MicroRNA-127 Promotes Mesendoderm Differentiation of Mouse Embryonic Stem Cells by Targeting Left-right Determination Factor 2

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Running title: Role of microRNA-127 in germ layer specification

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Abstract

Specification of the three germ layers is a fundamental process and is essential for the establishment of organ rudiments. Multiple genetic and epigenetic factors regulate this dynamic process; however, the function of specific microRNAs in germ layer differentiation remains unknown. In this study, we establish that microRNA-127 (miR-127) is related to germ layer specification via microRNA array analysis of isolated three germ layers of E7.5 mouse embryos and verified through differentiation of mouse embryonic stem cells. MiR-127 is highly expressed in endoderm and primitive streak. Overexpression of miR-127 increases, whereas inhibition of miR-127 decreases, the expression of mesendoderm markers. We further show that miR-127 promotes mesendoderm differentiation through the Nodal pathway, a determinative signaling pathway in early embryogenesis. Using luciferase reporter assay, left-right determination factor 2 (Lefty2), an antagonist of Nodal, is identified to be a novel target of miR-127. Furthermore, the role of miR-127 in mesendoderm differentiation is attenuated by Lefty2 overexpression. Altogether, our results indicate that miR-127 accelerates mesendoderm differentiation of mouse embryonic stem cells through Nodal signaling, by targeting Lefty2.

Introduction

The specification and patterning of three germ layers are crucial processes during mouse early development (1). The primitive streak (PS), through which epiblast cells move and form mesoderm and definitive endoderm, is a critical structure for specification of the three germ layers (2). The Nodal signaling pathway plays a central role in germ layer specification and PS formation (2,3). In this pathway, NODAL, a transforming
growth factor-beta (TGF-beta) superfamily protein, initially interacts with and activates Activin-like receptors that are serine/threonine kinases. Then, the activated Activin-like receptor phosphorylates SMAD2, which forms a Co-SMAD complex with SMAD4 and is transported to nuclei to regulate expression of target genes, eventually promoting PS formation (4-7). LEFTY2 is an antagonist of the Nodal pathway by competitive binding to the Activin-like receptors (8). In mice, LEFTY2 expresses in the anterior half of the primitive streak and the left part of the lateral plate mesoderm of the gastrulating embryo (9,10). Lefty2 mutant mouse embryos form enlarged PS and immoderate mesoderm (11). Thus, LEFTY2 is required for the formation of PS and its expression is precisely controlled during mouse gastrulation. However, an understanding of the mechanism of Lefty2 regulation during this process remains elusive.

MicroRNAs (MiRNAs) are 19- to 25-nt single-strand RNAs that are widespread in organisms and play important roles in diverse biological processes, including animal development, by controlling the expression of related genes (12,13). Mature miRNAs are generated from endogenous transcripts with a hairpin-shaped structure that functions as a complex known as the RNA-induced silencing complex (RISC), which inhibits protein translation and destabilizes mRNA transcripts (12,13). RNase III family member DICER and double-strand RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) play crucial roles in the production of mature miRNAs (12,14). Knockout of Dicer impairs the generation of both endo-siRNAs and miRNAs. The embryonic region of the E7.5 Dicer mutant embryo is obviously small and malformed, with decreased Oct4 expression (15). In addition, the differentiation potential of Dicer-deficient embryonic stem cells (ESCs) is severely destructed in vitro and in vivo (16,17). Similarly, knockout of Dger8 results in embryonic lethality at E6.5 and Dger8 knockout ESCs cannot form the cystic embryoid bodies (EBs) which consist of a group of cells representing the three germ layers of early mouse embryo (18). Thus, miRNAs are critical for mouse early embryonic development and may play roles in germ layer specification.

MiR-127 is located on mouse distal chromosome 12, and on human chromosome region 14q32, which are well known as a Dlk1/Gtl2 imprinted region. MiR-127 lies near a CpG island in Rtl1, a paternally expressed gene, and takes an antisense strand of Rtl1 as a template for transcription but is expressed from the maternal chromosome (19). Mice with disrupted miR-127 have placental defects in the labyrinthine zone (20). In addition, overexpression of miR-127 affects lung development in the fetal lung organ culture system (21). However, the role of miR-127 is still unclear during gastrulation of mouse early development.

In the present study, we explored the relationship between miR-127 and mouse germ layer differentiation during gastrulation, using microRNA array analysis of germ layers of E7.5 embryos. We found that miR-127 was enriched in endoderm and primitive streak of E7.5 mouse embryos. Overexpression of miR-127 accelerates, whereas miR-127 knockdown impedes mesendoderm differentiation. Furthermore, we showed that miR-127 directly down-regulated the expression of Lefty2, an antagonist of Nodal signaling and a regulator of PS formation. Thus, our results suggested that miR-127 promotes mesendoderm differentiation by down-regulating the expression of Lefty2.
Experimental Procedures

Mice maintenance; E7.5 mouse embryo germ layers separation and microarray

Mice were maintained under specific-pathogen-free (SPF) conditions (12 h light and 12 h dark) in the animal facilities of Institute of Zoology. All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Institute of Zoology at the Chinese Academy of Sciences.

E7.5 embryos were dissected from 30 pregnant ICR mice and stored in a 25mM HEPES-buffered DMEM solution containing 10% FBS. The Reichert’s membrane was removed. The embryonic region was separated using a needle, rinsed in serum-free medium, incubated in pancreatic enzyme solution at 4 °C for 10 minutes, and transferred into HEPES-buffered DMEM solution. The embryo was then gently sucked into a siliconized Pasteur pipette to remove the endoderm layer. Needle points were inserted under the mesoderm and each mesoderm wing was cut off. The remainder was collected as ectoderm. Total RNA was extracted with mirVana™ miRNA Isolation Kit (Ambion), and sent to the Bioassay Laboratory of CapitalBio Corporation for microarray and analysis.

Embryonic stem cell culture, transfection and EB differentiation

Mouse ESCs (CMTI-1, Millipore) were maintained on mouse embryonic fibroblasts (MEFs) treated by mitomycin C in DMEM containing 15% fetal bovine serum, 2 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μg/ml penicillin-streptomycin, 0.1 mM β-mercaptoethanol and 10 ng/ml leukemia inhibitory factor (LIF). The ESCs were cultured on gelatin-coated plates in N2B27 medium before transfection, as described previously (22). Mouse ESCs were plated in 12-well plates (1 × 10^5 cells/well) at the time of transfection. The miRNA mimics or inhibitors (Invitrogen) were transfected with indicated concentrations into ESCs using Lipofectamine 2000 reagent (Invitrogen). For EB formation, ESCs were trypsinized into single cells and cultured by seeding 1,000 ESCs in 30 μl hanging droplets of ES medium devoid of LIF.

RNA isolation, reverse transcription and quantitative real time PCR

Total RNA was extracted with RNAzol (Molecular Research Center). Synthesis of cDNA was performed with SYBR PrimeScript™ miRNA RT-PCR Kit (Takara) and PrimeScript™ RT-PCR Kit (Takara) respectively for miRNA and mRNA analysis. Real time PCR was carried out in the Roche LightCycler 480 II system. The primer sequences are listed in Table 1. Hprt and U6 were used as reference genes for normalization of mRNA and miRNA level. Data were processed using the 2^-△△Ct method.

Luciferase reporter assay

The 3’UTR sequences of Lefty2, Fstl3, Smad7 and Ppm1b, including the predicted miRNA binding site, and 3’UTR of Lefty2 with site-directed mutagenesis were amplified from mouse ESCs cDNA and then inserted into pGL3-control vector (Promega). HEK 293FT cells were grown in DMEM with 10% FBS and seeded in 24-well plates before transfection with Lipofectamine 2000 (Invitrogen). The cells were co-transfected with 500 ng firefly luciferase reporter vector pGL3 containing 3’UTR and 20 ng Renilla luciferase vector pRL-TK which was used to normalize luciferase activity. MiR-127 mimics at a concentration of 20 μM or scrambled oligonucleotides were added into each well. Luciferase reporter assay was conducted 48 h
after transfection using Dual-Luciferase Reporter Assay Kit (Promega).

Western blot analysis

Whole cell lysates were prepared by re-suspending cells in RIPA lysis buffer, which contained 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM Na3VO4 and 5 mM NaF. Proteins were separated with SDS-PAGE and subsequently to Western blot (Biorad). Western blot analysis was conducted with antibodies as follows: Rabbit anti-FOXA2 (1:1,000, #8186, Cell Signaling Technology); Goat anti-T (1:1,000, sc-17745, Santa Cruz); Mouse anti-GAPDH (1:1,000, DKM9002, Sungene); Rabbit anti-phosphor (Ser465/467)-SMAD2 (1:1,000, #3108, Cell Signaling Technology); Rabbit anti-SMAD2 (1:1,000, #5339, Cell Signaling Technology); Mouse anti-ACTIN (1:1,000, 214673, Cell Signaling Technology); Rabbit anti-β-CATENIN (1:1,000, #8814S, Cell Signaling Technology); and Rabbit anti-LEFTY2 (1:500, 13991-1-AP, Proteintech).

MiRNA oligonucleotide injection and whole-mount in situ hybridization in zebrafish embryos

Zebrafish embryos used in this study were derived from Tuebingen strain and raised in Holtfreter’s solution at 28.5°C. MiR-127 mimics were injected into one-cell stage zebrafish embryos. After 6 hrs, these embryos were collected. Whole-mount in situ hybridization was performed according to standard procedures with probes including chordin, goosecoid and lefty2 (23).

Statistical analyses

Quantitative analyses were performed at least 3 independent biological samples. Data were expressed as the mean ± SEM. Statistics were conducted by using SPSS 18.0 and data were subjected to student’s t-test or one-way analysis of variance (ANOVA). P<0.05 was considered as significant.

Results

MiR-127 is associated with mouse germ layer specification

To identify the miRNAs involved in germ layer differentiation, miRNA microarray profiling was conducted using isolated endoderm, mesoderm and ectoderm from the embryonic region of E7.5 ICR mice embryos. Quantitative RT-PCR (qRT-PCR) showed that the markers of each germ layer were specifically expressed in the separated sample counterparts (Fig. 1A), indicating effective separation and minimal contamination of each germ layer sample. MiRNA microarray profiling of the three germ layers was then performed, and the miRNAs expressed in each germ layer were clustered (Fig. 1B). Our results showed that most miRNAs are highly enriched in endoderm. Using a criterion of an expression fold change of >3, we screened out 30 miRNAs with comparatively high expression in endoderm.

To investigate the specific miRNAs involved in germ layer differentiation, we examined the expression of 30 miRNAs at day 0, 2, 4 and 6 during embryoid body (EB) formation, a model of ESC differentiation into germ layer lineages. Although expression of most miRNAs was unaltered, expression of miR-127 was significantly up-regulated during EB differentiation (Fig. 1C). These results indicated that miR-127 is likely associated with germ layer differentiation.

MiR-127 is highly expressed in endoderm and primitive streak in E7.5 embryos

We then measured miR-127 expression of during mouse embryonic development using qRT-PCR. The expression of miR-127 was
up-regulated progressively in E5.5 to E7.5 embryos (Fig. 1D) with expression in both the embryonic region and extra-embryonic tissue (Fig. 1E). Of the three germ layers of E7.5 embryo, miR-127 was enriched in the endoderm, cells of which were partially derived from PS (Fig. 1F). To further investigate the expression of miR-127 in E7.5 embryo, PS and neuro-ectoderm were isolated by micro-dissection. MiR-127 was highly expressed in PS (Fig. 1G). The results from in situ hybridization showed a relatively high expression level of miR-127 in endoderm and PS in E7.5 embryo (Fig. 1H).

MiR-127 promotes mesendoderm differentiation

To explore the function of miR-127, we transfected miR-127 mimics to mouse ESCs to exogenously overexpress miR-127. The expression level of miR-127 increased ~60-fold in ESCs transfected with mimics, and miR-127 overexpression did not affect the pluripotency of ESCs examined by the expressions of Oct4 and Nanog (Fig. 2A). Furthermore, the overexpression of miR-127 was consistently observed in day 1, 2 and 3 EBs during ESCs differentiation (Fig. 2B). Next, we detected lineage markers expression after spontaneous differentiation of ESCs into EBs. Compared with controls, expression of germ layer markers did not change in day 1 and 2 EBs (Fig. 2C and 2D); however, mesendoderm markers Foxa2, Brachyury (T) and Gsc were significantly up-regulated in day 3 EBs when miR-127 was overexpressed, with almost unchanged expression of ectoderm markers (Pax6 and Otx2; Fig. 2E). At protein level, mesendoderm markers (FOXA2 and T) were also increased in the day 3 EBs with miR-127 overexpression (Fig. 2F). These data suggested that miR-127 promotes mesendoderm differentiation.

We then performed a loss-of-function study in vitro by transfecting miR-127 inhibitors (Si-127) into ESCs. MiR-127 expression was reduced by ~90% in Si-127-transfected ESCs and pluripotent markers remained unaltered (Fig. 3A). The expression of miR-127 was consistently decreased during ESCs differentiation (Fig. 3B). Next, miR-127 knockdown ESCs were differentiated to EBs and germ-layer marker expression was measured. Compared with the control, miR-127 knockdown did not affect lineage markers expression in day 3 EBs (Fig. 3C). We speculated that the originally low expression of miR-127 during the early stages of ES differentiation (Fig. 1C) results in no effect of miR-127 knockdown in this system. As miR-127 peaked in day 6 EBs, we attempted to knockdown miR-127 expression in day 6 EBs and examined the expression of germ-layer markers 2 days later. The expression of miR-127 was decreased by ~70% (Fig. 3D), and mRNA of mesendoderm markers (Foxa2, T and Gsc) was significantly down-regulated in Si-127-transfected cells (Fig. 3E). Furthermore, FOXA2 and T protein were decreased in Si-127-transfected cells (Fig. 3F). The expression of ectoderm markers (Pax6 and Otx2) remained unaltered in the miR-127 knockdown cells (Fig. 3E).

To confirm the role of miR-127 in mesendoderm differentiation, we transfected miR-127 inhibitor into Epiblast stem cells (EpiSCs) and induced these cells to differentiation using Activin A. After the miR-127 inhibitor treatment, miR-127 expression decreased by ~80% in EpiSCs (Fig. 3G). Consistently, miR-127 knockdown decreased mesendoderm marker expression at RNA (Foxa2, T and Gsc) and protein (FOXA2 and T) levels during the differentiation of miR-127 knockdown EpiSCs (Fig. 3H and 3I). Collectively, these data implied that miR-127 specifically promotes mesendoderm differentiation.

MiR-127 functions by Nodal pathway
To unravel how miR-127 functions in mesendoderm differentiation, we carried out functional cluster analysis of the potential targets of miR-127 predicted from miRBase (www.mirbase.org). We found that these miR-127 targets were mainly clustered in two developmental pathways including Nodal and Wnt signaling. We showed that miR-127 overexpression promoted mesendoderm markers expression in day 3 EBs (Fig. 2E and F). Then we examined the expression of key effectors (active β-CATENIN and phosphorylated SMAD2) of these pathways in control and miR-127 overexpressed EBs at day 2. Overexpression of miR-127 increased phosphorylated SMAD2 (p-SMAD2), but not active β-CATENIN (Fig. 4A and 4B). These data suggested miR-127 may be involved in the Nodal pathway.

To investigate the pathways mediated by miR-127 in mesendoderm differentiation, we induced mouse ESC differentiation to mesendoderm with Activin A. We treated ESCs with 10 ng/ml Activin A, after 1 day culture in LIF-free medium (24,25). When ESCs were treated for 48 h, mesendoderm markers (FOXA2 and T) were up-regulated, indicating ESCs were successfully induced into mesendoderm (Fig.4C and 4D). We also examined the expression of miR-127 in this differentiation system. Our results showed that miR-127 was up-regulated accompanying the increased expression of mesendoderm markers (Fig.4D).

We then knocked down miR-127 when ESCs differentiated to mesendoderm. Upon Activin A stimulation, both p-SMAD2 and mesendoderm markers (FOXA2 and T) were down-regulated in miR-127 knockdown cells compared with the control (Fig. 4E). These findings suggested that miR-127 accelerates mesendoderm differentiation through Activin/Nodal signaling.

**MiR-127 is involved in the Nodal pathway through Lefty2 during mesendoderm differentiation**

As miR-127 promotes mesendoderm differentiation through the Activin/Nodal signaling pathway, we focused on the targets annotated to be associated with this pathway from the miRBase to identify the specific targets of miR-127. Among 15 predicted targets, four genes (Lefty2, Fstl3, Smad7 and Ppm1b) were reported to negatively regulate Nodal signaling (11,26-28), reminiscent of the positive function of miR-127 in this pathway as described above. Then we explored the potential links between miR-127 and these four genes. To do this, we constructed luciferase reporters containing wild-type 3′UTR of the four genes and performed dual-luciferase reporter assay. MiR-127 did not affect the luciferase activity of Fstl3, Smad7 and Ppm1b 3′UTR constructs (Fig. 4F). However, miR-127 significantly decreased the luciferase activity of Lefty2 3′UTR construct. The specificity was confirmed by the reporter in which the binding site of miR-127 in the 3′UTR of Lefty2 was mutant (Fig. 5A).

We then measured expression of Lefty2 during EB differentiation and noted that it gradually decreased, and this was accompanied by increasing miR-127, in the process of EB differentiation (Fig. 5B). In addition, Lefty2 was down-regulated in the miR-127 overexpressed ESCs at both RNA and protein level compared with controls (Fig. 5C and 5D). These data suggested that Lefty2 is a novel target of miR-127.

Next, we assessed whether overexpression Lefty2 decreased the effect of miR-127 on mesendoderm differentiation. To do this, we co-transfected control oligonucleotides or miR-127 mimics and Lefty2 overexpression vector into ESCs, and examined the expression of
specific markers in day 3 EBs. Compared with the cells transfected with miR-127 mimics, mesendoderm markers (F Paxa2 and T) were significantly decreased in the cells co-transfected with both miR-127 mimic and Lefty2 overexpression vector (Fig. 5E and 5F). A similar tendency of p-SMAD2 was observed in these cells by Western blot (Fig. 5F). These data show that the overexpression of Lefty2 could attenuate the role of miR-127 in promotion of mesendoderm differentiation.

Taken together, our data suggested that miR-127 is involved in Nodal signaling by down-regulating Lefty2 to accelerate mesendoderm differentiation.

**MiR-127 promotes embryonic shield development in zebrafish**

In zebrafish, lefty2 (lft2) possesses a conserved miR-127 targeting site. To investigate the function of miR-127 in vivo, we injected miR-127 mimics into one cell-stage embryos of zebrafish and analyzed the marker (goosecoid and chordin) expression of the embryonic shield area, which is the structure equivalent to the primitive streak in mice. Compared with the non-injected embryos, the expression of lft2, examined by in situ hybridization, was dramatically down-regulated, while the expression of chordin (chd) and goosecoid (gsc) was obviously up-regulated in embryos injected with 20 μM miR-127 mimics at 6 hours post-fertilization (hpf) (Fig. 6A). The changed expressions of these genes were confirmed by qRT-PCR results (Fig. 6B). These results were consistent with the analysis of EB differentiation in vitro. In summary, we proposed a model in which miR-127 controls the mesendoderm differentiation via involved in the Nodal signaling pathway by regulating the expression of Lefty2 (Fig. 7).

**Discussion**

MiRNAs play important roles in multiple physiological and pathological processes (29-32). The mutations of key regulators of miRNAs generation in mouse, such as DICER and DGCR8, result in early embryonic lethality and defects of ESC differentiation, suggesting an important role of miRNAs in germ layer specification during mouse early embryonic development (15-18). In this study, we found the expression of miR-127 enriched in endoderm and PS of E7.5 mouse embryo. Mouse EB differentiation assay showed that miR-127 overexpression led to elevated, while miR-127 knockdown decreased, mesendoderm markers expression. MiR-127 knockdown also down-regulated expression of mesendoderm markers during the direct differentiation of mouse EpiSCs into mesendoderm. In addition, overexpression of miR-127 up-regulated the expression of embryonic shield markers in zebrafish embryos. All these results suggest that miR-127 possibly promotes PS development during mouse gastrulation.

LEFTY2, an antagonist of Activin/Nodal signaling by competing to interact with the Nodal receptor, plays an important role in PS formation and L–R axis patterning during mouse gastrulation (33-35). The LEFTY2 asymmetric expression pattern is regulated by SPC4 during mouse L–R axis formation (36). However, it is still not clear how LEFTY2 is regulated in mouse PS development. We found that miR-127 directly regulated LEFTY2 expression, as suggested by luciferase reporter assay. LEFTY2 is down-regulated in miR-127 overexpressed ESCs. The decreased expression of LEFTY2 is accompanied by increased expression of miR-127 during the differentiation of ESCs into EBs. Importantly, the effect of miR-127 on mesendoderm differentiation can be attenuated by...
overexpressed Lefty2. All of these results consistently suggest that miR-127 directly regulates the expression of Lefty2 during mouse mesendoderm differentiation.

Although we find that miR-127 regulates mesendoderm differentiation, obvious developmental defects are not reported in miR-127 knockout mice (20). MiRNAs are regulators that fine-tune the expression of complementary mRNAs, and several miRNAs usually function together to regulate a given mRNA (37-39). Thus, other unknown factors may compensate the function of miR-127 in the knockout mice. Mice with disruption to many important genes involved in differentiation are similar to miR-127 knockout mice (22,40,41).

In summary, our data suggest that miR-127 is involved in mouse early embryonic development by controlling the expression of LEFTY2, a critical regulator of PS formation. Our research reveals a novel function of miR-127 in accelerating mesendoderm differentiation and provides new evidence for the function of specific miRNAs in germ layer specification during gastrulation.

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
H.-X.M designed and performed major experiments, analyzed the data and wrote the manuscript. Y.L. conducted the experiments of zebrafish. Z.-A.Z. performed mice embryo dissection and microarray. X.-K.L. constructed Lefty2 overexpression vector. Y.Y and X. Z. contributed to the results of EpiSCs. L.L. and Q.W. initiated and organized this project, analyzed the data and wrote the manuscript. All authors commented on the manuscript.

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**Footnote**
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The abbreviations used are: PS, primitive streak; miR-127, microRNA-127; *Lefty2*, Left-right determination factor 2; ESC, embryonic stem cell; EB, embryoid body; EpiSC, epiblast stem cell; *chd*, *chordin*; *gsc*, *goosecoid*.

**Figure Legends**
**Figure 1.** MiR-127 is associated with germ layer formation and is mainly expressed in PS and endoderm of E7.5 mouse embryo. (A) qRT-PCR shows lineage marker expression in detached germ layers of E7.5 embryos. (B) Heat map was generated by performing miRNAs specifically expressed in endoderm, mesoderm and ectoderm of E7.5 mice embryos, respectively. (C) qRT-PCR shows miR-127 expression during EB formation (one-way ANOVA, **p < 0.01**). (D) qRT-PCR shows miR-127 expression in gastrulating embryos from E5.5 to E7.5 (one-way ANOVA, **p < 0.01**). (E) qRT-PCR shows miR-127 expression pattern in extra-embryonic field and embryonic region of E5.5, E6.5 and E7.5 embryos. (F) qRT-PCR shows miR-127 expression in germ layers of E7.5 mouse embryo (one-way ANOVA, **p < 0.01**). (G) qRT-PCR shows miR-127 expression in PS and neuro-ectoderm (one-way ANOVA, **p < 0.01**). (H) Expression of miR-127 in E7.5 embryo, shown by *in situ* hybridization. Brown represents miR-127 expression. Scale bar, 300 μm.

**Figure 2.** Overexpression of miR-127 promotes mesendoderm differentiation. (A) qRT-PCR displays the expression of miR-127 and pluripotent markers in miR-127 overexpressed ESCs. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, ***p < 0.001**). (B) qRT-PCR displays the expression of miR-127 in miR-127 overexpressed day 1, 2 and 3 EBs. The data are presented as mean ± SEM (N = 3). (C) qRT-PCR displays lineage markers expression in miR-127 overexpressed day 1 EBs. The data are presented as mean ± SEM (N = 3). (D) qRT-PCR displays lineage markers expression in miR-127 overexpressed day 2 EBs. The data are presented as mean ± SEM (N = 3). (E) qRT-PCR displays lineage markers expression in miR-127 overexpressed day 3 EBs. Significant differences in expression between designated pairs were analyzed by student’s t-test with equal variance, and the data are presented as mean ± SEM (N = 3, *p < 0.05, **p < 0.01, ***p < 0.001). (F) Western blot shows mesendoderm marker expression in miR-127 overexpressed day 3 EBs. GAPDH was served as a loading control.

**Figure 3.** MiR-127 knockdown inhibits mesendoderm differentiation. (A) qRT-PCR shows expression of miR-127 and pluripotent markers in miR-127 knockdown ESCs. The significance of expression was
analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, *** p < 0.001). (B) qRT-PCR displays the expression of miR-127 in miR-127 knockdown day1, day2 and day3 EBs. The data are presented as mean ± SEM (N = 3). (C) qRT-PCR displays lineage markers expression in miR-127 knockout day 3 EBs. The data are presented as mean ± SEM (N = 3). (D) qRT-PCR displays the expression of miR-127 in day 6 and day 8 EBs following miR-127 knockdown in day 6 EBs. The data are presented as mean ± SEM (N = 3). (E) qRT-PCR shows miR-127 expression in day 6 knockdown EBs and lineage marker expression in day 8 EBs following miR-127 knocked down in day 6 EBs. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05). (F) Expression of mesendoderm markers in negative control and miR-127 knockdown day 8 EBs (NC and si-127), revealed by Western blot. GAPDH was served as loading control. (G) qRT-PCR shows miR-127 expression in miR-127 knockdown EpiSC cells. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, ** p < 0.01). (H) qRT-PCR shows lineage marker expression in EpiSC cells treated with 10 ng/ml Activin A followed by control and miR-127 inhibitor transfected (NC and Si-127). The significance of expression was analyzed using student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05). (I) Expression of mesendoderm markers (FOXA2 and T) was analyzed by Western blot. GAPDH was used as a loading control.

**Figure 4.** MiR-127 involves in Nodal signaling pathway. (A) Effectors of Nodal and Wnt pathway expression were measured in day 2 EBs by Western blot. GAPDH was used as loading control. (B) Analysis of gray scale scanning of Western blot results of signaling effectors in day 2 EBs. The data were from three independent experiments (*p < 0.05). (C) Mesendoderm markers expression in ESCs treated with Activin A for 0, 12, 24 and 48h, displayed by Western blot. (D) qRT-PCR shows Foxa2, T and miR-127 expression in ESCs treated with 10ng/ml Activin A. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05, ** p < 0.01, *** p < 0.001). (E) Expression of p-SMAD2 and mesendoderm markers in miR-127 knockdown ESCs under Activin A treatment, shown by Western blot. β-ACTIN was used as loading control. (F) Luciferase report vectors containing binding site of miR -127 in 3′UTR and either miR-127 mimics or negative control, co-transfected into 293FT cells, with relative luciferase activity measured. PGL3 vector acted as a control.

**Figure 5.** MiR-127 functions through Nodal signaling by down-regulating Lefty2. (A) Analysis of repression of Lefty2 gene by miR-127 at the sequence of 3′UTR through a luciferase reporter assay. PGL3 vector acted as a control. These results from three independent experiments are shown as mean ± SEM, * p < 0.05. (B) The expression levels of miR-127 and Lefty2, measured by qRT-PCR during EB differentiation. Data are presented as mean ± SEM (N = 3). (C) Lefty2 expression analyzed by qRT-PCR in miR-127 overexpressed ESCs. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05). (D) Western blot showing LEFTY2 expression in miR-127 overexpressed ESCs. β-ACTIN was used as loading control. (E) qRT-PCR reveals expression of mesendoderm markers in day 3 EBs, transfected with random sequence, miR-127 mimic or co-transfected with miR-127 mimic and Lefty2 overexpressed vector, respectively. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05). (F) Changes in expression of mesendoderm markers, p-SMAD2 and LEFTY2 are shown by Western blot, when miR-127
was overexpressed, or both miR-127 and Lefty2 were overexpressed. GAPDH was used as loading control.

**Figure 6.** MiR-127 accelerates the development of zebrafish embryonic shield. (A) Expression of embryonic shield markers and Lefty2, measured with whole-mount *in situ* hybridization in miR-127 mimic-injected zebrafish embryos at concentrations of 0 and 20 μM. Embryos were injected with miR-127 mimics at the one-cell stage and harvested at 6 hpf for probing with indicated probes. (B) qRT-PCR displaying embryonic shield markers and Lefty2 expression in miR-127 mimic-injected zebrafish embryos, at concentrations of 0 μM, 10 μM and 20 μM, respectively. The significance of expression was analyzed by student’s t-test and data are presented as mean ± SEM (N = 3, * p < 0.05).

**Figure 7.** Schematic representation depicts the mechanism of miR-127 in accelerating mesendoderm differentiation. Mir-127, through the complementary pairing, suppresses the expression of LEFTY2, which is referred to as antagonist to the Nodal pathway. Thus, miR-127 induces p-SMAD2 expression and finally up-regulates expression of mesendoderm markers.
| Gene   | Type   | Sequence (5’-3’)                      |
|--------|--------|---------------------------------------|
| miR-127| Forward| TCGGATCCGTCCTGAGCTTTGGCT              |
| U6     | Forward| CTCGCTTCCGCCACGACA                   |
|        | Reverse| AACGCCTTCAGAAAGTGGGTCG               |
| Sox17  | Forward| CTTTATGGGTGTGGACCAAACAG              |
|        | Reverse| TTCCAAGACTTGCTGGCAGAT               |
| Foxa2  | Forward| CCATCAGCACCACAAAATG                 |
|        | Reverse| CCAAGCTGCTGGCCATG                     |
| Foxf1  | Forward| GCATCCCTCGGTATCAGCTCAC               |
|        | Reverse| ATCCTCCGCCTGGGTGTATG                |
| T      | Forward| CCTTCATGTGCTGAGACCTTG               |
|        | Reverse| TCACAAAAAACCTGGGACCACA               |
| Flk-1  | Forward| GCCCTGCTGTGGTCTCAGCTAC              |
|        | Reverse| CAAAGCATGGCCATAGCTG                 |
| Gsc    | Forward| ACCATCTTCACCAGTGAGCAG                |
|        | Reverse| CTGGGCTCGGGGCTCCTAAAG               |
| Pax6   | Forward| TACGGGAAGCGACTCGGATGAGG              |
|        | Reverse| CGGGCAAAACACATCTGGATAATG            |
| Otx2   | Forward| CAGCGGCATCTCCCATCAC                 |
|        | Reverse| GTTGAGCCAGCATACCTTG                 |
| Sox2   | Forward| GCCAGCTACAGCATGATGCAGGAC             |
|        | Reverse| CTGGGTCTAGGGATGTACTGGCAGG            |
| Fgf5   | Forward| GAAATATTTTCTGTGTCTCAGGG             |
|        | Reverse| TAAATTTGCACTGGTATGGG                |
| Hprt   | Forward| GCCAGTAAAAATTAGCAGGTGTTCT            |
|        | Reverse| ATAGGGCTCATAGTGCAAAATCAAAG           |
Figure 4

A

| D2 EB    | NC  | mi-127 |
|----------|-----|--------|
| p-SMAD2  |     |        |
| SMAD2    |     |        |
| β-CATENIN|     |        |
| β-ACTIN  |     |        |

B

![Bar graph showing relative abundance of p-SMAD2, SMAD2, and β-CATENIN.](image)

C

| Activin A | 0 h | 12 h | 24 h | 48 h |
|-----------|-----|------|------|------|
| FOXA2     |     |      |      |      |
| T         |     |      |      |      |
| β-ACTIN   |     |      |      |      |

D

![Graph showing relative expression level of Foxa2, T, and miR-127.](image)

E

| Activin A | NC  | Si-127 |
|-----------|-----|--------|
| p-SMAD2   |     |        |
| SMAD2     |     |        |
| FOXA2     |     |        |
| T         |     |        |
| β-ACTIN   |     |        |

F

![Bar graph showing relative luciferase reporter activity.](image)
Figure 5

A

Mus-miR-127
UCGGUUCGAGUCGUCUGCCUAGGC
Lefty2-3’UTR
CAGTGA ACAGG TTGCAT CTGA
GGG(Mut)

Relative luciferase reporter activity

|            | NC  | mi-127 |
|------------|-----|--------|
| PGL3       | 0.6 | 0.9    |
| Lefty2-3’UTR| 0.6 | 1.2    |
| Lefty2-Mut | 0.9 | 1.1    |


B

miR-127
Lefty2

Relative expression level

D0 EB  | D2 EB  | D4 EB  | D6 EB  |
---     | ---    | ---    | ---    |
--      | --     | --     | --     |


C

Relative mRNA level

NC  | mi-127 |
--- | -------|
0.4 | 0.8     |


D

NC  | mi-127 |
--- | -------|
LEFTY2 |        |
NC  | mi-127 |
--- | -------|
LEF3Y2 |        |
NC  | mi-127 |
--- | -------|
β-ACTIN |       |


E

Foxa2

Relative mRNA level

ES  | NC | mi-127 | mi-127+Lefty2 |
--- | ---|-------|---------------|
5   | 7  | 22    | 22            |

T

Relative mRNA level

ES  | NC | mi-127 | mi-127+Lefty2 |
--- | ---|-------|---------------|
5   | 7  | 22    | 22            |


F

NC  | mi-127 | mi-127+Lefty2 |
--- | -------|---------------|
FOX42 |       |               |
T     |        |               |
LEFTY2 |       |               |
p-SMAD2 |       |               |
SMAD2  |        |               |
GAPDH  |        |               |
Figure 6

A

|        | lft2   | gsc    | chd    |
|--------|--------|--------|--------|
| 0 µM   | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| Dorsal | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| Lateral| ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| 20 µM  | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| Dorsal | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| Animal | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |

B

- **lft2**
  - ![Graph](image19.png)

- **gsc**
  - ![Graph](image20.png)

- **chd**
  - ![Graph](image21.png)
Figure 7

NODAL -> LEFTY2

Cytoplasm

SMAD2 -> Co-SMAD

Nucleus

SMAD2 -> Co-SMAD

miR-127

Lefty2

FOSFA2, T
MicroRNA-127 Promotes Mesendoderm Differentiation of Mouse Embryonic Stem Cells by Targeting Left-right Determination Factor 2
Haixia Ma, Yu Lin, Zhen-Ao Zhao, Xukun Lu, Yang Yu, Xiaoxin Zhang, Qiang Wang and Lei Li

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