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

Paralichthys olivaceus

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(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). 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.,...
The early development of teleosts. In Japanese flounder, the expression levels of miR-92a and miR-92b are significantly upregulated at the epiboly and somite stages (Bizuayehu et al., 2012), suggesting that miR-92 plays an important role in the early development of teleosts. In Atlantic halibut, 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 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 (Bizuayehu 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 involved 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.

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 set 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, 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 MicroCosm Targets 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 mirVana 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.
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Table 1. Primers used in this study

| Name                | Primer sequence (5′-3′) | Amplification target                                      |
|---------------------|------------------------|----------------------------------------------------------|
| Po-gata5-fw         | GGCCACTACCTTGTGCAAAAC  | Core fragment                                            |
| Po-gata5-rr          | GAGCTGAGTTCAGGAGAACAG  | Core fragment                                            |
| Po-gata5-fw1        | CGGTCGAGGAAGATATCCCTTTACCCGAC  | Reporter construction                                    |
| Po-gata5-rr1        | CGCGATCCCAACAAACGACTGTGGTGAT  | Reporter construction                                    |
| Po-gata5-3′RACE-fw1 | TTGTGAAATGTGGCTTACTACAGAGCG  | 3′ RACE                                                  |
| Po-gata5-3′RACE-fw2 | GTGACTGTCTTTAAGGGATACACACG  | 3′ RACE                                                  |
| Po-gata5-5′RACE-rr1  | GACTCACTGAGGGCTTACTACAGAGCG  | 5′ RACE                                                  |
| Po-gata5-5′RACE-rr2  | GTAAAAGAGAGCTGTCCGCA  | 5′ RACE                                                  |
| Po-gata5-3′mutant1-fw| TGTCCTCTGGTTACTGTGAATACACACG  | Mutational vector construction                           |
| Po-gata5-3′mutant1-rr1| AGCCATTTGGGGCTTACAGAGCGACTGTGGTGAT  | Mutational vector construction                           |
| Po-gata5-3′mutant2-fw| AGTTGAGCCACGGAGTCCGAGTGCTCTGTG  | Mutational vector construction                           |
| Po-gata5-3′mutant2-rr2| CACAGGAGCACTGGAGTCGCTTTACACGCAACG  | Mutational vector construction                           |
| Po-nlt-ISF-fw       | ATGACTTCTTAAAACCGCGCGCAACG  | ISH probe                                                |
| Po-nlt-ISF-rr        | TCAATAGTGTACCTTGAGGACGCAAG  | ISH probe                                                |
| Po-gata5-qPCR-fw    | GCTCATCAAAACCGACGACACAG  | Expression of *gata5*                                    |
| Po-gata5-qPCR-rr     | TACACACAGGTCTCTCTCTCT  | Expression of *gata5*                                    |
| Po-sox17-qPCR-fw    | CACATCTTCATTTGAGCAGAAAAGGGAAGAGC  | Expression of *sox17*                                   |
| Po-sox17-qPCR-rr     | CCCATGTAGGAGGAGGAGGAAGGGAAGAGC  | Expression of *sox17*                                   |
| Po-nlt-qPCR-fw       | CACATCTTCATTTGAGCAGAAAAGGGAAGAGC  | Expression of *nlt*                                     |
| Po-nlt-qPCR-rr       | TGATCCTCTGTTCTGTGTAAGGC  | Expression of *nlt*                                     |
| Po-miR-92a-fw        | TATACACATCTGTCCCAGCGCCTGTG  | Expression of miR-92a                                    |
| Po-miR-92b-fw        | CATTGCCACTTGTCCCAGCGCCTGTG  | Expression of miR-92b                                   |
| U6-SnRNA-fw          | TTGGAACGATAACAGAGAAAGATTAGCG  | Internal reference of miRNA                             |
| 18s-qPCR-fw          | GCTCTCTGTTTGCTTTACACGAGGG  | Internal reference of miRNA                             |
| 18s-qPCR-rr          | TGCTCTTCCATGGATGCTGTTG  | Internal reference of miRNA                             |

**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′-UAAUGACACUUCCCGCCUGCGGCUU-3′; miR-92b, 5′-UAAUGACACUUCCCGCCUGCGGCUU-3′; LNA-92, 5′-ACAGGCCGGGACAGTGAATA-3′; ctrl miRNA, 5′-UCUAGGCUUAACUCUAGGCUU-3′; ctrl LNA, 5′-TAACAGTCTCTATCAGCAGCC-3′. Equal amounts of miR-92a and miR-92b were mixed and then diluted to 0.2 μM for injection; LNA-92 was diluted to 2 μM. 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-mutant2-pmCherry* plasmid; (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 μl 2×SYBR Green qPCR Master Mix (Novoprotein, Shanghai, China), 0.6 μl of each primer (10 μM), 1 μl cDNA template, and 7.8 μl nuclease-free water, with a total reaction volume of 20 μl. 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−ΔΔCt 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 ± 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...
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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).

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**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 et al., 1986; 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
miR-92 causes 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 formation 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-
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 spatial 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...
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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 development 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., Fernandez, J. M., Johansen, S. D., and Babjak, 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., Sevignani, 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., Schier, 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., Itó, 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., Sijù, K. P., Knappmeyer, C., and Kadow, I. C. G. (2011) A new Prospero and *ntl* family regulate p21/CDKN1A and promote cell cycle progression. *J. Neurosci.* **31**, 15660–15673.

Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawaihara, 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., Merkenschlager, 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 TAP63α 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., Kanam, S., Tezuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. (2004) Identification and characterization of a novel gene, C13orf25, as a target for 13q31-32 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., Pilozzi, E., Liu, C.-G., Negrini, M., et al. (2008) E2F1-regulated microRNAs impair TGFβ-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.