was reduced (supplemental Fig. S1E). These results showed that the temporospatial expression of p38a mRNA corresponded to trunk muscle development.

Inhibition of p38a Caused Trunk Muscular Developmental Defects—To observe the effect of p38a on muscle development, p38a-MO was injected into one-celled fertilized eggs derived from the transgenic line Tg(α-actin:RFP), a muscle-specific red fluorescent transgenic line, and we found that the body axis of embryos changed from straight line (supplemental Fig. S2A) to tail-crooked (supplemental Fig. S2B). Observation of red fluorescence showed that somites did not present a

Kozak smad3b forward primer (GTGCAGCAGGACCATGTC-TATATTGCTTTCA) and NotI- dsnsmad3b reverse primer (GCGGCCGCTATGCACGCGGAGCGCGGCGGCGG) were used for PCR amplification of Sall-Kozak dsnsmad-NotI. The amplified products were ligated to pGEM-T, and the kdnp38a for PCR amplification of SalI-Kozak dsnsmad-NotI. The amplified products were ligated to pHSP70/4-EGFP, and Western blot analysis was performed when 20 μg of proteins were loaded. Compared with the control MO-injected embryos, the protein level of p38a from embryos injected with either control MO (lane 1), dkk3r-MO (lane 2), or p38a-MO (lane 3). Western blot analysis was performed when 20 μg of proteins were loaded. Compared with the control MO-injected embryos, the protein level of either p38a or phosphorylated p38a was reduced in the dkk3r-MO-injected group. D, protein amounts of the phosphorylated ERK (p-ERK), β-catenin, and c-Myc in the dkk3-MO-injected embryos. α-Tubulin and GAPDH were used as an internal control.

FIGURE 1. Inhibition of the translation of dkk3r mRNA resulted in reducing the protein amount of phosphorylated p38a. MO was designed to specifically inhibit the translation of either dkk3r mRNA (A) or p38a mRNA (B) resulting from the blockage at the translational start site (nucleotides marked in gray). C, the total proteins were extracted from 200 embryos at 16 hpf derived from the one-celled fertilized eggs injected with either control MO (lane 1), dkk3r-MO (lane 2), or p38a-MO (lane 3). Western blot analysis was performed when 20 μg of proteins were loaded. Compared with the control MO-injected embryos, the protein level of p38a from embryos injected with dkk3r-MO did not decrease. However, the amount of phosphorylated p38a (p-p38a) was reduced. Meanwhile, the protein level of either p38a or phosphorylated p38a was reduced in the p38a-MO-injected group. D, protein amounts of the phosphorylated ERK (p-ERK), β-catenin, and c-Myc in the dkk3-MO-injected embryos. α-Tubulin and GAPDH were used as an internal control.
FIGURE 2. Inhibition of p38a resulted in the abnormal expressions of myogenesis genes in zebrafish embryos, and this defect was rescued by injection of p38a mRNA. WISH was performed to detect the myogenesis genes of 16-hpf embryos injected with 1.2 ng of p38a-MO. Compared with embryos injected with control MO (n = 110) (A, E, and I), embryos injected with p38a-MO (n = 124) (B, F, and J) showed that myf5 was down-regulated (B), whereas myoD was expressed (F), and myogenin was unaffected (J). A rescue experiment of co-injection of p38a-MO and p38a mRNA (n = 119) (C, G, and K) showed that wp38a mRNA, which contained a normal phosphorylation domain, could rescue the expressions of myf5 (C). However, co-injection of p38a-MO and DN-p38a mRNA (n = 117) (D, H, and L), which contained a mutated phosphorylation domain, failed to rescue the abnormal expressions of the myf5 gene caused by p38a-MO (D), and these defects were even more severe than those of embryos injected with p38a-MO alone (B). The defects included the reduction of myf5 expression at PSM (parenthesis) and the disarray in the location of myf5 expression at somitic regions (D). The arrows refer to the somites with myf5 expression. Red arrows, stronger expression; black arrows, weaker expression.

normal V-shape (supplemental Fig. S2A’). Instead, somites presented an abnormal U-shape (supplemental Fig. S2B’), suggesting that p38a affects muscle development in zebrafish embryos.

p38a Positively Regulated myf5 Expression by Controlling the Promoter Activity—To investigate whether p38a is involved in the regulation of somitic myf5 expression, we injected embryos with p38a-MO to specifically inhibit the translation of p38a. These embryos were then subjected to WISH to examine the expression of myf5 at 16 hpf. In the wild-type (WT) embryos, myf5 was expressed in presomatic mesoderm (PSM) and five somites from −II, −I, 0, 1, to 2, among which −II, −I, and 0 somites showed a more intensive signal. Although myf5 was more strongly expressed in the forming somites, its intensity decreased toward the anterior (Fig. 2A). However, in the p38a-MO-injected embryos, myf5 was expressed normally in PSM, but the expression intensity decreased in somites. In −II forming somites, which typically had the strongest expression in WT, only low myf5 expression was shown in the p38a-MO-injected embryos (Fig. 2B). In addition, the somites in the p38a-MO-injected embryos became abnormally straight (Fig. 2B). All of the somites expressed myod in WT embryos (Fig. 2E) and p38a-MO-injected embryos (Fig. 2F). Moreover, neither the WT embryos nor the p38a-MO-injected embryos showed much difference in myogenin expression (Fig. 2, I and J). When p38a mRNA was synthesized and injected into zebrafish embryos to perform the rescue experiment, mRNA bound with p38a-MO and diluted the efficiency of p38a-MO because unmodified p38a was used. Therefore, we designed a wobble p38a (wp38a) mRNA, in which 7 nucleotides were mismatched to p38a-MO (supplemental Fig. S3). This allowed the rescue experiment to be performed without the translational inhibition effect of p38a-MO binding to wp38a mRNA, finally resulting in the down-regulation of myf5 expression. We also noticed that somites were normally formed on the basis of myod expression pattern (Fig. 2, E and F). This line of evidence showed that the effect of p38a-MO on myf5 mRNA expression was specific, not an off-target effect.

To investigate the possible effect of p38a phosphorylation on the activation of myf5 expression, the p38a phosphorylation domain TGY was mutated to NGF, a domain that could not be phosphorylated. This dominant negative form, wobble p38a (DN-p38a), competed with normal p38a to block the signal transduction of p38a phosphorylation (supplemental Fig. S3). The injection of DN-wp38a mRNA with p38a-MO showed that the mutation of the p38a phosphorylation domain did not rescue myf5 (Fig. 2D) expressional defects in somites induced by p38a-MO (Fig. 2B). Specifically, myf5 expression decreased, the expression location was disordered, and myf5 expression in PSM was decreased (Fig. 2D). However, somites were normally formed on the basis of myod expression pattern (Fig. 2, G and H).

Compared with the uninjected embryos derived from the transgenic line Tg(myf5(80K);GFP) in which GFP was driven by an upstream 80-kb segment of zebrafish myf5 (27), em-
bryos with p38a-MO injection showed a great decrease in GFP expression. On the other hand, co-injection of p38a-MO with wp38a mRNA restored GFP expression to a level similar to the control group (data not shown). When p38a-MO and DN-wp38a mRNA were co-injected into Tg(myf5(80K):GFP) embryos, DN-p38a mRNA did not rescue the GFP decrease caused by p38a-MO (data not shown). These results suggest that the phosphorylation of p38a may play an important role in myf5 expression. To further confirm the effects of p38a phosphorylation on myf5 expression, SB203580 was used to inhibit p38a phosphorylation and block the MAPK pathway. With 250–300 μM SB203580, somitic myf5 expression was decreased, but with 400–500 μM SB203580, 60–70% of embryos showed complete suppression of myf5 expression (supplemental Fig. S4).

The Expressions of smads and myf5 Were Co-localized in Tail Bud—To further understand the relationship between Smads and the downstream activation of the zebrafish myf5 promoter, we performed WISH on 16-hpf embryos to observe the spatial relationship between smad mRNA and myf5 mRNA expressions. At 16 hpf, myf5 was expressed in tail PSM and six somites from −II to 3 in WT (supplemental Fig. S5A). smad2 was expressed in the whole embryonic body, with stronger expression in eyes, brain, and tail (supplemental Fig. S5B). smad3a was expressed weakly in the whole embryonic body, but smad3a was noticeably expressed in eyes, tail epitelium, new somites, and PSM (supplemental Fig. S5C). smad3b expressed in the whole embryonic body but with stronger expression in eyes and tail (supplemental Fig. S5D). smad4 was also expressed in the whole embryonic body but more strongly in eyes, brain, and tail somites (supplemental Fig. S5E).

Overexpression of DN-Smad2, DN-Smad3a, DN-Smad3b, and DN-p38a Resulted in the Down-regulation of myf5 Expression—Because zebrafish Smad2 and Smad3a/b are maternal proteins (30–32), which might obstruct the defective phenotypes caused by MO injection, we designed dominant-negative forms of Smad2 and Smad3a/b to study the effects of Smad2:Smad3a:Smad3b on early embryonic development. The SXS motif at the C terminus of Smad2:Smad3 is mutated to AXA; therefore, neither Smad2 nor Smad3 is able to bind to TGF-B type I receptor (28, 29). In this study, we generated dominant-negative forms of Smad2 (DN-Smad2), Smad3a (DN-Smad3a), and Smad3b (DN-Smad3b), which were mutated at P445H, S465A, and S467A (32), respectively, from inducible plasmids pHSP70/4-dnsmad2, pHSP70/4-dnsmad3a, and pHSP70/4-dnsmad3b, respectively, after heat shock induction. In addition, because the Pro145 of Smad2:Smad3 was mutated to His145, Smad2:Smad3 was not able to attract Smad4 for the formation of a complex (33).

These three plasmids were injected into zebrafish embryos, and the embryos were heat-induced at 12.5 hpf for 30 min to produce DN-Smad2, DN-Smad3a, and DN-Smad3b. Embryos were then collected at 16 hpf for WISH experiments with myf5 and myod riboprobes. At 16 hpf, myf5 was expressed at 5 somites from −II to 2, with stronger expression at −II, −I, and 0 somites (Fig. 3B). With DN-Smad2 (Fig. 3D), DN-Smad3a (Fig. 3E), and DN-Smad3b (Fig. 3F) competing with endogenous corresponding Smads, 36, 44, and 18%, respectively, of embryos showed a decrease in myf5 expression, with

FIGURE 3. Overexpression of DN-p38a, DN-Smad2, DN-Smad3a, and DN-Smad3b caused myf5 down-regulation. A, plasmids of pHSP70/4-dn38a, pHSP70/4-dnsmad2, pHSP70/4-dnsmad3a, and pHSP70/4-dnsmad3b were separately microinjected into the one-cell stage of zebrafish embryos. When these embryos developed to 12.5 hpf (seven-somite stage), they were treated at 37 °C for 30 min for induction and then incubated at the reduced temperature of 28 °C to let them develop to 16 hpf. These embryos were collected to perform WISH with myf5 (B–F) or myod (G–K) riboprobe. In the WT, myf5 was expressed at five somites in a V-shape (B). In the embryos with overexpression of DN-p38a (C) (n = 91), DN-Smad2 (D) (n = 87), DN-Smad3a (E) (n = 94), and DN-Smad3b (F) (n = 81) reduced myf5 expression to 3-bandings and caused a decrease in quantity as well. myod probe was used as a somite marker to determine whether those somites were normally formed (G–K).

Overexpression of DN-Smad2, DN-Smad3a, DN-Smad3b, and DN-p38a resulted in the down-regulation of myf5 expression. Because zebrafish Smad2 and Smad3a/b are maternal proteins (30–32), which might obstruct the defective phenotypes caused by MO injection, we designed dominant-negative forms of Smad2 and Smad3a/b to study the effects of Smad2:Smad3a:Smad3b on early embryonic development. The SXS motif at the C terminus of Smad2:Smad3 is mutated to AXA; therefore, neither Smad2 nor Smad3 is able to bind to TGF-β type I receptor (28, 29). In this study, we generated dominant-negative forms of Smad2 (DN-Smad2), Smad3a (DN-Smad3a), and Smad3b (DN-Smad3b), which were mutated at P445H, S465A, and S467A (32), respectively, from inducible plasmids pHSP70/4-dnsmad2, pHSP70/4-dnsmad3a, and pHSP70/4-dnsmad3b, respectively, after heat shock induction. In addition, because the Pro145 of Smad2:Smad3 was mutated to His145, Smad2:Smad3 was not able to attract Smad4 for the formation of a complex (33).

These three plasmids were injected into zebrafish embryos, and the embryos were heat-induced at 12.5 hpf for 30 min to produce DN-Smad2, DN-Smad3a, and DN-Smad3b. Embryos were then collected at 16 hpf for WISH experiments with myf5 and myod riboprobes. At 16 hpf, myf5 was expressed at 5 somites from −II to 2, with stronger expression at −II, −I, and 0 somites (Fig. 3B). With DN-Smad2 (Fig. 3D), DN-Smad3a (Fig. 3E), and DN-Smad3b (Fig. 3F) competing with endogenous corresponding Smads, 36, 44, and 18%, respectively, of embryos showed a decrease in myf5 expression, with
three weak signals. Based on the myod expression pattern, we determined that somites were normally formed. Furthermore, we also injected another inducible plasmid, pHSP70/4-dnp38a, into embryos, and we obtained results similar to those based on the injection of pHSP70/4-dnsmad2/3a/3b, except that 74% of embryos exhibited a decrease in myf5 expression after competition between DN-p38a and endogenous p38a (Fig. 3C). Therefore, we concluded that overexpression of DN-Smads causes the down-regulation of myf5, a finding that further motivated us to ask whether Smad2-Smad3a can directly bind to the upstream region of myf5 to activate its promoter activity.

Smad2 and Smad3a Activated the myf5 Promoter—To further study the effect of Smad2, Smad3a, and Smad3b on myf5 promoter activity, we co-injected either smad2 or smad3 mRNA with pZMYF6.3R, an upstream 6.3-kb segment of myf5 fused with the Renilla luciferase gene, into zebrafish embryos to measure the ability of Smad2 and Smad3 to activate the myf5 promoter. Compared with the luciferase activity driven by pZMYF6.3R, results showed that the addition of 350 pg of smad2 mRNA increased myf5 promoter activity 3-fold (supplemental Fig. S6). Because the -fold increase in activity rose when the concentration of smad2 mRNA increased, we concluded that the activation effect of Smad2 on the myf5 promoter was dosage-dependent (supplemental Fig. S6). Although the addition of 50 pg of smad3a mRNA increased myf5 promoter activity 2-fold (supplemental Fig. S6), the addition of either 50 pg of smad3b mRNA or 300 pg of smad4 mRNA did not elevate the activity of the myf5 promoter (supplemental Fig. S6). Simultaneously adding 30 pg of smad2, 20 pg of smad3a, and 100 pg of smad4 mRNA increased myf5 promoter activity 2.5-fold (supplemental Fig. S6). This evidence indicated that the activation of myf5 promoter by Smad2 and Smad3a required the recruitment of Smad4.

The Upstream Segment of the myf5 Gene Contained Two Smad3a/4-binding Sites—To further investigate whether the effects of Smad2, Smad3a, and Smad4 are directly or indirectly involved in myf5 promoter activity, we employed the PROMO software (21) to search for the potential binding sites in the upstream 3-kb segment of zebrafish myf5 gene (supplemental Fig. S7). The results showed that there were two potential Smad-binding elements (SBEs), which are known to be Smad3/4 binding sites, located at −1156/−1146 (SBE1) and −258/−248 (SBE2). Adjacent to these sites, there were also binding sites for potential Smad cofactors, such as PEBP2, AP-1, and CAATT-binding factor (supplemental Fig. S7) (22, 23, 24). Thus, the in vivo ChIP assay was performed to observe whether Smad3a and Smad4 were bound to the upstream region of myf5 directly. After plasmid encoding His-tagged Smad2, Smad3a, or Smad4 was injected individually into the one-cell stage of zebrafish embryos, we performed ChIP assays in vivo by anti-histidine antiserum. PCR was then used to amplify the DNA fragments containing SBE1 and SBE2. No PCR product could be generated from embryos injected with pHis-Smad2 (supplemental Fig. S8). However, two PCR products with 218 bp containing SBE1 and SBE2 were found in embryos injected with either pHis-Smad3a or pHis-Smad4 (supplemental Fig. S8). This line of evidence suggested that Smad3a and Smad4 bind directly to the upstream region of myf5 and that the complex of Smad2, Smad3a, and Smad4 regulates the activity of the myf5 promoter.

Dkk3 Maintained the Stability of the Smad4 Protein through p38a—In Xenopus studies, Dkk3 was shown to affect TGF-β signal transduction by stabilizing the Smad4 protein and subsequently regulating the development of mesoderm (23). In addition, Smad2/Smad3a required the recruitment of Smad4 to enter the nucleus. It is therefore plausible that Dkk3 might stabilize Smad4 by regulating p38a, subsequently enabling Smad4 to form a complex with Smad2-Smad3a, enter the nucleus, and activate the myf5 gene. To investigate this hypothesis, dkk3- and p38a-MO were injected into zebrafish embryos to specifically knock down Dkk3 and p38a, respectively, allowing Smad4 protein expression to be detected. The endogenous Smad4 of zebrafish was negligible and thus undetectable (Fig. 4A). However, injection of 100 pg of smad4 mRNA into zebrafish embryos greatly enhanced the amount of Smad4.
protein, which was observed at the location of 68 kDa (Fig. 4A). Compared with the Smad4 level in the embryos injected 100 pg of smad4 mRNA, embryos co-injected either with 100 pg of smad4 mRNA and 6 ng of dkk3r-MO or with 100 pg of smad4 mRNA and 1.2 ng of p38a-MO showed a decrease in the protein level of Smad4 (Fig. 4A). On the contrary, the inhibition of Dkk3r did not affect the quantity of Smad3 protein (Fig. 4B). These results showed that Dkk3r does maintain the stability, but not the quantity, of Smad4 protein, through p38a.

Overexpression of Smad4 Enabled Embryos to Rescue the myf5 Down-regulation Defect Caused by either Dkk3r or p38a

Injection of excessive smad4 mRNA rescued the defect of myf5 down-regulation caused by either dkk3r-MO or p38a-MO. WISH was performed to detect myf5 expression in 16-hpf WT embryos and embryos co-injected with dkk3-MO and smad4 mRNA. A, in WT embryos, there were five somites with myf5 expression, three of which showed a V-shape of myf5 expression. B–E, in embryos co-injected with dkk3-MO and smad4 mRNA, four degrees of myf5 expression defects were exhibited: no defect, indicating that myf5 was expressed strongly in three somites with V-shaped somites (B); mild defect, indicating myf5 expression but with horizontally shaped somites (C); moderate defect, indicating that myf5 expression was lower than WT with horizontally shaped somites (D); and severe defect, indicating that myf5 expression had disappeared in somites (E). F, the percentages of defects in embryos injected with 100, 200, and 300 pg of smad4 mRNA along with 6 ng of dkk3r-MO were calculated according to the defect definitions above. G, the percentages of defects in embryos injected with 100 pg of smad4 mRNA along with 2 ng of p38a-MO were calculated. The arrows referred to the somites with myf5 expression. Red arrows, stronger expression; black arrows, weaker expression.

Dkk3r knockdown. Compared with WT embryos having a 150° angle between left and right somites (Fig. 5A), the angle of the dkk3r-MO-injected embryos was 180° (Fig. 5, B–D). In addition, the myf5 expression was down-regulated with various degrees of defect in dkk3r-MO-injected embryos. 66.7% (n = 112) of them exhibited a moderate defect, indicating that myf5 expression was lower than WT with horizontally shaped somites (D); whereas 33.3% of them exhibited a severe defect, indicating that myf5 expression had disappeared in somites (Fig. 5E). A rescue experiment was performed by co-injection of dkk3r-MO with smad4 mRNA. With 100 pg of smad4 mRNA, 92.6% (n = 124) of the embryos showed moderate defect, and 7.4% of the embryos showed severe defect. With 200 pg of
DKK3r Regulates Phosphorylated p38α-dependent Smad4 Activity

Smad4 mRNA, 92.3% (n = 112) of the embryos exhibited a mild defect, indicating that myf5 was normally expressed, although these embryos still showed 180° between left and right somites (Fig. 5C), and 7.7% of the embryos showed moderate defect. With 300 pg of smad4 mRNA, 24.1% (n = 127) of the embryos showed normal myf5 expression, with the angle between left and right somites restored to 150° (Fig. 5B), and 75.9% of the embryos showed mild defect. Fig. 5F summarizes the various rates of defects induced by the different smad4 mRNA concentrations we applied. Importantly, as smad4 mRNA concentration increased, the myf5 defect caused by dkk3r-MO was incrementally reduced, suggesting that the rescue of smad4 mRNA had a dosage-dependent effect.

We also asked whether overexpression of Smad4 would enable embryos to rescue myf5 defects caused by p38α knockdown. Compared with dkk3r-MO, injection of p38α-MO caused more toxicity to zebrafish embryos. The concentration of p38α-MO and smad4 mRNA could not be more than 2 and 100 pg, respectively. In the p38α-MO-injected embryos, 17% (n = 132) of the embryos exhibited no defect of myf5 expression, whereas 15, 23, and 45% of the embryos exhibited mild, moderate, and severe defect of myf5 expression, respectively (Fig. 5G). However, when we co-injected p38α-MO with smad4 mRNA, the percentage of embryos that exhibited no defect of myf5 expression increased to 40% (n = 111) (Fig. 5G), whereas the percentages of moderate and severe defect decreased to 12 and 32%, respectively (Fig. 5G). This evidence indicated that overexpression of smad4 mRNA could also rescue p38α-MO-induced defects. Taken together, we suggested that the addition of exogenous Smad4 complemented the endogenous Smad4, which was degraded by either Dkk3r or p38 inhibition, subsequently restoring myf5 promoter activity.

**DISCUSSION**

Phosphorylation of p38α Is Important for the Activation of the myf5 Promoter Activity—The signal transduction of p38α, a well known MAPK, is achieved by phosphorylation (21). The excessive mRNA of DN-p38α, which possesses a mutated phosphorylation domain, was unable to rescue the abnormal expression of myf5 caused by p38α-MO and was also unable to rescue the defect of green fluorescence shown in Tg(myf5/(80K):GFP) embryos caused by p38α-MO. Although injection of p38α-MO causes myf5 down-regulation only in somites, higher dosage of DN-p38α causes myf5 down-regulation in both somites and PSM. This phenomenon may be explained as follows: Although the p38α protein that has existed through maternal effect remains unaffected by p38α-MO, DN-p38α would have a competitive effect on either maternal p38α or p38α protein produced by embryos Thus, it is reasonable that additional DN-p38α with p38α-MO injection causes more severe defects in zebrafish embryos. Furthermore, injection of a p38 phosphorylation inhibitor results the decrease of somitic myf5 expression. Based on the p38α mRNA rescue and SB203580 treatment experiments, we concluded that p38α phosphorylation plays an important role in the activation of myf5 expression in somites.
receptor in order for Smad2-Smad3a to produce activation effects on the myf5 promoter.

Although there is a 94% similarity in amino acid sequence between Smad3b and Smad3a, Smad3b has a lower ability to activate myf5 promoter than Smad3a, based on the results from Tg(myf5(80K):GFP) and luciferase assay experiments. Although overexpression of Smad4 does not cause expression abnormality in fluorescence locations, somitic green fluorescence is enhanced. This might result from the fact that extra Smad4 continuously carried intrinsic Smad2-Smad3a into the nucleus to activate the myf5 promoter sustainably. Based on the results of experiments employing WISH, dominant negative forms of DN-Smad2/3a/3b, luciferase assay, and overexpression of smad2/3a/3b/mRNA to Tg(myf5(80K):GFP), Smad2 and Smad3a play an important role in the activation of the myf5 promoter. Although the ability of Smad2 and Smad3a to activate the myf5 promoter requires Smad4 to form a complex and enter the nucleus, Smad3b only plays a minor role in myf5 promoter activation.

Dkk3r and p38a Maintain the Stability of the Smad4 Protein, Enabling the Formation of a Smad2-Smad3a-Smad4 Complex to Enter the Nucleus and Activate the myf5 Promoter—In this study, we demonstrated that inhibition of zebrafish Dkk3r decreases Smad4 protein greatly. This result is consistent with the study described by Pinho and Niehrs (23), who reported that Dkk3 is shown to affect the stability of Smad4 protein in Xenopus. Dkk3 affects the p38a MAPK pathway, and inhibition of p38a also causes Smad4 protein to decrease. Thus, we concluded that Dkk3r and p38a are required to maintain the stability of the Smad4 protein. Furthermore, inhibition of Dkk3r does not alter the amount of Smad3 protein, indicating that the effect of Dkk3r on Smad4 protein is specific. We also found that inhibition of Dkk3r results in the reduction or even elimination of myf5 expression in somites, which is rescued by overexpression of smad4 mRNA. Therefore, it is reasonable to speculate that inhibition of Dkk3r might reduce phosphorylated p38a, which subsequently fails to stabilize Smad4 protein. Although there is some active Smad2-Smad3a in cytoplasm, Smad2-Smad3a is unable to enter the nucleus to activate the myf5 promoter without the assistance of Smad4 protein. Thus, overexpression of smad4 mRNA complements the insufficient endogenous Smad4 protein. This permits formation of the complex with Smad2-Smad3a in cytoplasm, Smad2-Smad3a is unable to enter the nucleus to activate the myf5 promoter without the assistance of Smad4 protein. Thus, overexpression of smad4 mRNA complements the insufficient endogenous Smad4 protein. This permits formation of the complex with Smad2-Smad3a and entry into the nucleus to activate the myf5 promoter. This line of evidence indicates that the myf5 defect induced by Dkk3r inhibition results from the loss of the stable Smad4 protein, not the loss of Smad2-Smad3a activity.

Recent studies have shown that many ubiquitin-like proteins, such as small ubiquitin-like modifier (Sumo), which is shown to modify proteins, are able to affect the functions of the target proteins, including transcriptional activity and the ability to enter and attract other transcriptional factors in the nucleus, protect against ubiquitination, and regulate mitosis (35, 36). The sumoylation of Smad4 protected Smad4 protein from being ubiquitinated for degradation in a cell line study; thus, sumoylation plays an important role in stabilizing Smad4 protein and subsequently enhancing TGF-β signal transduction (37, 38). At the same time, it has been shown that the p38 MAPK pathway is involved in Smad4 sumoylation (39). In this study, we found that Dkk3r regulates the phosphorylation of p38a and that inhibition of Dkk3r and p38a cause the degradation of Smad4 protein. Therefore, we
suggested that zebrafish Dkk3 might regulate p38a, which, in turn, affects Smad4 sumoylation, finally stabilizing Smad4 protein and enabling the Smad2-Smad3a-Smad4 complex to form, enter the nucleus, and activate the *myf5* promoter.

**CONCLUSIONS**

Based on the evidence shown in this study, we hypothesize a model to demonstrate the regulation of Dkk3r in the activation of zebrafish *myf5* in somites. Specifically, in the presence of Dkk3r, which is secreted outside of the cell, the phosphorylation of p38a in cytoplasm is enhanced through an unknown receptor located on the membrane. The phosphorylated p38a assists the stabilization of Smad4 protein by sumoylation. The stabilized Smad4 then helps to form a complex with Smad2 and Smad3a, which then enters the nucleus to activate the *myf5* promoter (Fig. 6A). In the absence of Dkk3r, however, the amount of phosphorylated p38a is reduced, which prevents Smad4 from being sumoylated. The unstable Smad4 protein is then incapable of helping form the Smad2-Smad3a complex to enter the nucleus, resulting in the eventual inactivation of the *myf5* promoter (Fig. 6B).

**REFERENCES**

1. Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002) *Annu. Rev. Cell Dev. Biol.* **18**, 747–783
2. Buckingham, M. (1992) *Trends Genet.* **8**, 144–148
3. Hacker, A., and Guthrie, S. (1998) *Nature Rev. Cell Dev. Biol.* **3**, 22–35
4. Coutelle, O., Blagden, C. S., Hampson, R., Halai, C., Rigby, P. W., and Hughes, S. M. (2001) *Dev. Biol.* **236**, 136–150
5. Lin, C. Y., Chen, Y. H., Lee, H. C., and Tsai, H. J. (2004) *Gene* **334**, 63–72
6. Ha, R. J., Lin, C. Y., Hoi, H. S., Zheng, S. K., Lin, C. C., and Tsai, H. J. (2010) *Nucleic Acids Res.* **38**, 4386–4393
7. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Thomsen, G. H. (1999) *Nature* **391**, 357–362
8. Kretzschmar, M., and Massague, J. (1998) *Annu. Rev. Biochem.* **67**, 753–791
9. Perdiguero, E., Ruiz-Bonilla, V., Gresh, L., Hui, L., Ballestar, E., Sousa-Victor, P., Baeza-Raja, B., Jardi, M., Bosch-Comas, A., Esteller, M., Cae-