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Journal of Genetics and Genomics 40 (2013) 515−521

JGG

ORIGINAL RESEARCH

Nodal Promotes *mir206* Expression to Control Convergence and Extension Movements During Zebrafish Gastrulation

Xiuli Liu [a], Yuanqing Ma [a], Congwei Zhang [b], Shi Wei [a], Yu Cao [a], Qiang Wang [a,*]

[a] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*
[b] *Department of Outpatient Surgery, Anguo City Hospital of Hebei Province, Baoding 071200, China*

Received 6 March 2013; revised 15 July 2013; accepted 22 July 2013
Available online 8 August 2013

ABSTRACT

Nodal, a member of the transforming growth factor β (TGF-β) superfamily, has been shown to play a role in mesendoderm induction and gastrulation movements. The activity of Nodal signaling can be modulated by microRNAs (miRNAs) as previously reported, but little is known about which miRNAs are regulated by Nodal during gastrulation. In the present study, we found that the expression of *mir206*, one of the most abundant miRNAs during zebrafish early embryo development, is regulated by Nodal signaling. Abrogation of Nodal signal activity results in defective convergence and extension (CE) movements, and these cell migration defects can be rescued by supplying an excess of *mir206*, suggesting that *mir206* acts downstream of Nodal signaling to regulate CE movements. Furthermore, in *mir206* morphants, the expression of cell adhesion molecule E-cadherin is significantly increased, while the key transcriptional repressor of E-cadherin, *snail1a*, is depressed. Our study uncovers a novel mechanism by which Nodal-regulated *mir206* modulates gastrulation movements in connection with the Snail/E-cadherin pathway.

KEYWORDS: Nodal; *mir206*; Convergence and extension; *snail1a*; E-cadherin

INTRODUCTION

Vertebrate germ layer progenitors are spatially rearranged with dorsoventral, anteroposterior, and left-right axes during gastrulation through numerous complex movements, including epiboly, internalization, and convergent extension (Solnica-Krezel et al., 1995; Carmany-Rampey and Schier, 2001). These body-plan patterning process require several conserved inductive signals, including Nodal, Wnt, Fgf and Bmp. Nodal belongs to the TGF-β superfamily, which transduces signal through type I and type II serine-threonine kinase receptors (Whitman, 2001). Two zebrafish nodal genes, *squint* (*sqt*) and *cyclops* (*cyc*), are expressed in blastulas, and *cyc;sqt* double mutant embryos lack endoderm and mesoderm tissues, suggesting an essential role of Nodal signaling in mesendoderm induction (Feldman et al., 1998; Sampath et al., 1998). The *Xenopus* Nodal ligands, *Xnr1* and *Xnr2*, promote cell migration *via* the direct regulation of key migratory genes, such as *papc*, *has2*, and *pdgfr* in gastrulating tissues (Luxardi et al., 2010), suggesting that Nodal signaling participates in the control of gastrulation movements. In zebrafish, Nodal controlled cortex tension plays a crucial role in germ layer organization (Krieg et al., 2008). However, the direct functional evidence linking Nodal signaling to zebrafish gastrulation movements remains absent.

MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression post-transcriptionally through various regulatory mechanisms, including messenger RNA (mRNA) deadenylation, degradation, and translation suppression (Bushati and Cohen, 2007; Erson and Petty, 2008; Filipowicz et al., 2008). miRNAs have been shown to play essential roles

* Corresponding author. Tel/fax: +86 10 6480 7895.
E-mail address: qiangwang@ioz.ac.cn (Q. Wang).

http://dx.doi.org/10.1016/j.jgg.2013.07.001

in cell fate determination, axis formation, cell differentiation, cell signaling, and tissue morphogenesis (Kloosterman and Plasterk, 2006).

Recently, miRNAs have emerged as major players in Nodal signaling. In early *Xenopus* embryos, the ventrally enriched *mir15* and *mir16* restrict the size of the organizer by targeting the Nodal type II receptor *Acvr2a* (Martello et al., 2007). In zebrafish embryos, *mir430* functions to achieve optimal Nodal signaling activity by inhibiting the expression of the Nodal agonist *sqt* and the antagonist *lefty* (Choi et al., 2007). In human embryonic stem cells, mir302s plays an important role in maintaining the balance between pluripotency and germ layer specification by negatively modulating Lefty expression (Barroso-delJesus et al., 2011). In addition, mir24 regulates human hematopoietic progenitor cell erythroid differentiation by targeting Nodal/activin type I receptor ALK4 (Wang et al., 2008). On the other hand, the expression of several other miRNAs is regulated by TGF-β signals. For example, *mir155* is regulated by the TGF-β/Smad pathway and contributes to TGF-β-induced epithelial-mesenchymal transition (EMT) and cell migration, while Dpp, another TGF-β superfamily ligand, promotes the transcription of the miRNA gene *bantam* to control growth in *Drosophila* tissues (Kong et al., 2008; Oh and Irvine, 2011).

We have reported that *mir206*, one of the skeletal muscle specific miRNAs, is an essential regulator for zebrafish convergence and extension (CE) movements by targeting *prickle1a* (*pk1a*) and regulating MAPK JNK signaling (Liu et al., 2012). Here we show that *mir206* expression is upregulated by Nodal signaling during zebrafish gastrulation. Abrogation of Nodal signal activity in zebrafish embryos by SB431542 treatment suppresses the expression of *mir206* and leads to defective CE movements. The defects of CE movements could be overcome by supplying an excess of *mir206*. In addition, we find that loss of *mir206* disrupts cell migration by diminishing Snail-mediated E-cadherin repression.

RESULTS

mir206 expression is regulated by Nodal signaling during early zebrafish embryonic development

To identify miRNA candidates that mediate Nodal effects during gastrulation, we analyzed the genome-wide profile of miRNAs in wild-type and *MZoep* mutant embryos at the end of gastrulation (bud stage). *MZoep* mutant embryos, which lack the organizer and mesendodermal tissues, are defective in Nodal signaling (Zhang et al., 1998; Gritsman et al., 1999). Among the 219 miRNAs examined, we only found 4 miRNAs (with an averaged signal intensity of more than 1000 in the control microarrays) that were up or downregulated more than 1.5-fold by Nodal signaling (Fig. 1A). As shown in Fig. 1A and B, *mir206* was highly expressed at the bud stage when compared with other Nodal-regulated miRNAs, and obviously decreased in *MZoep* mutants. These analyses suggest that *mir206* is a candidate to be further investigated in Nodal regulated gastrulation.

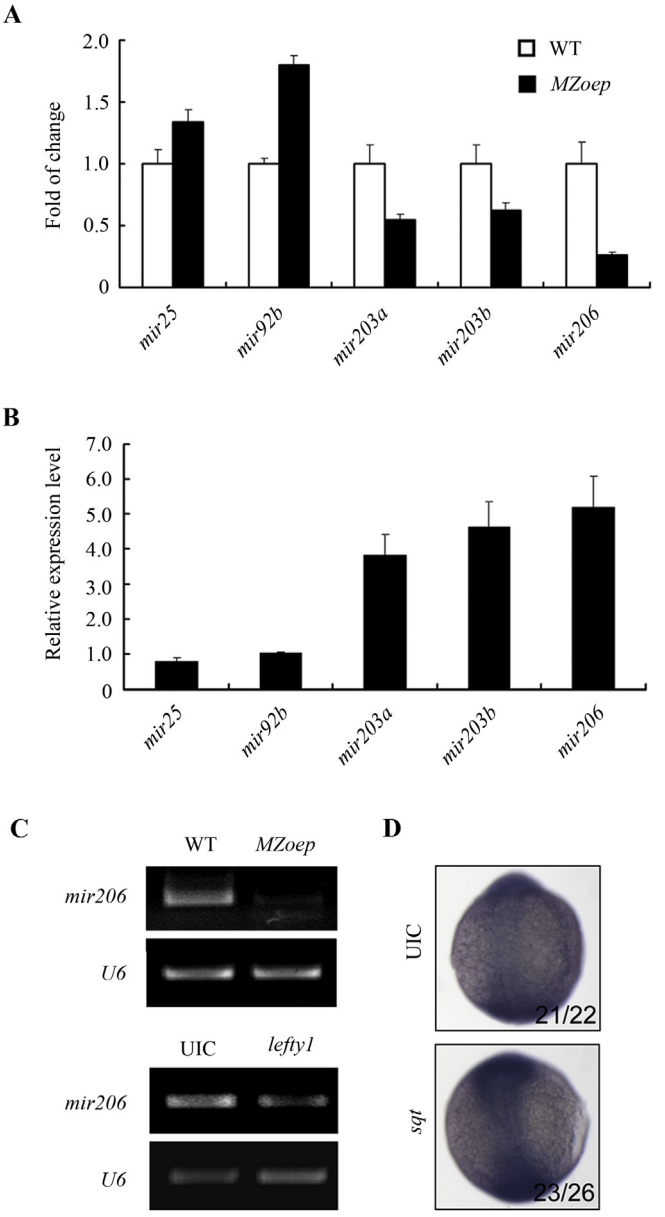

Fig. 1. *mir206* expression is regulated by Nodal signaling during early embryonic development.

A: identification of miRNAs regulated by Nodal signaling through microarray analysis on bud stage embryos. White bars indicate average signal intensity of the miRNAs in wild-type (WT) embryos, which were used as baseline, and black bars denote relative expression levels of miRNAs in *MZoep* mutants. **B:** the averaged expression of Nodal-regulated miRNAs in wild-type embryos at bud stage. **C:** *mir206* expression was detected in *MZoep* mutants and lefty1-overexpressed embryos at 75%-epiboly stage by semiquantitative stem-loop RT-PCR. *U6* expression was analyzed as a loading control. **D:** the expression of *mir206* in *sqt* overexpressing embryos at 6 somite stage. UIC, uninjected control. Dorsal view, anterior at the top. The ratios of affected embryos were indicated in the right bottom corners.

To verify the microarray results, we first performed stem-loop RT-PCR to assess *mir206* expression in *MZoep* mutants and after overexpression of the Nodal antagonist *lefty1* in embryos at mid-gastrulation (75%-epiboly). We found that the inhibition of Nodal signaling significantly downregulated

mir206 expression, which was consistent with the microarray data (Fig. 1C). *In situ* hybridizations were also performed to determine whether *mir206* expression was regulated by Nodal signaling after gastrulation. Overexpression of *sqt* was sufficient to induce ectopic expression of *mir206* at 12 hours post fertilization (hpf) (Fig. 1D). Thus, we conclude that *mir206* expression is regulated by Nodal signaling during and after gastrulation.

mir206 acts downstream of Nodal signaling to regulate CE movements

Xenopus Nodal family members *Xnr1* and *Xnr2* are required for convergent extension in the axial and paraxial mesoderm (Luxardi et al., 2010). In zebrafish, nodal gene mutant embryos lack endodermal and mesodermal tissues (Feldman et al., 1998; Sampath et al., 1998), making it difficult to investigate the physiological role of Nodal signaling in gastrulation movements. To examine whether Nodal signaling is indeed involved in zebrafish CE movements, wild-type embryos were treated with 75 μmol/L of SB431542, an inhibitor of Nodal receptor, to inhibit Nodal signal transduction (Liu et al., 2011). Stem-loop RT-PCR and *in situ* hybridization experiments showed that SB431542 treatment led to a dramatic reduction of *mir206* expression at mid-gastrulation and 24 hpf (Fig. 2A and B), and shorter body axis with more distant bilateral columns of adaxial cells (*myod1*) at the end of gastrulation (Fig. 2C). As loss function of *mir206* impairs cell migration during zebrafish gastrulation (Liu et al., 2012), the decrease of *mir206* expression in SB431542 treated embryos may be responsible for the Nodal deficiency-induced CE defects. To validate our hypothesis, embryos were injected with 50 pg synthetic *mir206* duplex at one cell stage and then treated with SB431542. Results showed that *mir206* duplex injection could partially rescue these CE defects (Fig. 2C).

To further clarify how Nodal signaling affects cell movements during gastrulation, we performed transplantation experiments. Lateral mesendodermal cells (LME) from the lateral blastoderm margin of donor embryos were transplanted to the same location of host normal embryos at the shield stage. We found that the convergence movements of LME donor cells treated with SB431542 were severely impaired, as the control LME donor cells had already migrated into the dorsal sides of host embryos at the end of gastrulation (Fig. 2D). We also tested the extension movements by transplanting axial mesendodermal cells (AME) from different donor embryos deeply into the embryonic shield of host embryos. The control AME donor cells were distributed in the dorsal axial hypoblast along the entire length of the AP axis at 10 hpf, while the SB431542 treated AME cells showed obviously reduced extension ability with a shorter distributed region (Fig. 2E). Importantly, *mir206* duplex injection rescued these convergence and extension defects in the transplantation experiments (Fig. 2D and E). These results together indicate that *mir206* regulates CE movements at downstream of Nodal signaling.

mir206 controls E-cadherin expression by Snail-mediated repression

Generally, cell movement defects are caused by unbalanced cell adhesion, either in excess or deficit (Hammerschmidt and Nusslein-Volhard, 1993; Hammerschmidt and Wedlich, 2008; Speirs et al., 2010). Cadherin adhesion molecules, particularly E-cadherin, also known as epithelial Cdh1, play key roles in tissue morphogenesis during vertebrate gastrulation (Solnica-Krezel, 2005; Hammerschmidt and Wedlich, 2008). TGF-β induces the expression of snail1 transcription factor and decreases E-cadherin expression during epithelial-mesenchymal transition, which is essential for organogenesis and tumor metastasis (Vincent et al., 2009; Fuxe et al., 2010). Since *mir206* expression is regulated by Nodal signaling (Fig. 1C and E), which belongs to the TGF-β superfamily, we first tried to explore whether the expression of zebrafish *snail1a* is regulated by *mir206*. Injection of 206-MOs, which can effectively block *mir206* activity (Liu et al., 2012), dramatically reduced the expression of *snail1a* transcripts (Fig. 3A). We next performed gain-of-function experiments using synthetic *mir206* duplex and found that overexpression of *mir206* also resulted in obvious decrease in *snail1a* expression (Fig. 3B). Subsequently, we found that both knockdown and overexpression of *mir206* induced a significant increase in E-cadherin expression (Fig. 3C).

To investigate whether the decrease of *snail1a* expression was responsible for the CE defects in *mir206* morphants, 100 pg zebrafish *snail1a* mRNA was co-injected with 8 ng 206-MOs per embryo at one cell stage. The expression patterns of *myod1* and *ntl* were almost recovered to normal as their wild-type controls by overexpression of *snail1a* in *mir206* morphants (Fig. 3D and E). Taken together, these results suggest that Snail-mediated E-cadherin repression is involved in the cell migration defects in *mir206* morphants.

DISCUSSION

The formation of the three germ layers (endoderm, mesoderm, and ectoderm) is a fundamental process in the vertebrate body plan resulted from coordinated gastrulation movements. Nodal signals are essential for the induction and patterning of mesoderm and endoderm during vertebrate embryogenesis (Schier, 2003, 2009). Nodal-deficient zebrafish embryos failed in gastrulation, as they lack the tissues that normally undergo internalization, suggesting that Nodal signaling may also function in the control of gastrulation movements.

Evidence for a direct role of Nodal signaling in gastrulation movements comes from *Xenopus*. There are four *Xenopus* Nodal family members (*Xnr1*, *Xnr2*, *Xnr5*, and *Xnr6*) that are expressed in the deep vegetal cells of the blastula. *Xnr1* and *Xnr2* are required for convergent extension in the axial and paraxial mesoderm, while *Xnr5* and *Xnr6* are essential for the early mesendoderm induction (Luxardi et al., 2010). In zebrafish, two nodal genes, *sqt* and *cyc*, are expressed in the blastula, and double mutant embryos lack endodermal and mesodermal tissues (Feldman

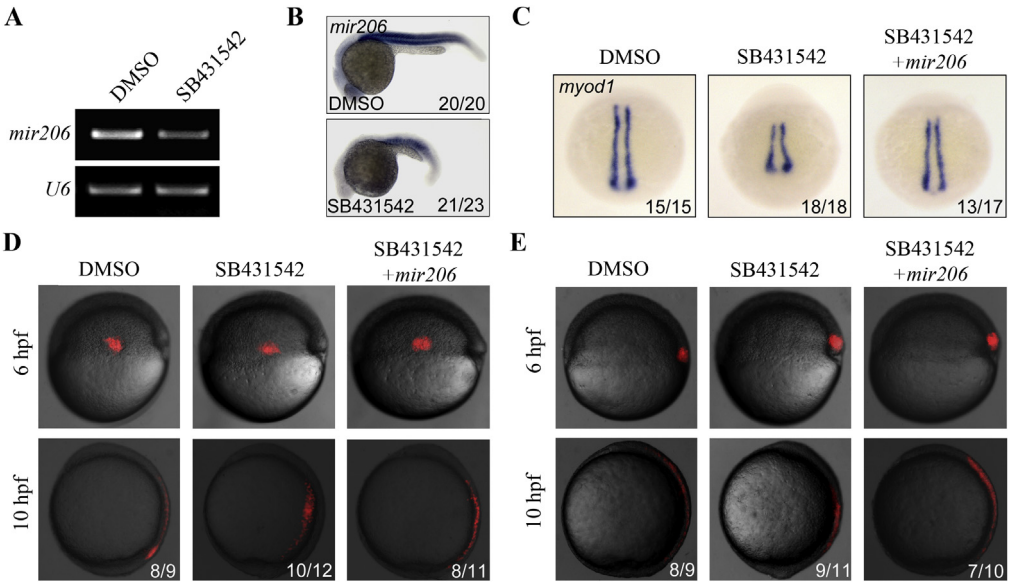

Fig. 2. Nodal signal regulated cell movements through *mir206*.

A and **B:** the expression of *mir206* was detected in DMSO or SB431542 treated embryos at 75%-epiboly stage by semiquantitative stem-loop RT-PCR (**A**) and 24 hpf by whole-mount *in situ* hybridization (**B**). Embryos were viewed laterally. **C:** the expression patterns of *myod1* in SB431542 treated embryos injected with or without 100 pg *mir206* duplex at the tailbud stage (10 hpf). Embryos were viewed dorsally with anterior to the top. The ratios of affected embryos were indicated in the right bottom corners. **D** and **E:** overexpression of *mir206* could rescue Nodal deficiency-induced CE defects. Lateral (**D**) or axial (**E**) mesendodermal cells (red) derived from SB431542 treated embryos injected with or without 100 pg *mir206* duplex were transplanted into the corresponding regions of the hosts at the shield stage (6 hpf) and were observed at the tailbud stage (10 hpf). All embryos were lateral views with dorsal to the right. The ratios of affected embryos were indicated in the right bottom corners.

et al., 1998; Sampath et al., 1998), making it difficult to investigate the physiological role of Nodal signaling in zebrafish gastrulation movements. In the current study, we present evidence that *mir206* is a Nodal-regulated gene and acts as a pivotal modulator of cell migration during zebrafish gastrulation. To our knowledge, this is the first report describing the role of *mir206* as a downstream Nodal effector on cell migration during gastrulation. Importantly, our study also implies that Nodal signaling is involved in zebrafish gastrulation movements.

We previously reported that *mir206* modulates JNK2 phosphorylation by targeting *pk1a* to regulate CE movements

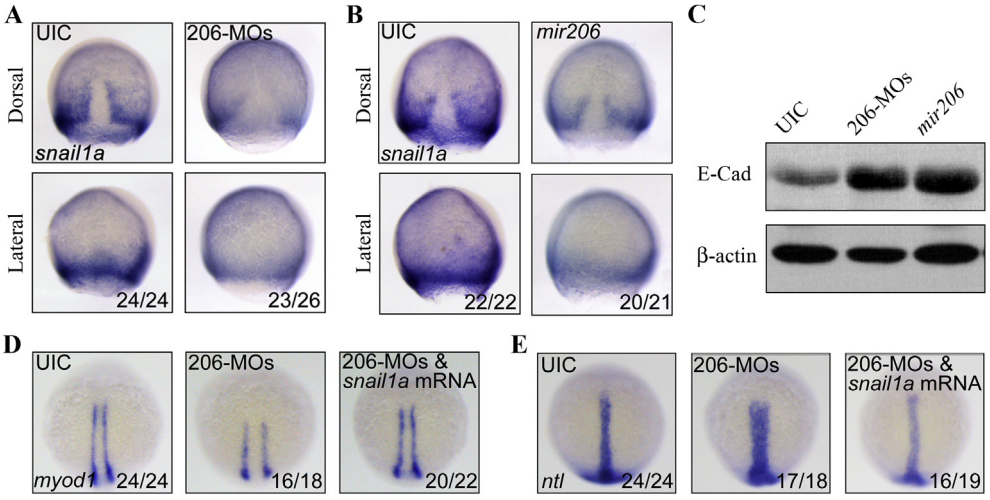

Fig. 3. *mir206* modulates E-cadherin expression by controlling *snail1a* transcript levels.

A and **B:** whole-mount *in situ* hybridization with *snail1a* probe in 8 ng 206-MOs (**A**) or 200 pg *mir206* duplex (**B**) injected embryos at 75%-epiboly stage. **C:** detection of E-cadherin expression by Western blotting at 75%-epiboly stage. The expression of β-actin was examined as loading control. **D** and **E:** injection of zebrafish *snail1a* mRNA could rescue CE defects of *mir206* morphants. Embryos were injected with 8 ng 206-MOs alone or together with 100 pg zebrafish *snail1a* mRNA at the one-cell stage. The expression of *myod1* (**D**) and *ntl* (**E**) were assayed at the bud stage. Dorsal views with anterior to the top. The ratios of affected embryos were indicated in the right bottom corners.

(Liu et al., 2012). However, overexpression of JNK2α2, which encodes a constitutively active JNK2 isoform, cannot fully rescue the CE defects in *mir206* morphants (Liu et al., 2012), suggesting that other migration-related factors are involved in *mir206*-regulated CE movements. E-cadherin, which belongs to a member of the classic cadherin family, is known to be involved in adhesion-dependent morphogenetic processes in mouse, chick, and zebrafish (Takeichi, 1988; Shimizu et al., 2005; Ulrich et al., 2005). Furthermore, Snail1 is a transcriptional repressor of the *cdh1* gene that binds the E-boxes in the *cdh1* promoter to inhibit its transcription (Barrallo-Gimeno and Nieto, 2005). We found that the expression of E-cadherin is significantly increased in both *mir206* overexpressed embryos and *mir206* morphants, while *snail1a* is depressed. These findings are consistent with our previous observation that the correct amount of *mir206* is important for proper cell migration during zebrafish gastrulation (Liu et al., 2012). More importantly, injection of *snail1a* mRNA into *mir206* morphants rescued CE defects, indicating that Snail/E-cadherin pathway is also essential for the mir206/Snail/E-cadherin signaling in the control of CE movements.

miRNAs in generally suppress gene expression at post-transcription level (Bushati and Cohen, 2007; Erson and Petty, 2008; Filipowicz et al., 2008). It is unlikely that *snail1a* is the direct target of *mir206* because knockdown of *mir206* resulted in decreased rather than increased *snail1a* expression. The identification of *mir206* direct targets which regulate *snail1a* expression will be valuable to illuminate the genetic interaction between *mir206* and Snail/E-cadherin pathway. It has been recently reported that interference with JNK signaling pathway suppressed the motile capacity of vincristine-resistant human oral cancer KB cells (KB/VCR) through decreasing snail and twist expression (Zhan et al., 2013). p-JNK2 and snail1a are decreased in both *mir206* overexpressed embryos and *mir206* morphants as we observed in our previous and current reports. Therefore, the expression of *snail1a* may be regulated by mir206-Pk1a-JNK2 pathway during zebrafish gastrulation. In conclusion, our results collectively indicate that Nodal-regulated *mir206* may modulate gastrulation movements through regulating Snail/E-cadherin expression.

MATERIALS AND METHODS

Fish maintenance

Wild-type embryos used in this study were derived from the Tuebingen strain. *MZoep* embryos were generated by crossing $oep^{-/-}$ female with $oep^{-/-}$ male as described before (Jia et al., 2008). Embryos were maintained in Holtfreter's solution at 28.5°C, and staged according to Kimmel et al. (1995).

miRNA microarray analysis

Total RNA was extracted from bud stage wild-type and *MZoep* mutant embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA of 5 μg was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA, USA) and miRNA microarray assay based on Sanger miRNA database 12.0 version was performed using a service provider (LC Sciences, Houston, TX, USA). The up-regulated or down-regulated genes were determined by the signal log ratio.

Morpholinos, microinjection and whole-mount *in situ* hybridization

The *mir206-1* morpholino (206-MO1) (5′-ACCACA CACTTCCTTACATTCCATAACTTG-3′) and *mir206-2* morpholino (206-MO2) (5′-GCCACACACTTCCTTACATTCCA TAGATTA-3′) were designed complementary to the miRNA guide strand and the Dicer nucleolytic processing sites respectively according to the sequences of *mir206-1* and *mir206-2* precursors (Liu et al., 2012). All morpholinos were purchased from Gene Tools and resuspended in nuclease free (NF) water. 2 ng of 206-MO1 combined with 2 ng 206-MO2 (206-MOs) were injected into the yolk of one-cell stage embryos. *In situ* hybridizations were performed as previously described (Wienholds et al., 2005; Liu et al., 2011).

RNA synthesis

mir206 duplex was designed and synthesized by Shanghai GenePharma Co, Ltd., China. For making *mir206* duplex, synthesized two single-stranded RNA sequences were: sense, 5′-UGGAAUGUAAGGAAGUGUGUGG-3′; antisense, 5′-AGACAUGCUUCCUUAUAUGCCCA-3′.

Stem-loop RT-PCR

Stem-loop RT-PCR was performed as previously described (Kloosterman et al., 2006; Wang et al., 2008). Total RNAs were reversely transcribed using the *mir206* RT primer (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACCCACAC-3′) and the *U6* RT primer (5′-AAAAA TATGGAGCGCTTCACG-3′). The PCR primers were listed as follows: for *U6*, forward primer with 5′-TTGGTCTGATCTGG CACATATAC-3′, and reverse primer with 5′-AAAAA TATGGAGCGCTTCACG-3′; for *mir206*, forward primer with 5′-GCGTCTGGAATGTAAGGAAGTG-3′, and reverse primer with 5′-GTGCAGGGTCCGAGGT-3′.

Immunoblotting

Dechorionated embryos were isolated at 75% epiboly and embryonic extracts were prepared in TNE buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, and 0.5% NP-40) with protease inhibitors. Embryonic extracts were centrifuged to remove membrane debris and yolk lipids and then were subjected to SDS-PAGE. The expression of E-cadherin was visualized by Western blotting using anti-cdh1 antibody (55527, ANA SPEC, USA).

Cell transplantation

For cell transplantation assay, donor embryos were firstly co-injected with 1 nL of 0.5% rhodamine−dextran (MW 10,000, Molecular Probes, USA) together with or without 50 pg of synthesized *mir206* precursor RNA at one-cell stage. Then the donor and host embryos were treated with 75 μmol/L SB431542 from 256-cell stage to shield stage. Lateral or axial mesendodermal cells (30−50 cells) from the lateral margin or embryonic shield of donor embryos were transplanted to the relevant region of hosts at the same developmental stage (shield stage, about 6 hpf), and SB431542 treatment was continued into tailbud stage.

ACKNOWLEDGEMENTS

This work was supported by the grants from the Major Science Programs of China (Nos. 2011CBA01101 and 2011CB943904) and the National Natural Science Foundation of China (Nos. 30971656 and 90919011).

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