The role of miR-92 in regulating early development and metamorphosis of Japanese flounder *Paralichthys olivaceus*

Xuemei Li, Hongshuang Hu, Rui Li, Zhenwei Wang, Jie Qi and Zhigang Wang*

Key Laboratory of Marine Genetics and Breeding, Ministry of Education, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

(Received 2 September 2018, accepted 20 May 2019; J-STAGE Advance published date: 14 December 2019)

MicroRNAs are a class of short non-coding RNAs that contain approximately 22 nucleotides and play a regulatory role in RNA silencing and translational repression. miR-92 belongs to the miR-17-92 family and has a regulatory effect on cell proliferation, apoptosis, and expression of proto-oncogenes and tumor suppressor genes. However, its function in flatfish is unclear. In this study, we used farmed Japanese flounder, *Paralichthys olivaceus*, and showed that *gata5* is a target gene of miR-92. Experiments on miR-92 overexpression indicated that *gata5* and *sox17* were downregulated, while the transcription level of *ntl* increased. By contrast, depletion of miR-92 resulted in increased *gata5* and *sox17* levels and reduced *ntl* level. Moreover, thiourea treatment indicated that miR-92 may inhibit the metamorphic development of Japanese flounder. Our study suggests that miR-92 regulates the fate of endoderm and mesoderm by controlling *gata5*.

Key words: embryonic development, metamorphic development, miR-92, *Paralichthys olivaceus*

INTRODUCTION

MicroRNAs (miRNAs) are a family of 21–23-nucleotide endogenous non-coding RNAs, which are derived from monocistronic, bicistronic or polycistronic transcripts. They play a regulatory role in gene expression by binding to specific sequences in the 3′ untranslated region (UTR) (Bartel, 2004), 5′ UTR (Lytle et al., 2007) or open reading frame (ORF) (Duursma et al., 2008) region of target genes after transcription. MicroRNAs such as miR-263, miR-14 and miR-2 play a key role in cell proliferation, development, differentiation and apoptosis (Kloosterman and Plasterk, 2006). miR-8 and miR-200 are notch-induced inhibitors of overgrowth and tumor metastasis, and miR-279 regulates the formation of carbon dioxide receptors (Hartl et al., 2011). In addition, miRNA is a class of oncogenes or tumor suppressor genes, whose abnormal expression leads to various human diseases, especially cancer. To date, most studies on miRNAs have focused on mammals, amphibians, birds, insects, plants and teleosts. However, the limitations of high-throughput sequencing technology impede miRNA research (Fu et al., 2011). Therefore, miRNAs have been screened and

identified using microarray techniques to study their differential expression (Bizuyehu et al., 2012).

The small RNA cluster of miR-17-92 produces an 800-nucleotide polycistronic primary transcript, resulting in the production of six mature human miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. The cluster is located in an intron, and this region is frequently expanded in various lymphomas and solid tumors (Ota et al., 2004; Hayashita et al., 2005). The miR-17-92 cluster also has a transcriptional activation function, which is regulated by c-Myc and E2F3 (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007). The multiple downstream targets of the miR-17-92 cluster contribute to its tumorigenic effects, and include *E2F1* (O'Donnell et al., 2005; Woods et al., 2007), *p21/CDKN1A* (Ivanovska et al., 2008; Petrocca et al., 2008), *Bim/BCL2L11*, *PTEN* (Koralov et al., 2008; Ventura et al., 2008; Xiao et al., 2008), *Tsp1* and *CTGF* (Dews et al., 2006). The specific biological role of miR-92 has been reported in zebrafish. During the development of zebrafish pharyngeal cartilage, miR-92a, whose target sequence is located within *noggin3*, can indirectly regulate the BMP signaling pathway, and its overexpression can cause pharyngeal dysplasia (Ning et al., 2013). During the proliferation of endothelial cells, both in vivo and in vitro experiments have confirmed that miR-92a greatly affected the function of endothelial cells (Bonauer et al.,

Edited by Rimantas Kodzius

* Corresponding author. E-mail: zgwang@ouc.edu.cn

DOI: <http://doi.org/10.1266/ggs.18-00047>

2009; Wu et al., 2011), and pharmacological inhibition of miR-92a promoted angiogenesis, but the capillary density was not much affected. These studies not only confirm the regulatory effect of miR-92 on multiple genes at post-transcriptional levels, but also imply the diversity and complexity of its function.

The study of miR-92 in teleosts is mainly focused on zebrafish and Atlantic halibut. miR-92 plays a key role in the endomesoderm and gastrulation development by targeting *gata5*. It is also involved in the formation of left-to-right asymmetric patterning of zebrafish (Li et al., 2011). In Atlantic halibut, the expression levels of miR-92a and miR-92b are significantly upregulated at the epiboly and somite stages (Bizuyehu et al., 2012), suggesting that miR-92 plays an important role in the early development of teleosts. In Japanese flounder, the expression level of miR-92 shows significant differences during metamorphosis (Fu et al., 2011). This finding prompted us to consider the role of miR-92 in the early developmental stage, especially in the development of the embryo, as well as its relationship with the asymmetric mechanism during the metamorphosis of flounder.

The early development stage of Japanese flounder involves a critical process, metamorphosis. During this stage, the body structure and living habits of the flounder change significantly through cell proliferation and differentiation, and many larvae cannot adapt to such dramatic change, resulting in death. This has caused great difficulties for early fish breeding. Therefore, a deeper understanding of metamorphosis of Japanese flounder is vital for flounder breeding and the whole industry.

In this study, we revealed the relationship between miR-92 and *gata5* during the early developmental stages of Japanese flounder. The function of miR-92 in the process of early germ layer development and metamorphosis is considered through target gene verification and the overexpression and inhibition of miR-92. Our findings lay the groundwork for further research.

MATERIALS AND METHODS

Sample preparation of Japanese flounder and zebrafish Japanese flounder were reared in a commercial hatchery in Haiyang City, China. The water temperature was maintained at 17 °C. Artificially fertilized eggs were incubated in an incubation tank with seawater and continuous aeration. Embryos at different stages of development, including 1–4-cell stages, were collected for injection, and 50% epiboly (midgastrula), tailbud and hatching stages were collected for observation. Eighteen days after fertilization, the eggs were randomly divided into two groups. Sample numbers for each group were about 500 zygotes. The thiourea (TU) treatment group and the control group were reared in a glass fish tank with a length of 30 cm, a width of 14 cm, a height of

40 cm and a depth of 10 cm. The room temperature was maintained at 20 °C. The TU concentration in the water was 30 mg/l. Artemia was provided as food, the water was replaced at 09:00 and 14:00 daily, and the TU was replenished after changing the water in the TU treatment group. AB line wild-type zebrafish were maintained at 28 °C under standard fish-rearing conditions with a 14-h light/10-h dark cycle, and fed with live Artemia twice a day. Embryos from different development stages, including 1–4-cell stages, were collected for injection, while 70% epiboly stage embryos were collected for observation.

Sequence alignment and target gene prediction Multiple sequence alignment of miR-92a and miR-92b sequences was performed using the ClustalX2 program. The online target site prediction software platforms TargetScan and MicroCosmTargets were used to search for potential targets of miR-92. Target sites of miR-92 in target gene 3' UTRs were identified using RNAhybrid software.

Isolation of RNA and cDNA synthesis Total RNA was extracted from samples using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. miRNA was extracted from samples taken at days 20, 22, 27 and 35 after hatching, using the mir-Vana miRNA Isolation Kit (Thermo Fisher Scientific, MA, USA). A total of 1 µg RNA from each sample was reverse-transcribed according to the instructions of the PrimeScript RT reagent kit with the gDNA Eraser (Takara, Dalian, China). The final reaction volume was set at 20 µl.

Molecular cloning of *gata5* Primers, *P. olivaceus* (*Po*)-*gata5*-fw/rv (Table 1), were designed for core fragment PCR amplification. 5' and 3' RACE were employed to obtain full-length cDNA of *Po-gata5* using the Smart RACE cDNA amplification kit (Clontech, CA, USA) according to the manufacturer's instruction. Based on the central fragment, *Po-gata5*-3'RACE-fw1, *Po-gata5*-3'RACE-fw2, *Po-gata5*-5'RACE-rv1 and *Po-gata5*-5'RACE-rv2 (Table 1) were designed for 5' and 3' RACE amplification using Touchdown and Nest PCR. The PCR products were identified through agarose gel electrophoresis and purified using the Gel DNA Recovery Kit (Zymoclean, CA, USA) for sequencing.

Construction of reporter plasmids The predicted target sequences of *gata5* were cloned downstream of the mCherry coding sequence (XhoI/BamHI sites) in the plasmid pmCherry-C1, yielding the *gata5*-3'UTR-pmCherry vector. Mutant primers, *Po-gata5*-3'mutant1-fw, *Po-gata5*-3'mutant1-rv, *Po-gata5*-3'mutant2-fw and *Po-gata5*-3'mutant2-rv (Table 1), were designed using QuikChange Primer Design software.

Table 1. Primers used in this study

Name	Primer sequence (5'-3')	Amplification target
<i>Po-gata5</i> -fw	GGCCACTACCTGTGCAAAAC	Core fragment
<i>Po-gata5</i> -rv	GACGTGAGTTTCAGGAGAACAG	Core fragment
<i>Po-gata5</i> -fw1	CCGCTCGAGGAAGATTATCCCTTTACCCCGAC	Reporter construction
<i>Po-gata5</i> -rv1	CGCGGATCCCCACAACGACTGGTCCTGAT	Reporter construction
<i>Po-gata5</i> -3'RACE-fw1	TGTTGAACATGTCTCATTACAAGCG	3' RACE
<i>Po-gata5</i> -3'RACE-fw2	GTGACTTGCTTTAGAGGGATACAGC	3' RACE
<i>Po-gata5</i> -5'RACE-rv1	GA CTCACGTAGGCGTAAGG	5' RACE
<i>Po-gata5</i> -5'RACE-rv2	GTAAAAGAGGAGCTGTCCGC	5' RACE
<i>Po-gata5</i> -3'mutant1-fw	TGTCCCTCGTGATTCTGTAAAGCCCCAAATGGCT	Mutational vector construction
<i>Po-gata5</i> -3'mutant1-rv	AGCCATTTGGGGCTTACAGAATCACGAGGGACA	Mutational vector construction
<i>Po-gata5</i> -3'mutant2-fw	AGTTGAGCCACCGAGTCCAGGTGTCCTGTG	Mutational vector construction
<i>Po-gata5</i> -3'mutant2-rv	CACAGGACACCTGGACTCGGTGGCTCAACT	Mutational vector construction
<i>Po-ntl</i> -ISH-fw	ATGACTTCTTCCAACCCCG	ISH probe
<i>Po-ntl</i> -ISH-rv	TCAGTAGCTCTGAGCCACAGG	ISH probe
<i>Po-gata5</i> -qPCR-fw	GCTCATCAAACCGCAGAAAC	Expression of <i>gata5</i>
<i>Po-gata5</i> -qPCR-rv	TACACACAGGTTCTCCCTCT	Expression of <i>gata5</i>
<i>Po-sox17</i> -qPCR-fw	CCAGTCTCCTTCAGCAAAGT	Expression of <i>sox17</i>
<i>Po-sox17</i> -qPCR-rv	CCCATGTAGGAAGGAGGAAGA	Expression of <i>sox17</i>
<i>Po-ntl</i> -qPCR-fw	CCAGTCTCCTTCAGCAAAGT	Expression of <i>ntl</i>
<i>Po-ntl</i> -qPCR-rv	TGATCTCTTCGTTCTGGTAAGC	Expression of <i>ntl</i>
<i>Po-miR-92a</i> -fw	TATTGCACTTGTCCCGGCCTGT	Expression of miR-92a
<i>Po-miR-92b</i> -fw	CATTGCACTTGTCCCGGCCTGA	Expression of miR-92b
U6-SnRNA-fw	TTGGAACGATACAGAGAAGATTAGC	Internal reference of miRNA
18s-qPCR-fw	GGTAACGGGGAATCAGGGT	Internal reference
18s-qPCR-rv	TGCCTTCCTTGATGTGGT	Internal reference

Microinjection Embryos were injected at the one-cell stage with miRNAs and reporter plasmids.

miR-92 and control (ctrl) miRNA were synthesized by GenePharma (Shanghai, China). LNA-92 and ctrl LNA were synthesized by Exiqon (Vedbaek, Denmark), as follows: miR-92a, 5'-UAUUGCACUUGUCCCGGCCUGU-3'; miR-92b, 5'-UAUUGCACUCGUCCCGGCCUCC-3'; LNA-92, 5'-ACAGGCCGGGACAAGTGCAATA-3'; ctrl miRNA, 5'-CUCUAGGUUAAACUCCUGGUU-3'; ctrl LNA, 5'-TAACACGTCTATACGCCCA-3'. Equal amounts of miR-92a and miR-92b were mixed and then diluted to 0.2 μ M for injection; LNA-92 was diluted to 2 μ M. Unless otherwise indicated, 0.5 ng of miR-92a and 0.5 ng of miR-92b were injected. Control miRNAs were injected at 1 ng per embryo. LNA-92 and ctrl LNA were injected at 10 ng per embryo of Japanese flounder. *gata5*-3'UTR-pmCherry plasmids were divided into the following eight groups and injected (1.5 ng/embryo) into zebrafish embryos at the one-cell stage. (i) *gata5*-3'UTR-pmCherry plasmid; (ii) *gata5*-3'UTR-pmCherry plasmid+miR-92; (iii) *gata5*-3'UTR-mutant1-pmCherry plasmid; (iv) *gata5*-3'UTR-mutant1-pmCherry plasmid+miR-92; (v)

gata5-3'UTR-mutant2-pmCherry plasmid; (vi) *gata5*-3'UTR-mutant2-pmCherry plasmid+miR-92; (vii) *gata5*-3'UTR-mutant1, 2-pmCherry plasmid; (viii) *gata5*-3'UTR-mutant1, 2-pmCherry plasmid+miR-92.

Quantitative real-time PCR (qRT-PCR) RT-PCR amplification was carried out using 10 μ l 2 $\times$ SYBR Green qPCR Master Mix (Novoprotein, Shanghai, China), 0.6 μ l of each primer (10 μ M), 1 μ l cDNA template, and 7.8 μ l nuclease-free water, with a total reaction volume of 20 μ l. The expression levels of *gata5* and two genes related to germ layer development of *P. olivaceus* (*ntl*, *sox17*) were identified at 50% epiboly and tailbud stages. The expression levels of miR-92 in response to TU treatment were observed after hatching. Primers are described in Table 1. The data were calculated using the $2^{-\Delta\Delta Ct}$ method, and significant difference was analyzed using one-way ANOVA. All samples were run in triplicate.

Whole-mount in situ hybridization (ISH) Injected samples were collected at the tailbud stage, fixed in

4% paraformaldehyde overnight, dehydrated through a methanol series (30%, 50% and 70%), and stored in 100% methanol at 4 °C. *Po-ntl*-ISH-fw/rv (Table 1) were used to synthesize RNA probes. Whole-mount ISH was performed using digoxigenin-labeled antisense RNA probes (Roche, Basel, Switzerland) to examine the effect of *ntl* on the development of *P. olivaceus* at the tailbud stage.

Statistical analysis Expression data were analyzed using SPSS 19.0 software. Statistical differences were tested through one-way ANOVA and considered significant at $P < 0.05$. All data are expressed as mean $\pm$ standard error of the mean.

Image collection Images of the ISH samples were acquired using a Nikon SMZ 1500 stereo microscope. Fluorescently labeled embryos were documented using a Nikon ECLIPSE Ci microscope. The projection images

were exported in TIFF format, assembled into figures, and processed using Adobe Photoshop CS2 software.

RESULTS

gata5 is a target of miR-92 in Japanese flounder

Alignments of miR-92a and miR-92b of flounder with those of other species indicate that miR-92s share a highly conserved nucleotide sequence except for one or two base differences at the 5' and 3' ends. The seed region, AUUGCAC, is identical among different species (Fig. 1B). The cDNA sequence of a predicted miR-92 target gene in flounder, *gata5*, was obtained. The full-length cDNA of *gata5* is 2,741 bp, including a 1,140-bp ORF, a 442-bp 5' UTR and a 1,159-bp 3' UTR, and encodes 379 amino acids (Fig. 1A). Two miRNA recognition elements (MREs) of miR-92 in the 3' UTR of the *gata5* gene were identified: one is 104–129 bases downstream of

A

```

1  gcaattatcagcgacgaacactatctccagatataccgacaggaagagaacacccaggagc
61  aggcacaagacaaaaaacaagttgcagattccagttatccagttacacaggagagccgca
121  acttttacgacaaaagtggagagagttggagaggctccgctgcgcgtccctcgctcc
181  gtcctcatctgagcgagtgacagggtttttctgctgcgtccctgctgaggtctggaagg
241  accggactgctttatgcggccgacgctccttattctggacaggactctccgcaacattaat
301  cctagccctgttcttcaagaacagaataatcatcaagtggaagcgtcttcttcaagc
361  ggacaacacccctccacgggagtgctgttaagaacatctctcattatttttccacatccct
421  cgaggagctcaacgagtgaaATGTACCAAGACGAGTCCCTTACGGCAGCAGACCGG
481  M Y Q S Q S P Y A H D T G
GAATTACATCCACCCCTGGCCAGCTCCCGGTTACGTCCCAACACAGAGTCCCGG
541  N V L P S A S P V V V P T T R V P L A
GATGCTGCCACCCCTGCTCTACGTGACAGCTGCGACTCGCTCACCAGTCCCAACACAG
601  M L P T L S Y L O T C D S P H Q S H H S
CCTCGCGGGGACACCGGCTGGCCAGTCCGCGGGACAGCTCTCTTTTACGCCGG
661  L G G H H G W P Q S G A D S S S F T P G
CAGCCCTCACCCCTATCAGGCTTGTCTACTCGACAGCCCGCGGTACGACGACAC
721  S P F H P H H G L S Y H S P F V S S N T
GGCGCGGAGCTGACCTACAGAGCCCGTGGTTCGGAACGGCGCGCGGACAGATCA
781  G R D V T Y Q S P L V L G N G A R A D Q
GTACGAGGCGCCCTGATGCTCGGTCGAGGCTCCTACTCCAGCCCTTACGCCCTACGT
841  Y G G A L M R S V G G S Y S S P Y A Y V
GAGTCGGAGATGGCAGCTCTCTCTGACACCGGGCCCTTCGAGAGGGGGTGATCAG
901  S P E M A T S S W T P G P E S S V I S
TCTGCGAGGCGGAGGGGCTCTGTCCGGGAATTTCCAGCTGGAAGTGAAGACGA
961  L Q G R Q G A L S G R I S S L E L M D D
CATGCAAGTGGAGGAGGAGTGGCTCACTCGGCTCCGTCCGTCACGCGCGCTGGCG
1021  M Q C E G R E C V N C G S V S T P L W R
CAGAGAGGACCGGCTACTGTCAGAGCTCGGCGCTGTACACACAGATGAAGCG
1081  P D G T G H Y L C N A C G L Y H K M N G
CATCAACAGGCGCTCATCAACCGCAGAAAGCTGACAGACACACCCCGCAGAGCGG
1141  I N R P L I K P Q K R L Q T T P R R A G
CCTGTGCTGACTAAGTCCACACACGACACACACACACTGTGGAGGCGCAACGAGAGGG
1201  C C T N C H T S T T L W R R N A E G
AGAACCTGTGTAAAGCTCGGCGCTCTACATCAAGTGGGTGCGCCAGACCTCT
1261  E P V C N A C G L Y N K L H G V P R P L
GGCCATGAAGAAAGAGCATTCACCAAGAGAGCGCAACCCAGTTGCTAAGATAA
1321  A M K K E S I Q T R K R K P K L P K N K
AACATCCACAGGAGATCAGCACTCTGACAGCGGCTCGCCACGCGCCCTGTCAAGTTC
1381  T S T S A T S D S S P T P L S V S
CGAACATGCTTCAACATCAAGAGTGAACGACATGGCCCGCTCACTTACCTGAGCA
1441  E H A F T I K S E P S M A P S P Y A G Q
GACGCTCATCCGCTCTCAGACGACTTCTAGCTGGACTGCACAGGACACGACATGT
1501  T V S S A S Q T T S Q L D C T G P G H V
GGACATTAATATGAAGATTATCCCTTACCCCGACCTCCATGGCCCGCAGAGATCCCTG
1561  D I K Y E D Y P F T P T S N A P Q N S W
GTCCGCTCTGTCACAGCTTGAAGAGCTGACGAGCTGACGAGCTGCTGTGACGAGCAGTA
1621  C A L S Q A *
cacaactcactttgctgggacaggagcactgttccgctcgcagctgtccctgtg
1681  attctgggctgtgggaaacagtggttataagcccaaatgtgtatgggtgtctga
1741  tggcgacatgttccacactcacttgagctgagagacatctgattgtctgactatgtata
1801  aactatctgtgtcagctgtggcggtggaatgaaagggtccacagagtgctacactggac
1861  cgaacttaactcaacaggacacagagcactgtgacagcagcttacaataatgacaggt
1921  gcaactgtgtgtcactgtgacgaaagatccacagctgagcgagacactgacgctgttc
1981  tcttgaactcagctccagcagcctcggcaccatgacaaactaactataactaaaagaa
2041  atctctattttgggaaacacttgacacaaataatgatgtggaagacaaatcaaggggaa
2101  tgtaaaaaacagctgactcctcttttaaaagtattttatttttttttttttttttttttt
2161  ttctgggtgtcattacagagaacacagctcaactgttactatcatcgcaggaagaagaa
2221  cataaaatggagagaaataccctttttatgtgtgtgaacatgtcttactacagagctg
2281  tagatgattaaactttatgtgtgattttatgttgagataaagacagtttaagctcag
2341  gtggacactgaaacagcagttataacacctgtcgtcgtgtgtgtgtgtgtgtgtgtgtgt
2401  agctatcatcatatgctgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt
2461  ctgagttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt
2521  atcgttaacatttgagatgaatgaagagcaggaatgattctgtcatattatcatcatt
2581  tttattgtccgtatgtgtgcaacatgtgaaggaattttattgtgtactgttcttaagat
2641  ctctctgtttctcttttttttttttttttttttttttttttttttttttttttttttttt
2701  ttccattcccaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

```

B

```

pol-miR-92a  UAUUGCACUUGUCCGGCCUUGU
ola-miR-92a  -----
dre-miR-92a  -----
ccr-miR-92a  -----
bbe-miR-92a  -----U-
xtr-miR-92a  -----
mmu-miR-92a  -----
hsa-miR-92a  -----

pol-miR-92b  UAUUGCACUUGUCCGGCCUCC
ola-miR-92b  -----C
dre-miR-92b  -----
ccr-miR-92b  -----
bbe-miR-92b  -----GA
mmu-miR-92b  -----

```

C

Fig. 1. Multiple alignment of nucleotide sequences of miR-92a and miR-92b, and their target sites in *gata5* of Japanese flounder. (A) The cDNA sequence of *gata5* is shown and its coding sequence is represented in upper-case letters and the flanking non-coding sequences in lower-case letters; the amino acids are exhibited below the coding sequence region, and the asterisk marks the stop codon. MREs in the 3' UTR are underlined. (B) The seed regions of miR-92a and miR-92b are underlined, and identical bases in different species are shown by dashes (–). pol: *Paralichthys olivaceus*; ola: *Oryzias latipes*; dre: *Danio rerio*; ccr: *Cyprinus carpio*; bbe: *Branchiostoma belcheri*; xtr: *Xenopus tropicalis*; mmu: *Mus musculus*; hsa: *Homo sapiens*. (C) The mCherry reporter fused to the 3' UTR of *gata5*. Base pairing between miR-92 and the two MREs is shown.

the termination codon, and the other is 314–343 bases downstream (Fig. 1C). To determine whether Japanese flounder *gata5* is a true target of miR-92, we analyzed the interaction between the *gata5* 3' UTR and miR-92 using mCherry reporter assays. Three constructs were established, with the intact 3' UTR of *gata5* and with one or both of the MREs deleted, each integrated downstream of the mCherry ORF. Reporter plasmids were injected into one-cell zebrafish embryos in the presence or absence of miR-92. At 70% epiboly, mCherry expression levels were monitored by fluorescence microscopy.

Injections of single reporter plasmids with one or two miR-92 MREs yielded red fluorescence in more than 90% of the embryos (Figs. 2A, 2B and 2C). This was similar to the result for deletion of both MREs (Fig. 2D). In contrast, miR-92 co-injection with plasmids harboring either or both miR-92 MREs resulted in red fluorescence in only

about 20% of the embryos (Figs. 2E, 2F and 2G). Therefore, mCherry expression was suppressed by miR-92 co-injection when at least one miR-92 MRE was present in the attached *gata5* 3' UTR. Together, these results indicate that *gata5* is a true target of miR-92.

Overexpression and inhibition of miR-92 result in larval deformity The influence of miR-92 injection on larval phenotype was explored. A stereo microscope was used to observe the phenotype of Japanese flounder larvae at the tailbud stage. Compared with the control group, the overexpression and inhibition of miR-92 resulted in torso swelling. Curved spines were observed in the miR-92-injected group compared with those in the control group, and the tail of the LNA-92-injected group was deformed (Fig. 3).

Fig. 2. *gata5* is a target gene of miR-92. The expression of mCherry was observed as red fluorescence at 12 h after injection of one-cell zygotes of zebrafish. Sample numbers for each group were about 100 zygotes. (A–H) Embryos were injected with reporters containing either the full-length *gata5* 3' UTR (full) or a construct in which MRE(s) were deleted (D1: MRE 1 deleted; D2: MRE 2 deleted; D12: MRE 1 and MRE 2 deleted). (A–D) Reporter plasmids were injected alone. (E–H) Reporter plasmids were co-injected with miR-92. (I) The proportion of fluorescent embryos in (A–D). (J) The proportion of fluorescent embryos in (E–H). Fluorescence-positive and -negative embryos are depicted in red and blue, respectively.

miR-92 regulates the formation of endomesoderm during early embryonic development The effect of miR-92 on the early embryonic development of Japanese flounder was investigated. qRT-PCR was used to compare the expression level of the miR-92 target gene *gata5*, the endodermal marker gene *sox17* and the mesodermal marker gene *ntl* through gain-of-function and loss-of-function assays for miR-92. The expression levels of *gata5* and *sox17* during the miR-92 overexpression experiment were lower than that in the control group ($P < 0.05$) at the 50% epiboly and tailbud stages (Fig. 4A and 4C). By contrast, the expression levels of *gata5* and *sox17* were higher in the LNA-92 injection group (Fig. 4B and 4D), indicating defects in endoderm formation. Hence, miR-92 is required for proper endoderm specification. Furthermore, the expression pattern of the mesodermal marker gene *ntl* was opposite to that of *gata5* and *sox17* (Fig. 4E and 4F). After miR-92 microinjection, both ISH and qRT-PCR results showed a significantly higher level of *ntl* expression than was seen in the control group (Fig. 5D). In the LNA-92 injection group, the expression level of *ntl* was lower than that in the control group, and the scope of the expression was narrowed (Fig. 5B). Hence, miR-92 may affect the fate of the endomesoderm by regulating particular target genes.

Expression of miR-92 after TU treatment Previous studies on metamorphosis of Japanese flounder confirmed that exogenous TU treatment inhibited metamorphosis and caused deflection of both the body and the eye (Inui and Miwa, 1985; Miwa and Inui, 1991). To explore whether the expression of miR-92 was changed upon inhibition of metamorphosis, we treated Japanese flounder larvae with TU, beginning at 18 days post-hatching (dph). The qRT-PCR results for miR-92a were consistent with those for miR-92b (Fig. 6), with a decrease in expression at 22 dph and a peak at 27 dph in the untreated group. Meanwhile, the expression levels steadily increased until 27 dph and then decreased in the TU-treated group.

DISCUSSION

The miR-17-92 family controls cell proliferation and apoptosis, and the microRNAs of this family play a role in the regulation of proto-oncogenes and the expression of tumor suppressor genes. miR-92 belongs to the miR-17-92 family and has a regulatory effect on cell proliferation. Its overexpression may alter the abundance of p63 (Li et al., 2012), thereby promoting bone marrow cell proliferation. miR-92 also promotes the proliferation of endothelial cells and osteoblasts (Bonauer et al., 2009; Wu et al., 2011). In this study, the injection of

Fig. 3. Effects on larval phenotype after overexpression and inhibition of expression of miR-92. Representative tailbud-stage embryos of untreated (A), miR-92 overexpression (B, C) and miR-92 inhibition (D–F) groups. miR-92-overexpressing embryos (B, C) showed an intumescent body with sinuous spine compared to untreated embryos (A), while inhibition of miR-92 (D–F) resulted in an intumescent body and abnormal tail. Sample numbers for each group were about 30 zygotes.

Fig. 4. Relative gene expression at the 50% epiboly and tailbud stages after overexpression and inhibition expression of miR-92. (A, B) Relative expression of *gata5* in the miR-92 group (A) and LNA-92 group (B). (C, D) Relative expression of *sox17* in the miR-92 group (C) and LNA-92 group (D). (E, F) Relative expression of *ntl* in the miR-92 group (E) and LNA-92 group (F). All data are shown as mean $\pm$ SEM (n = 3). *P < 0.05; **P < 0.01. Sample numbers for each group were about 30 zygotes.

miR-92 caused swelling of the body trunk of Japanese flounder. ISH results show that miR-92 overexpression resulted in a curved spine compared with the control group, which was probably due to excessive proliferation of mesoderm cells caused by *ntl* overexpression.

During the developmental process of endoderm forma-

tion in zebrafish, *gata5* expression was upregulated under the regulation of phosphorylated *SMAD2* (Shivdasani, 2002). Moreover, the overexpression and inhibition of *gata5* can alter the number of endodermal cells in the late stages of gastrulation. As a downstream effector of Nodal signaling and a regulator of endoderm specifica-

Fig. 5. Tailbud ISH analysis of *ntl* after overexpression and inhibition of miR-92. About 25 zygotes were treated for each group, and representative embryos are shown. (A) Untreated group. (B) LNA-92 injection group. (C) TE buffer injection group. (D) miR-92 injection group. The red arrow marks the region of *ntl* expression.

Fig. 6. Expression profiles of miR-92a and miR-92b after treatment of larvae with TU. The time was calculated from larval hatching of the untreated group. 20 dph: pre-metamorphosis (metamorphosis of the larva has not begun); 22 dph: early metamorphosis (the right eye of the larva begins to move); 27 dph: mid-metamorphosis (the right eye of the larva has moved to the top of the head); 35 dph: the larva is settled. All data are shown as mean $\pm$ SEM ($n = 3$). $**P < 0.01$. Sample numbers for each group were about 500 zygotes.

tion, *gata5* needs to be precisely controlled, both temporally and spatially (Li et al., 2011). In a previous study, we have verified that *gata5* is the target gene of miR-92 in Japanese flounder. Hence, miR-92 may play a vital role in the establishment of the proper temporal and spa-

tial expression profile of *gata5*.

Based on previous research, *sox17* was selected to mark the number of endodermal cells. In this study, miR-92 overexpression significantly downregulated the expression of *sox17* during the gastrulation period, contrary to

the inhibition of miR-92 expression, which upregulated *sox17* expression (Li et al., 2011). These results show the regulatory function of miR-92 on *sox17* expression during gastrulation, and suggest that miR-92 plays a role in development of the endoderm of Japanese flounder.

The mesoderm originates from the common precursor cells. Some cells are specialized from mesodermal cells to form the endoderm when the nodal signal becomes stronger in the later developmental stages (Schier et al., 1997). However, it is still unclear whether the increase in the number of endodermal cells is at the expense of a reduced number of mesodermal cells. Therefore, we examined *ntl* expression under miR-92 overexpression and inhibition. The qRT-PCR and ISH results show that miR-92 overexpression significantly upregulated *ntl* expression, which was the opposite of the *sox17* test results. Therefore, miR-92 may regulate the fate of the mesoderm by regulating *gata5*.

In zebrafish, abnormal expression of miR-92 altered the number and length of cilia in Kupffer's vesicle (KV). Also, the left-to-right asymmetry and flow in KV were interrupted. Moreover, a previous study confirmed that 66 flounder miRNAs were differentially expressed at two metamorphic stages (17 and 29 dph) (Fu et al., 2011). This finding prompted us to consider the role of miR-92 in the metamorphosis of Japanese flounder.

We examined the expression of miR-92 during metamorphosis. The expression levels of miR-92a and miR-92b were elevated in mid-metamorphosis and remained relatively high thereafter. This high expression level suggests that miR-92 plays an important part in the mid and late stages of Japanese flounder metamorphosis. miR-92 expression (including miR-92a and miR-92b) was upregulated after TU treatment, indicating that miR-92 expression levels were tightly correlated with the status of metamorphosis. Interestingly, when metamorphosis was blocked by TU treatment, miR-92a and miR-92b expression was higher than in untreated larvae at 22 dph but lower at 27 dph. A possible reason for this result is decreased concentration of TU. With the growth of larvae, the relative concentration decreases because the larvae are bigger than before but concentration is constant. Also, the regulation of miR-92 during development may be dynamic. Overall, these findings suggest that miR-92 acts as a regulator during metamorphosis.

CONCLUSIONS

We confirmed that *gata5* is a target of miR-92 and explored the function of miR-92 during the early development and metamorphosis of Japanese flounder. Our study revealed that miR-92 may regulate the fate of the mesoderm by controlling the expression of the target gene *gata5*. Furthermore, our study demonstrated a potential regulatory function of miR-92 in the metamorphic devel-

opment of Japanese flounder. However, further studies are needed to confirm these findings.

This study was supported by the National Science Foundation of China (Grant No. 31372511) and Fundamental Research Funds for the Central Universities (No. 201822026). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

- Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297.
- Bizuayehu, T. T., Lanes, C. F. C., Furmanek, T., Karlsen, B. O., Fernandes, J. M., Johansen, S. D., and Babiak, I. (2012) Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. *BMC Genomics* **13**, 11.
- Bonauer, A., Carmona, G., Iwasaki, M., Mione, M., Koyanagi, M., Fischer, A., Burchfield, J., Fox, H., Doebele, C., Ohtani, K., et al. (2009) MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* **324**, 1710–1713.
- Dews, M., Homayouni, A., Yu, D., Murphy, D., Seignani, C., Wentzel, E., Furth, E. E., Lee, W. M., Enders, G. H., Mendell, J. T., et al. (2006) Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat. Genet.* **38**, 1060–1065.
- Duursma, A. M., Kedde, M., Schrier, M., le Sage, C., and Agami, R. (2008) miR-148 targets human DNMT3b protein coding region. *RNA* **14**, 872–877.
- Fu, Y., Shi, Z., Wu, M., Zhang, J., Jia, L., and Chen, X. (2011) Identification and differential expression of microRNAs during metamorphosis of the Japanese flounder (*Paralichthys olivaceus*). *PLoS One* **6**, e22957.
- Garcia-Cao, I., Song, M. S., Hobbs, R. M., Laurent, G., Giorgi, C., de Boer, V. C. J., Anastasiou, D., Ito, K., Sasaki, A. T., Rameh, L., et al. (2012) Systemic elevation of PTEN induces a tumor-suppressive metabolic state. *Cell* **149**, 49–62.
- Hartl, M., Loschek, L. F., Stephan, D., Siju, K. P., Knappmeyer, C., and Kadow, I. C. G. (2011) A new Prospero and *microRNA-279* pathway restricts CO₂ receptor neuron formation. *J. Neurosci.* **31**, 15660–15673.
- Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y., and Takahashi, T. (2005) A polycistronic microRNA cluster, *miR-17-92*, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res.* **65**, 9628–9632.
- Inui, Y., and Miwa, S. (1985) Thyroid hormone induces metamorphosis of flounder larvae. *Gen. Comp. Endocrinol.* **60**, 450–454.
- Ivanovska, I., Ball, A. S., Diaz, R. L., Magnus, J. F., Kibukawa, M., Schelter, J. M., Kobayashi, S. V., Lim, L., Burchard, J., Jackson, A. L., et al. (2008) MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. *Mol. Cell. Biol.* **28**, 2167–2174.
- Kloosterman, W. P., and Plasterk, R. H. A. (2006) The diverse functions of microRNAs in animal development and disease. *Dev. Cell* **11**, 441–450.
- Koralov, S. B., Muljo, S. A., Galler, G. R., Krek, A., Chakraborty, T., Kanellopoulou, C., Jensen, K., Cobb, B. S., Merckenschlager, M., Rajewsky, N., et al. (2008) Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* **132**, 860–874.
- Li, F., Lu, Y., Ding, M., Wu, G., Sinha, S., Wang, S., and Zheng,

- Q. (2012) Putative function of TAP63 α during endochondral bone formation. *Gene* **495**, 95–103.
- Li, N., Wei, C., Olena, A. F., and Patton, J. G. (2011) Regulation of endoderm formation and left-right asymmetry by miR-92 during early zebrafish development. *Development* **138**, 1817–1826.
- Lytle, J. R., Yario, T. A., and Steitz, J. A. (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc. Natl. Acad. Sci. USA* **104**, 9667–9672.
- Miwa, S., and Inui, Y. (1991) Thyroid hormone stimulates the shift of erythrocyte populations during metamorphosis of the flounder. *J. Exp. Zool.* **259**, 222–228.
- Ning, G., Liu, X., Dai, M., Meng, A., and Wang, Q. (2013) *MicroRNA-92a* upholds Bmp signaling by targeting *noggin3* during pharyngeal cartilage formation. *Dev. Cell* **24**, 283–295.
- O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839–843.
- Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. (2004) Identification and characterization of a novel gene, *C13orf25*, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res.* **64**, 3087–3095.
- Petrocca, F., Visone, R., Onelli, M. R., Shah, M. H., Nicoloso, M. S., de Martino, I., Iliopoulos, D., Pilozi, E., Liu, C.-G., Negrini, M., et al. (2008) E2F1-regulated microRNAs impair TGF β -dependent cell-cycle arrest and apoptosis in gastric cancer. *Cancer Cell* **13**, 272–286.
- Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S., and Driever, W. (1997) The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail*. *Development* **124**, 327–342.
- Shivdasani, R. A. (2002) Molecular regulation of vertebrate early endoderm development. *Dev. Biol.* **249**, 191–203.
- Sylvestre, Y., De Guire, V., Querido, E., Mukhopadhyay, U. K., Bourdeau, V., Major, F., Ferbeyre, G., and Chartrand, P. (2007) An E2F/miR-20a autoregulatory feedback loop. *J. Biol. Chem.* **282**, 2135–2143.
- Ventura, A., Young, A. G., Winslow, M. M., Lintault, L., Meissner, A., Erkeland, S. J., Newman, J., Bronson, R. T., Crowley, D., Stone, J. R., et al. (2008) Targeted deletion reveals essential and overlapping functions of the *miR-17-92* family of miRNA clusters. *Cell* **132**, 875–886.
- Woods, K., Thomson, J. M., and Hammond, S. M. (2007) Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. *J. Biol. Chem.* **282**, 2130–2134.
- Wu, W., Xiao, H., Laguna-Fernandez, A., Villarreal, G. Jr, Wang, K.-C., Geary, G. G., Zhang, Y., Wang, W.-C., Huang, H.-D., Zhou, J., et al. (2011) Flow-dependent regulation of Krüppel-like factor 2 is mediated by microRNA-92a. *Circulation* **124**, 633–641.
- Xiao, C., Srinivasan, L., Calado, D. P., Patterson, H. C., Zhang, B., Wang, J., Henderson, J. M., Kutok, J. L., and Rajewsky, K. (2008) Lymphoproliferative disease and autoimmunity in mice with increased miR-17–92 expression in lymphocytes. *Nat. Immunol.* **9**, 405–414.