4384–4393 *Nucleic Acids Research, 2010, Vol. 38, No. 13* *Published online 17 March 2010*
doi:10.1093/nar/gkq148

Novel intronic microRNA represses zebrafish *myf5* promoter activity through silencing *dickkopf-3* gene

Ren-Jun Hsu, Cheng-Yung Lin, Hao-Seong Hoi, Shu-Kai Zheng, Chiu-Chun Lin and Huai-Jen Tsai*

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

Received January 23, 2010; Revised January 27, 2010; Accepted February 18, 2010

ABSTRACT

A strong, negative *cis*-element located at the first intron +502/+835 (I300) of zebrafish *myf*5 has been reported. To elucidate the molecular mechanism underlying this repression network, we microinjected zebrafish single-cell embryos with I300 RNA, resulting in the dramatic reduction of luciferase activity driven by the *myf*5 promoter. Within this I300 segment, we identified an intronic microRNA (miR-In300) located at +609/+632 and found that it was more highly expressed in the older mature somites than those newly formed, which negatively correlated with the distribution of zebrafish *myf5* transcripts. We proved that miR-In300 suppressed the transcription of *myf*5 through abolishing *myf5* promoter activity, and we subsequently identified the long isoform of the Dickkopf-3 gene (*dkk3*) as the target gene of miR-In300. We further found that injection of the *dkk3*-morpholinos (MOs) resulted in downregulation of *myf5* transcripts in somites, whereas co-injection of *myf5* mRNA with *dkk3*-MO1 enabled rescue of the defects induced by *dkk3*-MO1 alone. Finally, injection of *miR-In300*-MO enhanced both *myf*5 transcripts in somites and the level of Dkk3 protein in zebrafish embryos. Based on these findings, we concluded that miR-In300 binds to its target gene *dkk3*, which inhibits the translation of *dkk3* mRNA and, in turn, suppresses zebrafish *myf5* promoter activity.

INTRODUCTION

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 which is expressed in mammals (2), birds (3,4) and fish (5) during early embryogenesis. In zebrafish, *myf*5 is primarily detectable in the somites and segmental plates (5,6). The transcription level of *myf5* elevates substantially until 16 h post-fertilization (hpf), gradually declines to undetectable levels by 33 hpf, and is strictly repressed after somitogenesis (5). The *cis*-element –82/–62 segment enables direct somite-specific expression of zebrafish *myf*5 (7), which is directly bound by *FoxD3* (8). Thus, the cell lineage-specific expression of *myf5* is delicately regulated. More importantly, Lin *et al*. (9) had previously reported that a strong, negative *cis*-regulatory motif located at the first intron of zebrafish *myf*5, + 502/ + 835 (I300), specifically represses the promoter activity of *myf*5. Interestingly, they found that the sense strand of I300 is capable of repressing GFP expression driven by the upstream region of zebrafish *myf*5, but that the I300 with antisense strand substantially loses its repressive ability. Furthermore, when the repressive segment is placed in the upstream 3 kb of *myf5*, repression is nearly abolished. Thus, the I300 repressive function appears to behave in an orientation-, position- and *myf5*-dependent manner. However, the molecular mechanism underlying this I300-mediated repression of zebrafish *myf5* transcription is totally unknown. Based on these lines of evidence, we hypothesized that I300 could function as a self-regulatory, microRNA-triggered modulator whereby

*To whom correspondence should be addressed. Tel: +886 2 33662487; Fax: +886 2 2363 8483; Email: hjtsai@ntu.edu.tw

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

negative feedback regulation is initiated by RNA-mediated silencing.

MicroRNAs are single-stranded RNA molecules, about 19–30 nt in length, which repress gene expression through sequence-specific base-pairing with target mRNA. Mature microRNA molecules are partially complementary to one or more mRNA molecules, and their main function is to downregulate gene expression. MicroRNAs were first described by Lee and colleagues (10) in *Caenorhabditis elegans* in *lin-4*. Subsequently, hundreds of microRNAs have been identified in other organisms, such as *Drosophila*, zebrafish, *Xenopus*, mammals and plants, by using molecular cloning and bioinformatics (11–16). MicroRNAs are generated either from specific RNA genes or from intronic splicing. While microRNAs derived from intronic splicing require the presence of RNA polymerase II and spliceosomal components (17), this is not the case for microRNAs derived from small RNA genes. An estimated one-fourth of all microRNAs are intronic, and, although several of these have been identified in worm, mouse and in human cells (18), the target gene and function of intronic microRNAs remain largely unknown. One exception is miR-208 whose target gene, THARP-1, regulates cardiac growth (18).

In this study, we demonstrated that a novel intronic microRNA, miR-In300, which is derived from I300 of the first intron of zebrafish *myf5,* enables significant repression of *myf5* promoter activity through silencing the long isoform of the Dickkopfs-3 gene (*dkk3*), indicating that miR-In300 is an effective negative regulator of *myf5* expression.

MATERIALS AND METHODS

Knockdown experiments

The morpholinos (MOs) designed specifically for knocking down *myf*5 and *dkk*3 were TACCTGCATAAGAGGTGT AGGGTCT (*myf5*-MO) and GAGGCTGAATCCGAGC AGAAACATG (*dkk3*-MO1), respectively. A negative control MO designed for *dkk*3 was GACGCTCAATCC GACCACAAAGATG (*dkk3*-control-MO), in which the mutated-mismatched nucleotides are underlined. In order to study the efficacy and specificity of *dkk3*-MO in blocking the translation of the *dkk*3 mRNA, we designed *dkk3*-MO2, (ATGATGCAAGACTCTCGTACCTTTA), in which the matching nucleotide sequences were located at the boundary between exon 2 and intron 2. In addition, we also constructed a plasmid containing a *dkk3-GFP,* in which the 27th nucleotide after ATG of *dkk3* mRNA was fused with GFP cDNA. The MO designed for knocking down miR-In300 was AAAATCTGCATTCA AAATGCTTTTATCTACC (*miR-In*300-MO), in which *miR-In300*-MO bound to the miR-In300 motif within the pre-miR-In300 sequence to avoid forming a mature miR-In300 by the lack of hairpin structure. All MOs were prepared at a stock concentration of 1 mM and diluted to the desired concentration for microinjection into each embryo.

Northern blot analysis of small RNAs by polyacrylamide gel electrophoresis

Total RNAs at an amount of 70 μg were separated on an 6% and 15% polyacrylamide/8M urea gel (Amersham Pharmacia) to analyze I300 RNA and miR-In300, respectively, by a Protein III Apparatus (BioRad). Gel was preheated to 55°C by electric current and water bath circulation before loading sample. The separated RNA on gel was electro-blotted onto a Hybond-N^+ membrane (Amersham) by Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad). After ultraviolet (UV) cross-linking and air drying, the blotted membrane was prehybridized with hybridization buffer (0.02% SDS, 5× SSC, 50% Fromamide deionized, 0.1% N-lauroylsarcosine, 2% Blocking solution and 20 μg/ml salmon sperm DNA) at 37–42°C for 60 min, hybridized with radiolabeled I300 or miR-In300 and incubated at 37–42°C for 3–4 h or overnight. The I300 was ^{32}P-labeled, and miR-In300 was DIG-labeled and LNA-incorporated (Exiqon). The membrane was washed two to four times at 40°C with 2× SSC and 0.5% SDS for 15 min and exposed to an X-ray film (Kodak, NY) at −80°C.

Searching for the target gene of miR-In300

To determine the target gene of miR-In300, we used our novel labeled microRNA pull-down (LAMP) assay system in which the pre-microRNA was labeled with digoxigenin (DIG) and then mixed with cell extracts as described previously (19). Briefly, DIG-labeled pre-miRNA was synthesized by using the DIG RNA labeling kit (Roche). After cell extracts were incubated with 70 μg of digoxigeninylated RNA at 4°C for 30 min, the total volume was adjusted to 1 ml with binding buffer (25 mM Tris-base pH 7.4, 60 mM KCl, 2.5 mM EDTA, 0.2% Triton X-100, 80 U of rRnasin), and the mixture was incubated at 30°C for 60 min. The purified RNA was collected when DNase I (20 U) was added and incubated at 37°C for 30 min before the phenol/chloroform extraction was performed. The putative target genes were precipitated by anti-DIG antiserum, cloned out by reverse transcriptase-polymerase chain reaction (RT-PCR) or collected by a pull-down assay. Then, we analyzed these cDNAs or putative clones for miR-In300 targeting.

(Other experimental procedures are available in Supplementary Data).

RESULTS

I300 segment of zebrafish *myf*5 specifically suppresses *myf*5 promoter activity

A plasmid containing –6300/–1 of zebrafish *myf*5 fused with a luciferase gene and a segment of I300 (+502/+835 of *myf*5) DNA were either transfected into the cell lines or injected into the zebrafish embryos. Results showed that the expression of luciferase reporter gene was dramatically reduced. Luciferase activity was decreased by 50% and 80% in the cell lines and zebrafish embryos, respectively (Supplementary Figure S1), which enabled

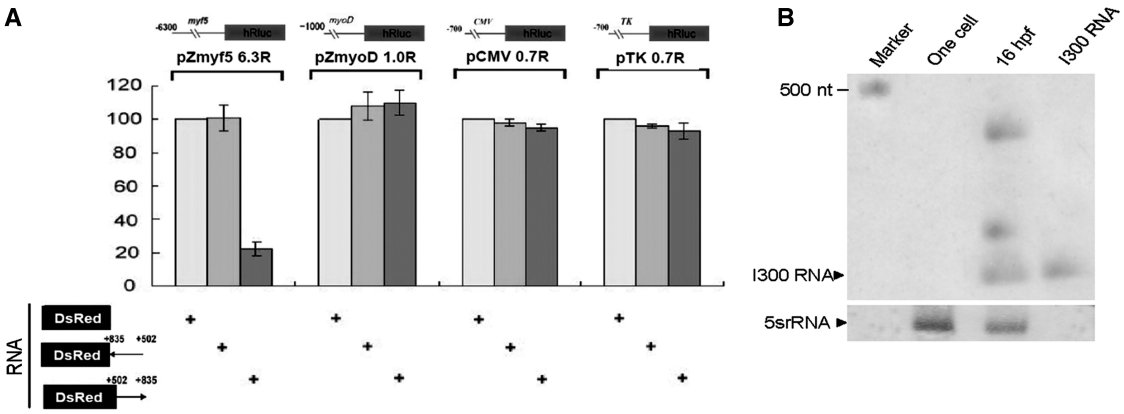

Figure 1. Intron 1 RNA is a repressive element for *myf5*-specific expression. (**A**) Plasmids used for the transient luciferase assay were microinjected into the one-cell stage of fertilized embryos. The luciferase gene was under-controlled in different promoters, including the *myf5* promoter (pZmyf5 6.3R), *myod* promoter (pZmyoD 1.0R), *CMV* promoter (pCMV 0.7R) and *TK* promoter (pTK 0.7R). RNAs used for microinjection into zebrafish embryos were mRNA encoding red fluorescence protein (DsRed), sense strand of the first intron +502/+835 (I300) of zebrafish *myf5* followed by DsRed mRNA (DsRed +502-> +835), and antisense strand of I300 followed by DsRed mRNA (DsRed +835-> +502). Luciferase activity was reduced only in the embryos co-injected with DsRed fused with sense I300 and the plasmid containing *myf5* promoter. (**B**) Detection of the existence of primary transcript of I300 RNA by northern blot analysis. Using I300 RNA as the positive control, a positive signal (333 nt) was shown in the 16-hpf embryos, but not in the one-cell stage (0 hpf) embryos. The 5S rRNA (5srRNA) was served as a loading control.

the sense strand of I300 DNA to repress the *myf5* promoter activity.

To accomplish this, RNA encoding I300 fused with red fluorescence protein (RFP, DsRed) was synthesized *in vitro* (DsRed +502/+835) and co-injected with four plasmids consisting of –6300/–1 of *myf5* (pZmyf5 6.3), −1014/–1 of *myod* (pZmyoD 1.0), cytomegalovirus promoter (pCMV), and thymidine kinase promoter (pTK). Results showed that I300 RNA did, in fact, enable the downregulation of luciferase activity driven by the *myf5* promoter in the RFP-expressing embryos by 20% from that of control (Figure 1A). These results are consistent with those obtained from co-injection with sense I300 DNA (+502/+835). However, the decrease of luciferase activity was neither from promoters of *myod*, CMV or TK, nor from injection of antisense I300 RNA (DsRed +835/+502), indicating that the I300 RNA-mediated repression is *myf5* promoter- and sense strand-specific. As expected, since the I300 RNA is spliced from intron 1 of *myf5*, Northern blot analysis showed that I300 RNA was predominantly detected in the somites of embryos at the 16 hpf stage when *myf5* was highly expressed, whereas I300 RNA was not detected at the one-celled stage when *myf5* was not expressed (Figure 1B). As this evidence demonstrated that I300 RNA is endogenous in zebrafish embryos, we were motivated to investigate whether RNA-mediated regulation is involved in *myf5* promoter activity.

The first intron of zebrafish *myf5* contains miR-In300

I300 RNA is derived from the first intron of the primary mRNA of zebrafish *myf5*. Using computer analysis, we predicted that the pre-miR-In300 sequence may locate at +546/+644 and that the mature miR-In300 may locate at +609/+632 (Figure 2A). In order to establish the soundness of these predictions, we first used northern blot to confirm the ability of pre-miR-In300 to process normally to a mature miR-In300 in the presence of endogenous Dicer of cell extracts (lanes 1 and 3, Supplementary Figure S2A). However, when Dicer was completely depleted from the cell extracts (Supplementary Figure S2B), the pre-miR-In300 was not processed to mature miR-In300 (lanes 2 and 4, Supplementary Figure S2A). Therefore, we next used anti-biotin beads to perform immunoprecipitation, and the complex of biotin-labeled miR-In300 and miRNP was obtained from cell extracts. When we used anti-AGO antiserum to perform western blot analysis, we found that AGO was detected in that complex (Supplementary Figure S2C). This result was consistent with the control group when miR-1 was used in parallel (Supplementary Figure S2C). Since miR-In300 was picked up by AGO, we are confident that it had indeed been processed correctly from pre-miR-In300 by Dicer. Furthermore, northern blot analysis also demonstrated the existence of a 22-nt miR-In300 in the somites of embryos during 16–30 hpf when the *myf5* transcripts were expressed (Figure 2B). However, among these stages, the miR-In300 signal appeared at its highest levels after 20 hpf (lane 3 of Figure 2B).

Based on whole-mount *in situ* hybridization, we found that miR-In300 was highly expressed in the older mature somites, but much less so in the newly formed somites (Figure 2D). This asymmetrical distribution stood in exact contrast to the distribution of the expression of *myf5* mRNA (Figure 2C and D), which is substantially greater at the early somite stages. This did not result from hybridization to the genomic DNA because no signal appeared when the control probe, which was complementary to the first intron at +1341/+1361 of *myf5*, was used (Figure 2F). Moreover, the positive control, miR-206, was shown in mature somites (Figure 2E).

In order to further confirm that the zebrafish miR-In300 motif functions as an RNAi, we next designed a plasmid construct, TK-Luciferase-5 X PT, in which a perfectly matched complementary sequence of miR-In300 in five

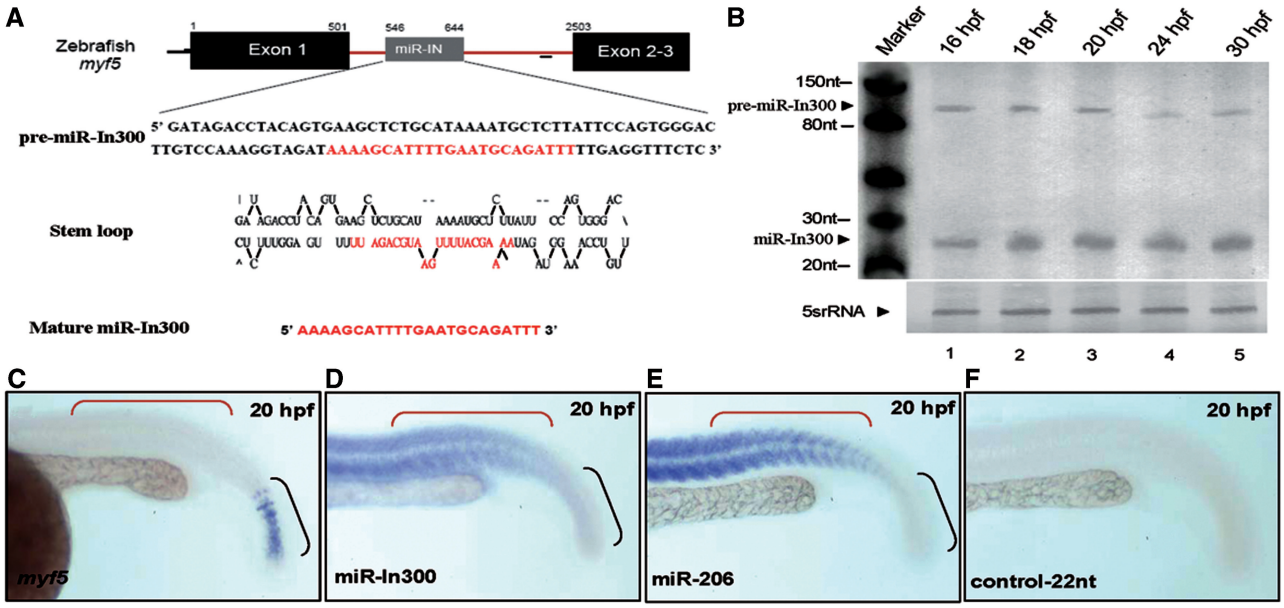

Figure 2. Expression patterns of zebrafish miR-In300. (**A**) miR-In300 is generated from intron 1 (+502/+2502) of the zebrafish *myf5* gene. Pre-miR-In300 (+546/+644) and mature microRNA sequences (indicated in red; +609/+632) are presented. The predicted secondary structure of pre-miR-In300 is also illustrated. (**B**) Detection of the existence of miR-In300 transcript by northern blot analysis. The total RNAs extracted from the various stages of zebrafish embryos. The RNA level of miR-In300 was gradually increased in the embryos from 16 hpf to 20 hpf. The 5S rRNA (5srRNA) was served as a loading control. (**C–F**) Expression patterns of *myf5*, miR-In300 and miR-206 in somites at 20 hpf were detected by whole-mount *in situ* hybridization. *myf5* was expressed only in the newly formed somites and in the presomitic mesoderm (PSM) at 20 hpf (C), whereas miR-In300 was predominant in the older formed somites, but only mildly present in the newly formed somites at 20 hpf (D). A muscle-specific microRNA in zebrafish, miR-206, was used as a positive control and was detected in mature muscle, but not in PSM at 20 hpf (E). In contrast, the antisense strand of *myf5* intron 1 (A, red line) served as negative control and did not present any signal (F).

copies (5 X PT) was inserted in the 3′ untranslated region (UTR) of luciferase cDNA and driven by the TK promoter (Figure 3A). Zebrafish embryos were co-injected with I300 RNA and TK-Luciferase-5 X PT. Results showed that the luciferase activity of these embryos was dramatically reduced, down to 25% of that observed in the embryos co-injected with I300 RNA and a control construct, TK-Luciferase, which does not contain a 5 X PT-inserted motif at the 3′ UTR of luciferase cDNA (Figure 3B). This result was consistent with embryos co-injected with I300 RNA and another control construct, TK-Luciferase-5 X mT, in which a mismatched complementary sequence of miR-In300 in five copies (5 X mT) was inserted at the 3′ UTR (Figure 3A). This evidence clearly demonstrated that miR-In300 does function as a microRNA by its repression of reporter gene expression through sequence-specific base-pairing with target mRNA at the 3′ UTR.

Either overexpression or knockdown of miR-In300 causes misexpression of *myf5* in somites

To further confirm whether miR-In300-mediated repression affects zebrafish *myf5* promoter activity *in vivo*, we microinjected 300 pg of miR-In300 dsRNA into single-cell fertilized eggs. Results showed that *myf5* expression was decreased, both in the newly formed somites of S-II, S-I, S0 and S1, as well as the presomitic mesoderm (PSM) at 15 hpf (Figures 4A and B versus 4C and D). On the other hand, we employed an antisense MOs which specifically blocks miR-In300 (*miR-In300*-MO) to examine *myf5* promoter activity at 16 hpf. First, we used northern blot analysis to prove that *miR-In300*-MO caused a decrease in the amount of miR-In300 (Figure 4I). Second, when *miR-In300*-MO was injected, we found that the expression of *myf5* was enhanced not only in the somites at S-II, S-I, S0 and S1, but also extended to S3 in the embryos (Figures 4E and F versus 4G and H). This evidence suggests that miR-In300 may suppress the transcription of *myf5* mRNA through abolishing the *myf5* promoter activity in the older formed somites at 16 hpf.

miR-In300 targets the long isoform of *dkk3* mRNA

To determine the target gene of miR-In300, we used our novel LAMP assay system (19). Accordingly, we obtained around 3000 putative clones for miR-In300 targeting. Among them, 667 clones were screened, and 20 putative clones potentially containing sequences complementary to miR-In300 were selected. Nine out of 20 contained the EST sequences, while the remaining 11 clones contained an undefined DNA sequence. Seven of the nine clones contained the same EST sequences. The full length of cDNA corresponding with this EST was cloned and identified as a zebrafish long-isoform *dkk3* gene (NM_001159283.1), also named *dkk3*-related gene, encoding a 293-amino-acid polypeptide with Cys-rich domain located at amino acid positions 134–182 and 170–275 (Figure S3A). The EST sequence was complementarily matched with three miR-In300-binding sites at 3′ UTR by miRanda v.1.0b software analysis (Figure S3B). Using RT-PCR, we revealed that the *dkk3* transcript was maternally inherited

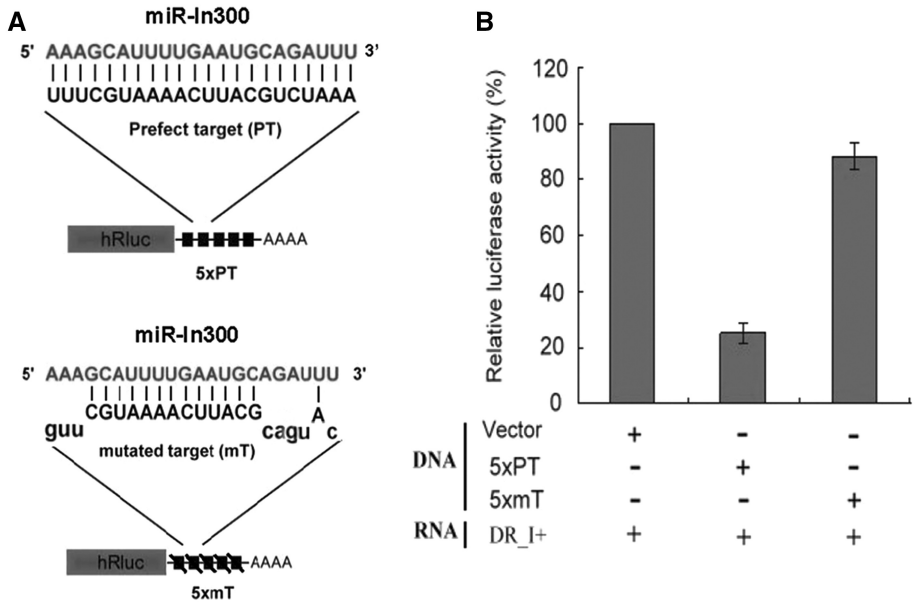

Figure 3. Injection of the sense strand of I300 RNA containing miR-In300 repressed the target mRNA expression in embryos. (**A**) Two plasmids containing a luciferase reporter gene (hRluc) fused with five copies (5 X) of either a perfectly matched target sequence (PT) or a mutated-mismatched target sequence (mT) for miR-In300 were constructed. (**B**) Relative luciferase activities of zebrafish embryos microinjected with the materials as indicated. Co-injection of the sense strand of I300 RNA (DR_I+) with a vector DNA containing hRluc gene served as a standard for comparison to the luciferase activities driven by co-injection of DR_I+ with a plasmid DNA containing either 5 X PT or 5 X mT. *In vivo* transgenesis enabled the sense strand of I300 RNA to repress the gene expression of luciferase in the 5 X PT construct, but not in the 5 X mT construct.

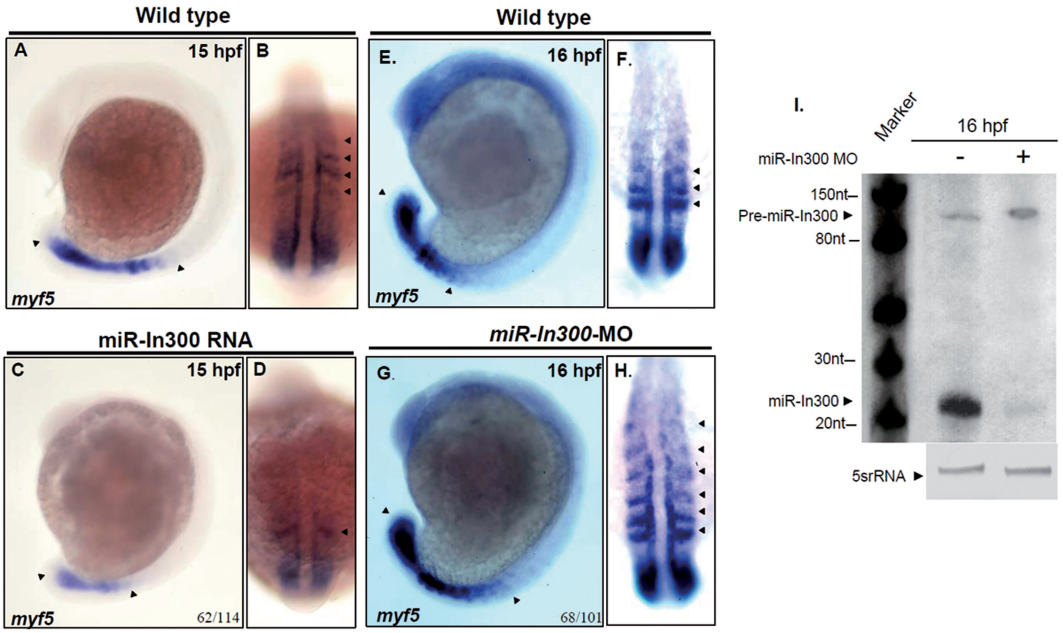

Figure 4. Change of miR-In300 level resulted in abnormal expression patterns of *myf5*. Expression patterns of *myf5* (**A** and **B**) in somites at the 12–14 somite stage, as indicated. However, when miR-In300 dsRNA was injected, the expression of *myf5* was greatly reduced in the newly formed somites and in the presomitic mesoderm (PSM), which became smaller (**C** and **D**). In wild-type embryos, *myf5* was expressed in S-II to S1 somites (**E** and **F**), while in the *miR-In300*-MO-injected embryos, *myf5* was expressed in S-II to S3 somites (**G** and **H**). Detection of the existence of miR-In300 transcripts by northern blot analysis. Results showed that miR-In300 was decreased in the *miR-In300*-MO-injected embryos at 16 hpf (**I**). The 5S rRNA (5srRNA) was served as a loading control.

and expressed in the somites from 16 to 30 hpf (Supplementary Figure S4). The remaining two clones contained another EST sequence, which was part of a sequence located at the 3′ UTR of another undefined target gene. When we used it as a probe to detect the expression pattern, results showed that it was also co-localized with miR-In300 in trunk somites (data not shown). Although similar, this clone was not the focus of this study.

Therefore, based on computer analysis, the putative miR-In300 binding site in the 3′ UTR of the *dkk*3

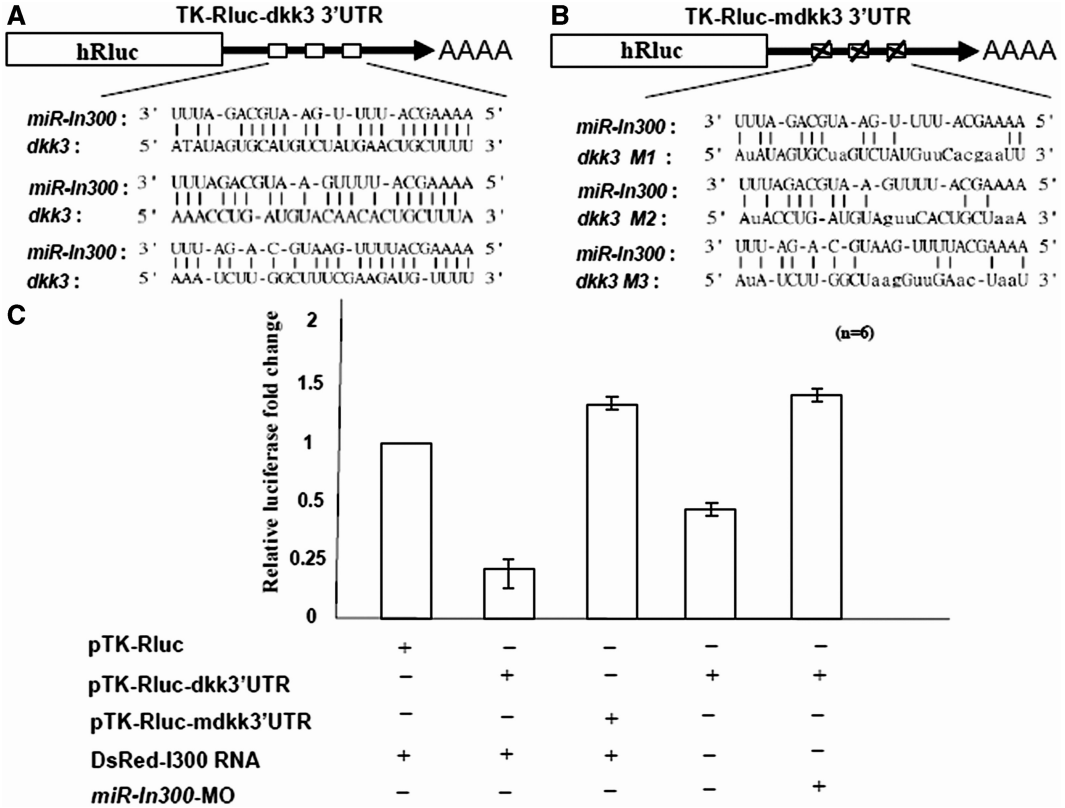

Figure 5. miR-In300 represses the translation of *dkk3* mRNA. Schematic illustration of plasmid constructs used for microinjection. (**A**) Plasmid Tk-Rluc-dkk3 3′ UTR, in which the *dkk3* 3′ UTR was fused with the 3′ UTR of the luciferase gene and driven by TK promoter. Three putative miR-In300 binding sites, which are located at *dkk3* 3′ UTR, are indicated by empty boxes, and their possible targeted sequences are also presented. (**B**) Plasmid TK-Rluc-mdkk3 3′ UTR, which was the same as plasmid Tk-Rluc-dkk3 3′ UTR, except that the mutated target sequences (M1, M2 and M3) of *dkk3* 3′ UTR were included, are indicated by crossed boxes and the lowercase alphabet. (**C**) Luciferase activities in the microinjected embryos. TK-Rluc was constructed without ligating any sequence into the 3′ UTR region of the luciferase gene, which served as basal control. DsRed-I300 (+) was a sense strand RNA of I300. Compared to the embryos injected with TK-Rluc and I300 RNA, the luciferase activity of embryos injected with Tk-Rluc-dkk3 3′ UTR and I300 RNA decreased greatly, which was only 1/4th of the luciferase activity induced by injection of TK-Rluc and I300 RNA. However, the luciferase activities of embryos injected with I300 RNA and TK-Rluc-mdkk3 3′ UTR, which contained mutated sequences, remained unchanged, compared to the luciferase activity driven by I300 RNA and TK-Rluc. Co-injection of *miR-In300*-MO with Tk-Rluc-dkk3 3′ UTR showed that the luciferase activity was increased 3-fold over that of embryos injected with Tk-Rluc-dkk3 3′ UTR alone, without co-injection of *miR-In300*-MO.

mRNA was highly complementary with the miR-In300. However, in order to confirm that the miR-In300 target sequence was located at the 3′ UTR of *dkk*3 and enabled mediation of translational repression, we monitored the luciferase activity in embryos injected with a construct in which the 3′ UTR of *dkk*3 was inserted downstream of the luciferase cDNA driven by TK promoter (TK-Rluc-dkk3 3′ UTR) (Figure 5A). Results showed that luciferase activity was greatly decreased in the embryos injected with I300 RNA and TK-Rluc-dkk3 3′ UTR, compared to that of embryos injected with I300 RNA with a plasmid in which the luciferase cDNA did not contain 3′ UTR of dkk3 (TK-Rluc) (Figure 5C). If embryos received injection with I300 RNA using a plasmid in which the luciferase cDNA contained mutated sequences of *dkk3* 3′ UTR (TK-Rluc-mdkk3 3′ UTR) (Figure 5B), the luciferase activity was not responsive to miR-In300 (Figure 5C). Furthermore, if *miR-In*300-MO was used to reduce the endogenous miR-In300 and co-injected with TK-Rluc-dkk3 3′ UTR to embryos, the luciferase activity was initially increased, but then reduced when embryos did not receive *miR-In*300-MO (Figure 5C). This evidence strongly supports the implication that the recognition sequence of zebrafish miR-In300 is specifically bound at the 3′ UTR of *dkk3* mRNA in a canonical binding assay.

The specific defects induced by the injected MOs

Two different MOs were specifically designed to inhibit the translation of *dkk3* mRNA. One was complementary to 25 bp after AUG (*dkk3*-MO1), and another, *dkk3*-MO1, contained five mutated nucleotides (*dkk3*-control-MO). Injection of 5 ng *dkk3*-MO1 resulted in defective embryos characterized by reduced head size and wide, U-shaped somites with irregular boundaries and the expression of *myf5* was downregulated in the somites when the *myf5* probe was used to perform whole-mount *in situ* hybridization and the embryos were observed at 13 hpf and 16 hpf (Supplementary Figure S5). However, injection of *dkk3*-control-MO did not result in any defective embryos, even when the *dkk3*-control-MO was injected as high as 8 ng per embryo (Supplementary

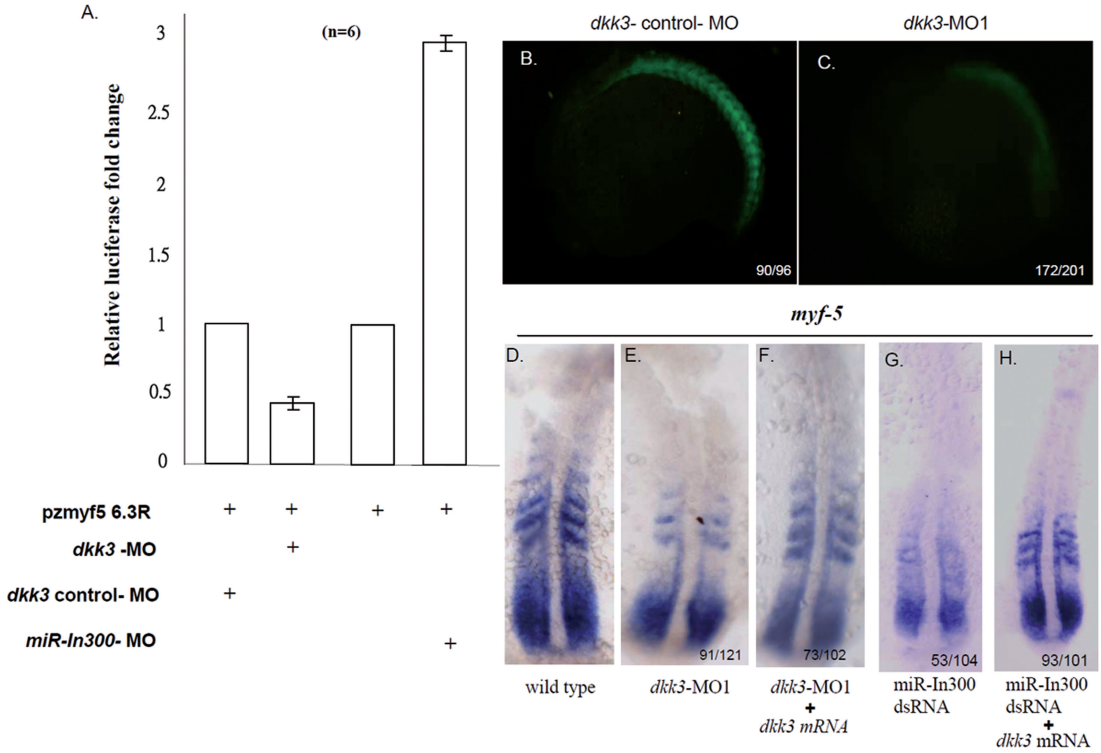

Figure 6. The *myf*5 promoter activity is controlled by the *dkk3* gene. (**A**) Plasmid and MO, as indicated by +, were co-microinjected into the one-celled stage of fertilized embryos to carry out the transient luciferase assay. The luciferase activity driven by the upstream 6.3 kb of zebrafish *myf5* promoter (pZmyf5 6.3R) and the co-injected *dkk3*-control-MO was measured in average ($n = 6$) and served as 100%. Compared to the embryos injected with pZmyf5 6.3R and *dkk3*-control-MO, the luciferase activity was greatly reduced in the embryos injected with pZmyf5 6.3R and *dkk3*-MO1. Meanwhile, the average of luciferase activity driven by pZmyf5 6.3R was also measured and served as 100%. Compared to the embryos injected with pZmyf5 6.3R, the luciferase activity was dramatically increased for the embryos in which the endogenous miR-In300 production had been inhibited by injection of *miR-In300*-MO. Zebrafish embryos derived from the transgenic line *Tg* (*myf5* (*80K*): *GFP*), whose somites display GFP reporter, were used. (**B**) When *dkk3*-control-MO was injected, the GFP expression in somites remained unchanged at 16 hpf, whereas (**C**) the GFP was greatly reduced in somites when *dkk3*-MO1 was injected. (**D–H**) Whole-mount *in situ* hybridization of *myf5* transcripts in zebrafish embryos at 16 hpf. Compared to the control (D), the *myf5* signal in the somites of either *dkk3*-MO1-injected embryos (E) or miR-In300-dsRNA-injected embryos (G) was decreased. Co-injection of either *dkk3*-MO1 with *dkk3* mRNA (F) or excess miR-In300-dsRNA with *dkk3* mRNA (H) enabled embryos to rescue the defects induced by injecting either *dkk3*-MO1 alone (E) or miR-In300-dsRNA alone (G). The numbers shown in the lower-right corner of panels B, C, E, F, G and H indicate the number of phenotypes out of the number of embryos examined.

Figure S5).To confirm that the phenotypes of morphants were specific as a result Dkk3 loss of function, we constructed a *dkk3-GFP* and microinjected 0.1 ng of *dkk3-GFP* RNA in the presence of 1.5 ng *dkk3*-MO1, which resulted in the absence of GFP expression (Supplementary Figure S6). In contrast, there was no effect on the expression of GFP in embryos co-injected with 0.1 ng of *dkk3-GFP* RNA and 1.5 ng *dkk3*-control-MO (Supplementary Figure S6). Furthermore, we co-injected synthetic *dkk*3 mRNA and *dkk*3-MO1, which enabled synthetic *dkk*3 mRNA to rescue the morphological defects induced by 5 ng *dkk*3-MO1 (Figure 6E versus 6F). In contrast, by injection of *dkk3* mRNA alone, no defective phenotype was observed. To further study whether the phenotypes induced by *dkk*3-MO1 were specific, we designed *dkk3*-MO2, whose corresponding sequences were located at the boundary between exon 2 and intron 2. Results showed that the defective phenotypes caused by injection of 4 ng *dkk3*-MO2 were identical to those caused by *dkk3*-MO1 (Supplementary Figure S5). Therefore, we concluded that the phenotypes of *dkk3*-MO1-injected embryos were specific.

The promoter activity of *myf*5 is modulated by *dkk3* expression

To study whether *dkk3* affects zebrafish *myf5* expression, we first co-injected pZmyf5 6.3R with *dkk3*-MO1, which resulted in the repression of luciferase activity (Figure 6A). However, co-injection of pZmyf5 6.3R with *dkk3*-control-MO had no effect on the luciferase activity in embryos (Figure 6A), indicating that the absence of *dkk3* reduces the expression of zebrafish *myf5 in vivo*. Next, we injected either *dkk3*-MO1 or *dkk3*-control-MO into embryos derived from the zebrafish transgenic line *Tg* (*myf5* (*80K*): *GFP*) in which the upstream 80 kb segment of *myf*5 fused with the GFP reporter (20). Results showed that the expression of GFP was repressed dramatically in transgenic embryos injected with *dkk3*-MO1 (Figure 6C), but not in embryos injected with *dkk3*-control-MO (Figure 6B). Therefore, our data suggest that *dkk3*-MO1 specifically inhibits the expression of *myf5* promoter activity in zebrafish embryos. Furthermore, the *myf5* signal in the somites of miR-In300-dsRNA-injected embryos was decreased (Figure 6G). Co-injection of

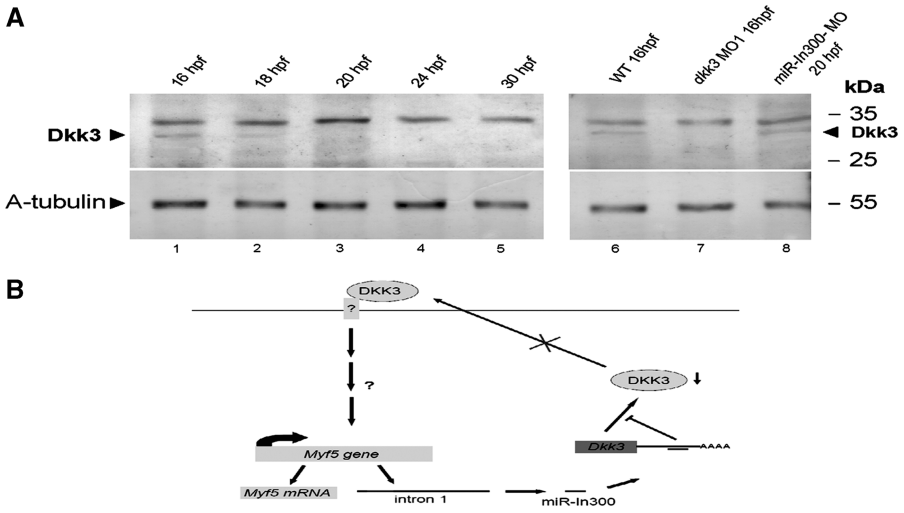

Figure 7. Western blot analysis of Dkk3 protein during embryogenesis. (**A**) Total protein lysates were extracted from zebrafish embryos at 16, 18, 20, 24 or 30 hpf, as indicated. The molecular weight of protein markers and the positions of positive reactive bands for antiserum against Dkk3 and alpha-tubulin (A-tubulin) are also indicated. The protein level of Dkk3 was greatly reduced in the protein lysates extracted from embryos at 18 hpf. The Dkk3 protein became undetectable at 20, 24 and 30 hpf. The Dkk3 protein was reduced in the lysates extracted from the *dkk3*-MO1-injected embryos (lane 7), whereas the Dkk3 protein was greatly enhanced in the lysates extracted from the *miR-In300*-MO-injected embryos at 20 hpf (lane 8). The intensity of positive bands for antiserum against A-tubulin served as a loading control. (**B**) Possible model that illustrates the modulation of *myf5* expression through miR-In300 mediation during somitogenesis in zebrafish embryos. The miR-In300 is an intronic microRNA within the first intron of zebrafish *myf*5, and *dkk3* is the target gene of miR-In300. The miR-In300 blocks the target sequences located at the 3′ UTR of *dkk3* mRNA, which results in repressing the translation of *dkk*3 mRNA. The absence of Dkk3 protein in zebrafish embryos then causes the downregulation of *myf5* promoter activity through an unknown signal transduction pathway. Consequently, *myf5* mRNA expression is gradually decreased at the later stages of zebrafish embryogenesis.

excess miR-In300-dsRNA with *dkk3* mRNA enabled embryos to rescue the defects induced by injecting miR-In300-dsRNA alone (Figure 6H). Interestingly, when pZmyf5 6.3R and *miR-In300*-MO were co-injected into embryos, the luciferase activity was increased, suggesting that the *myf5* promoter activity was enhanced by the inhibition of endogenous miR-In300 (Figure 6A).

To further validate *in vivo* the correlation between *dkk3* and miR-In300, the expressions of Dkk3 protein and mRNA, as well as miR-In300, during embryonic development of zebrafish, were investigated. Cell extracts of embryos from different developmental stages were prepared for western blot analysis by using anti-Dkk3 antibody. Results showed that the protein level of Dkk3 was greatly reduced in the *dkk3*-MO1-injected embryos (lane 7, Figure 7A), suggesting that the anti-Dkk3 antibody could recognize the Dkk3 protein. Furthermore, we found that Dkk3 protein was highly produced at the 16 hpf stage (lane 1, Figure 7A) when, at the same time, *myf5* was highly expressed, but miR-In300 was weakly expressed. We found that the level of Dkk3 protein was greatly decreased at 18 hpf (lane 2, Figure 7A) and undetectable at 20 hpf, as well as later stages examined, such as 24 hpf and 30 hpf (lane 4 and lane 5, Figure 7A). On the other hand, miR-In300 was highly expressed after 20 hpf (Figure 2B). Interestingly, when the protein lysates were extracted from wild-type embryos at 16 hpf and from the *miR-In300*-MO-injected embryos at 20 hpf, the protein level of Dkk3 in the *miR-In300*-MO-injected embryos at 20 hpf was as high as the protein level of Dkk3 in the wild-type embryos at 16 hpf (lane 8, Figure 7A). At 20 hpf, however, the protein level of Dkk3 became undetectable in the wild type (lane 3, Figure 7A). Taken together, these data therefore suggested that 1) the miR-In300 level is negatively correlated with the protein level of Dkk3 protein and 2) the absence of endogenous miR-In300 causes an increase of Dkk3 protein in zebrafish embryos. From these lines of evidence, we can, in turn, conclude that miR-In300 specifically binds to its target gene *dkk3*, which leads to three interacting consequences: (i) inhibiting the translation of *dkk3* mRNA, thus (ii) decreasing the Dkk3 protein level, an event ultimately (iii) suppressing the promoter activity of zebrafish *myf5*.

DISCUSSION

Around three-fourths of identified microRNAs are intergenic, whereas the remainder belongs to the class known as intronic microRNAs. To date, about 90 intronic microRNAs have been predicted among humans, mice, and *C. elegans,* but most of them have originated from bioinformatics BLASTing and have not yet been validated (21). Recently, an intronic microRNA known as miR-208 was confirmed as a cardiac-specific microRNA, which is encoded by an intron of the α-myosin heavy chain (αMHC) gene and is reported to be required for cardiomyocyte hypertrophy, fibrosis and expression of βMHC in response to stress and hypothyroidism (22). In this study, we are the first to report a *de novo* intronic microRNA, miR-In300, to isolate its cognate target gene, *dkk3*, and to provide evidence that it functions as a negative modulator to reduce *myf*5

promoter activity in somites of zebrafish by silencing *dkk3* during embryonic stage.

As development progresses, we observed that the expression of *myf5* is decreased in the somites at 24 hpf and is not detectable at 30 hpf. Interestingly, it is during the same developmental stages that the expression level of miR-In300 is lower in the newly formed somites where *myf*5 starts its transcription, but gradually appears in somites where *myf*5 had previously been expressed. As a result, up to 30 hpf, almost all somites have the same expression level of miR-In300, but after 30 hpf, we noticed that miR-In300 remains highly expressed. Therefore, we hypothesize that only pre-miR-In300, but not mature miR-In300, is generated when *myf*5 is transcribed initially in the newly formed somites. After mature miR-In300 is processed, the miR-In300 is accumulated in the older mature somites by the long half-life of microRNA, a phenomenon which is in agreement with Rooij *et al*. (18) who reported that the half-life of miR-208 is around 21 days. This hypothesis is also supported by Giraldez *et al*. (22) who reported that mature miR-430 of zebrafish is not shown until 4 hpf, but pre-miR-430 starts to present at 2.5 hpf.

In this study, we discovered that *dkk3* mRNA is the target of miR-In300. The Dkk family is composed of four main members (Dkk1, 2, 3, 4 and Soggy). Many reports have described that all family members, except Dkk3, typically regulate Wnt/β-catenin signaling (23,24). Recently, however, Yue *et al*. (25) demonstrated that Dkk3 is involved in Wnt signaling. Whether Dkk3 plays the same role in the simitogenesis of zebrafish embryos is open to further investigation; however, this study has presented evidence suggesting that it does modulate zebrafish *myf5* promoter activity. This is noteworthy in view of the fact that *dkk3* is known as a tumor suppressor gene in higher vertebrates (26) Specifically, evidence clarifying the roles and relationships between miR-In300 and *myf5* promoter activity was provided by our two luciferase assays, which first indicated that either I300 DNA or I300 RNA is capable of silencing zebrafish *myf*5 promoter activity (Supplementary Figure S1 and Figure 1A). We next demonstrated that the recognition sequence of zebrafish miR-In300 is specifically located at the 3′ UTR of *dkk3* mRNA and inhibits luciferase activity (Figure 5), suggesting that miR-In300 binds to the *dkk3* 3′ UTR, thereby validating *dkk3* as a target gene of miR-In300. Although the detailed regulatory pathway involved in Dkk3-mediated *myf*5 expression requires further study, we have conclusively shown that the following sequence of events leads to the gradual disappearance of *myf5* mRNA expression in late-stage somites. First, during *myf5* mRNA transcription, an I300 segment +502/+835 is generated from the first intron of primary zebrafish *myf*5 mRNA transcripts. Second, within this I300 segment, an intronic microRNA, miR-In300, is processed to maturity from +609/+632. Third, miR-In300 inhibits *myf5* promoter activity by silencing the *dkk3* gene and blocking the expression of *myf5* mRNA at mature somite regions.

The decrease of Dkk3 protein level, which becomes undetectable after 20 hpf (Figure 7A), seems to be correlated to the down-regulation of *myf5* mRNA after 16 hpf, whereas the level of *dkk3* mRNA remains unchanged (Supplementary Figure S4F and G). Importantly, this is also the period when, as noted above, miR-In300 gradually appears in somites in which *myf*5 had previously been expressed. This evidence suggests that the distribution of miR-In300 is negatively correlated with the protein level of Dkk3 during zebrafish embryogenesis. Furthermore, as shown in Figure 7A, inactivation of Dkk3 expression by miR-In300 is further demonstrated by up-regulation of *dkk3* in the *miR-In300*-MO-injected embryos. For example, those 20-hpf embryos injected with *miR-In300*-MO to abolish the endogenous miR-In300 displayed a high protein level of Dkk3, which is similar to the level of Dkk3 protein observed in the 16 hpf embryos (Figure 7A). Taken together, these lines of evidence support our hypotheses that *dkk3* is a target gene of miR-In300, that microRNA is indeed responsible for the post-transcriptional silencing of *dkk3* expression, and, finally, that miR-In300 is a strong negative modulator of zebrafish *myf5* transcription (Figure 7B).

In summary, we have demonstrated that (i) miR-In300 is derived from the first intron of *myf*5; (ii) the miR-In300-mediated *myf*5 promoter activity is gene-specific and regulates *dkk*3 mRNA translation; and (iii) the transfection of exogenous miR-In300 can significantly reduce *myf*5 transcription through the silencing of *dkk3* gene translation, an event which most likely occurs with optimum efficiency during *myf*5 transcription.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

National Science Council of Republic of China under the grant of NSC 97-2313-B-002-036-MY3, ROC.

Conflict of interest statement. None declared.

REFERENCES

1. Pownall,M.E., Gustafsson,M.K. and Emerson,C.P. Jr (2002) Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell. Dev. Biol.*, **18**, 747–783.
2. Buckingham,M. (1992) Making muscle in mammals. *Trends Genet.*, **8**, 144–148.
3. Hacker,A. and Guthrie,S. (1998) A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. *Development*, **125**, 3461–3472.
4. Hirsinger,E. (2001) Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. *Development*, **128**, 107–116.
5. Chen,Y.H., Lee,W.C., Liu,C.F. and Tsai,H.J. (2001) Molecular structure, dynamic expression, and promoter analysis of zebrafish (*Danio rerio*) myf-5 gene. *Genesis*, **29**, 22–35.
6. Coutelle,O., Blagden,C.S., Hampson,R., Halai,C., Rigby,P.W. and Hughes,S.M. (2001) Hedgehog signalling is required for maintenance of *myf5* and *myoD* expression and timely terminal differentiation in zebrafish adaxial myogenesis. *Dev. Biol.*, **236**, 136–150.

7. Chen,Y.H., Lee,H.C., Liu,C.F., Lin,C.Y. and Tsai,H.J. (2003) Novel regulatory sequence -82/-62 functions as a key element to drive the somite-specificity of zebrafish *myf5*. *Dev. Dyn.*, **228**, 41–50.
8. Lee,H.C., Huang,H.Y., Lin,C.Y., Chen,Y.H. and Tsai,H.J. (2006) Foxd3 mediates zebrafish *myf5* expression during early somitogenesis. *Dev. Biol.*, **290**, 359–372.
9. Lin,C.Y., Chen,Y.H., Lee,H.C. and Tsai,H.J. (2004) Novel cis-element in intron 1 represses somite expression of zebrafish *myf5*. *Gene*, **334**, 63–72.
10. Lee,R.C., Feinbaum,R.L. and Ambros,V. (1993) C. *elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, **75**, 843–848.
11. Lagos-Quintana,M., Rauhut,R., Yalcin,A., Meyer,J., Lendeckel,W. and Tuschl,T. (2002) Identification of tissue-specific microRNAs from mouse. *Curr. Biol.*, **312**, 735–739.
12. Ambros,V. and Lee,R.C. (2004) Identification of microRNAs and other tiny noncoding RNAs by cDNA cloning. *Methods Mol. Biol.*, **265**, 131–158.
13. Lim,L.P., Glasner,M.E., Yekta,S., Burge,C.B. and Bartel,D.P. (2003) Vertebrate microRNA genes. *Science*, **299**, 1540.
14. Llave,C., Xie,Z., Kasschau,K.D. and Carrington,J.C. (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. *Science*, **297**, 2053–2056.
15. Reinhart,B.J. and Bartel,D.P. (2002) Small RNAs correspond to centromere heterochromatic repeats. *Science*, **297**, 1831.
16. Watanabe,T., Takeda,A., Mise,K., Okuno,T., Suzuki,T., Minami,N. and Imai,H. (2005) Stage-specific expression of microRNAs during *Xenopus* development. *FEBS Lett.*, **579**, 318–324.
17. Lin,S.L., Miller,J.D. and Ying,S.Y. (2006) Intronic microRNA (miRNA). *J. Biomed. Biotechnol.*, **2006**, 26818.
18. Rooij,Van.E., Sutherland,L.B., Qi,X., Richardson,J.A., Hill,J. and Olson,E.N. (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science*, **316**, 575–579.
19. Hsu,R.J., Yang,H.J. and Tsai,H.J. (2009) Labeled microRNA pull-down assay system: an experimental approach for high-throughput identification of microRNA-target mRNAs. *Nucleic Acids Res.*, **37**, e77.
20. Chen,Y.H., Wang,Y.H., Chang,M.Y., Lin,C.Y., Weng,C.W., Westerfield,M. and Tsai,H.J. (2007) Multiple upstream modules regulate zebrafish *myf5* expression. *BMC Dev. Biol.*, **3**, 7:1.
21. Rodriguez,A., Griffiths-Jones,S., Ashurst,J.L. and Bradley,A. (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Res.*, **14**, 1902–1910.
22. Giraldez,A.J., Mishima,Y., Rihel,J., Grocock,R.J., Van Dongen,S., Inoue,K., Enright,A.J. and Schier,A.F. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, **312**, 75–79.
23. Krupnik,V.E., Sharp,J.D., Jiang,C., Robison,K., Chickering,T.W., Amaravadi,L., Brown,D.E., Guyot,D., Mays,G., Leiby,K. *et al.* (1999) Functional and structural diversity of the human Dickkopf gene family. *Gene*, **238**, 301–313.
24. Mao,B., Wu,W., Li,Y., Hoppe,D., Stannek,P., Glinka,A. and Niehrs,C. (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature*, **411**, 321–325.
25. Yue,W., Sun,Q., Dacic,S., Landreneau,R.J., Siegfried,J.M., Yu,J. and Zhang,L. (2008) Downregulation of Dkk3 activates beta-catenin/TCF-4 signaling in lung cancer. *Carcinogenesis*, **29**, 84–92.
26. Kuphal,S., Lodermeyer,S., Bataille,F., Schuierer,M., Hoang,B.H. and Bosserhoff,A.K. (2006) Expression of Dickkopf genes is strongly reduced in malignant melanoma. *Oncogene*, **25**, 5027–5036.