miRNA-181 regulates embryo implantation in mice through targeting leukemia inhibitory factor

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Embryo implantation is a crucial step in mammalian reproduction. However, little is known regarding the physiological roles of microRNAs in the regulation of embryo implantation. Here we show that a minimum uterine expression of miR-181 is essential for the onset of embryo implantation. Both transient and prolonged transgenic expression of miR-181 led to impaired implantation, which can be rescued by exogenous administration of leukemia inhibitory factor (LIF). Mechanistically, miR-181 is able to directly target LIF and downregulate LIF expression, thereby inhibiting embryo implantation. We also show that miR-181 expression is regulated by the transcriptional factor Emx2, and the Emx2–miR-181 axis plays an important role in regulating embryo implantation. Taken together, these results reveal a novel function of miR-181 in embryo implantation through the regulation of LIF, and also indicate a potential link between miR-181 dysregulation and human embryo implantation defects.

Keywords: miR-181, LIF, Emx2, embryo implantation

Introduction

In mammals, embryo implantation is a crucial step in the reproductive process (Wang and Dey, 2006). At this stage, the blastocyst becomes closely associated with the maternal endometrial tissues. It is evident that ovarian hormones, including estrogen and progesterone, initiate this process, making the uterus receptive to accept a blastocyst for implantation (Carson et al., 2000; Paria et al., 2002; Dey et al., 2004). In preparation for embryo implantation, estrogen and progesterone act directly and indirectly in promoting distinct cycles of cell proliferation and differentiation in the uterus tissues (Finn and Martin, 1969; Martin et al., 1970, 1973). The indirect actions of these hormones are mediated by the production of growth factors and cytokines such as epidermal growth factor (EGF) and insulin-like growth factors 1 (IGF1) (Ignar-Trowbridge et al., 1992; Hana and Murphy, 1994; Curtis et al., 1996; Adesanya et al., 1999). It has been demonstrated that the cytokine leukemia inhibitory factor (LIF) is absolutely essential for mouse embryo implantation and critical for human embryo implantation (Bhatt et al., 1991; Shen and Leder, 1992; Stewart et al., 1992; Cullinan et al., 1996; Vogiagis and Salamonsen, 1999; Lass et al., 2001). In the mouse, LIF is transiently expressed in the uterine endometrial glands on Day 4 of pregnancy (Bhatt et al., 1991). Female mice lacking a functional LIF gene are fertile, but their blastocysts fail to implant and do not develop, suggesting the critical role of LIF for embryo implantation (Stewart et al., 1992). It has been recently shown that p53 regulates embryo implantation through regulating LIF expression (Hu et al., 2007). Although many factors have been identified and postulated to be involved in the regulation of embryo implantation (Dey et al., 2004; Cha et al., 2012), the underlying molecular mechanisms still remain largely unknown.

MicroRNAs (miRNAs) are 18–24 nucleotide long non-coding RNAs that regulate gene expression at the posttranscriptional level. miRNAs have been implicated in numerous biological processes, including cell proliferation, cell death, cell differentiation, and tumorigenesis (Hwang and Mendell, 2007; Zhang et al., 2007; Rottiers and Naar, 2012); however, less is known with regard to how microRNAs regulate embryogenesis. Despite previous studies reporting that some miRNAs including miR-101a, miR-199a*, miR-21, let-7a, and miR-320 may be important for embryo implantation (Chakrabarty et al., 2007; Hu et al., 2008; Xia et al., 2010a, b), these investigations are mostly correlative studies and little is known regarding the underlying mechanism or physiological significance of miRNAs in the regulation of
embryo implantation.

Results

miR-181 inhibits embryo implantation

To identify novel miRNAs involved in embryo implantation, we performed custom microRNA microarray analysis to compare the miRNA expression profile in mouse uterus between non-pregnant and 4-day pregnant mice. By this analysis, entire miR-181 family members, including miR-181a-1, miR-181a-2, miR-181a, miR-181b, miR-181c, and miR-181d, were revealed to be downregulated in mouse uterus on Day 4 of pregnancy (Supplementary Table S1). The subsequent real-time RT–PCR analysis further confirmed that levels of both primary and mature forms of miR-181a and miR-181b were indeed dramatically decreased on Day 4 of pregnancy (Figure 1A) and miR-181a and miR-181b levels returned back to normal on Day 6 and 7 (Figure 1B), demonstrating the close association of decreased expression of miR-181a and miR-181b with the onset of implantation that occurs in mice in the evening of Day 4 post coitum.

We next evaluated whether miR-181 could regulate embryo implantation. To overexpress miR-181a and miR-181b in mice, mice were given repeated administrations of nanoparticles packed with either miR-181a or miR-181b mimics on Day 2 and 3 of pregnancy as previously described (Liu et al., 2011). This method was used because the nanoparticle has better circulating capability due to its particular surface structure and even more importantly, nanoparticle-packed siRNA enters the cell more easily than naked siRNA (Liu et al., 2011). The effectiveness was confirmed by the fact that siRNA packed with nanoparticles was much more stable than free siRNA when administered into mice (Supplementary Figure S1). After 4 days of pregnancy, miR-181a and miR-181b were efficiently enriched in mouse uterus (Figure 1C), and the number of implanted embryos was significantly lower in miR-181a mimics- and miR-181b mimics-treated mice than control mice (Figure 1D). However, if miR-181a and miR-181b mimics were administered into mice on Day 4 of pregnancy, the embryo implantation was less affected (Figure 1E). Together, these data indicate that miR-181a and miR-181b may inhibit embryo implantation.

To further investigate the possible inhibitory functions of miR-181a and miR-181b in embryo implantation in vivo, we generated miR-181a- and miR-181b-transgenic mice. These transgenic mice indeed expressed high levels of miR-181a/b in uterus during pregnancy than control mice (Figure 2A). Both miR-181a- and miR-181b-transgenic mice showed a decreased pregnancy rate and smaller litter size compared with control mice (Table 1). It has been shown that miRNAs have important roles in pituitary and ovarian functions (Otsuka et al., 2008; Hasuwa et al., 2013). To exclude the possibility that the reproductive defects in miR-181a/b-transgenic mice were due to changes in other organs such as ovary and pituitary, we measured serum levels of estrogen and examined ovarian luteinization in miR-181a/b-transgenic and control mice. The results showed that serum estrogen levels showed no difference in these mice (Supplementary Figure S2A). Also, transgenic expression of miR-181a/b showed little effect on ovarian luteinization (Supplementary Figure S2B). These data suggest that miR-181a/b does not affect pituitary and ovarian functions.

Ovulation, fertilization, embryonic growth, implantation, and decidualization are critical processes involved in early pregnancy. By counting the number of 2-cell embryos flushed from oviducts of miR-181a/b-transgenic and control mice on Day 2 of pregnancy, we found that miR-18a/b overexpression affected neither ovulation nor fertilization (Figure 2B). In vitro culturing of these 2-cell embryos revealed no obvious difference in embryonic growth between miR-181a/b-transgenic and control mice (Figure 2B). Also, transgenic expression of miR-181a/b showed minimal effect on decidualization, as revealed by the slightly lower weight of deciduas from miR-181a/b-transgenic mouse uterus on Day 8 of pregnancy (Figure 2C). By staining the implantation sites in 5-day pregnant mouse uterus with the blue dye, we found that the number of implanted embryos was greatly lower in miR-181a/b-transgenic mice (Figure 2D). Similar results were observed in mouse uterus on Day 7 of pregnancy (Figure 2E). Nevertheless, the number of non-implanted blastocysts in miR-181a/b-transgenic mice was much higher than that in control mice (Figure 2F). By performing embryo transfer experiments, where pseudopregnant miR-181a/b-transgenic and control mice were served as recipients for wild-type embryos, we showed that pseudopregnant miR-181a/b-transgenic mice had less implanted embryos than control mice (Figure 2G). Taken together, these data strongly suggest that miR-181 plays an inhibitory role in the regulation of embryo implantation.

miR-181-impaired embryo implantation is rescued by LIF

It has been well recognized that LIF plays a critical role in embryo implantation. Here, we found that the pattern of LIF mRNA expression was virtually opposite to that of miR-181a and miR-181b expression during pregnancy (Figures 1B and 3A). In addition, administration of miR-181a or miR-181b mimics into mice led to the decreased LIF mRNA and protein levels in their uteri on Day 4 of pregnancy (Figure 3B and C). Similarly, miR-181a/b-transgenic mice exhibited lower LIF expression in uteri on Day 4 of pregnancy than control mice (Figure 3D). These data suggest that miR-181 and LIF may have contrasting roles in the regulation of embryo implantation.

We therefore evaluated whether miR-181 regulated embryo implantation through LIF. It has been shown that embryo implantation can be restored in LIF–/– mice by the administration of exogenous LIF on Day 4 of pregnancy (Chen et al., 2000). To determine whether low expression of uterine LIF in female mice treated with miR-181a/b mimics contributed to the impaired implantation, LIF was administered into these mice on Day 4 of pregnancy. LIF injection indeed significantly improved the embryo implantation in these mice (Figure 3E). Similarly, LIF injection was able to greatly improve the impaired embryo implantation in both miR-181a- and miR-181b-transgenic mice (Figure 3F), suggesting miR-181a and miR-181b inhibit embryo implantation through LIF. Taken together, these results demonstrate that miR-181 lowers the functional levels of uterine LIF, and administration of LIF improves embryo implantation in mice with increased expression of miR-181.
To explore whether miR-181a and miR-181b could directly target LIF and downregulate its cellular levels, we inspected the 3′-untranslated region (3′UTR) of the LIF mRNA and found that it contained a site (nucleotides UGAAUGU) that could be recognized by miR-181a and miR-181b (Figure 4A). The luciferase reporter constructs containing either wild-type or mutant 3′UTR of LIF were then transfected into H1299 cells, which had high transfection efficiency.

Figure 1 Treatment with miR-181 mimics decreases the number of implanted embryos. (A) Real-time RT–PCR analysis of the expression of primary and mature forms of miR-181 in mouse uterus on Day 0 and 4 of pregnancy. The day on which the plug was observed was considered to be Day 1 of pregnancy. Female proestrus mice were used as non-pregnant controls. (B) Real-time RT–PCR analysis of the expression of mature forms of miR-181a and miR-181b in mouse uterus on Day 0, 4, 6, and 7 of pregnancy. Female proestrus mice were served as non-pregnant controls. (C) miR-181a or miR-181b mimics were repeatedly administrated into mice on Day 2 and 3 of pregnancy. Uteri were collected on Day 4 of pregnancy and analyzed for RNA levels of miR-181a and miR-181b. (D) miR-181a or miR-181b mimics were repeatedly administrated into mice on Day 2 and 3 of pregnancy. Uteri were collected on Day 7 of pregnancy. The number of implanted embryos was counted. (E) miR-181a or miR-181b mimics were repeatedly administrated into mice, with a 12-h interval, on Day 4 of pregnancy. Uteri were collected on Day 7 of pregnancy. The number of implanted embryos was counted. Data are mean ± SD from three, if not otherwise specified, independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

miR-181 downregulates LIF expression

To explore whether miR-181a and miR-181b could directly target LIF and downregulate its cellular levels, we inspected the 3′-untranslated region (3′UTR) of the LIF mRNA and found that it contained a site (nucleotides UGAAUGU) that could be recognized by miR-181a and miR-181b (Figure 4A). The luciferase reporter constructs containing either wild-type or mutant 3′UTR of LIF were then transfected into H1299 cells, which had high transfection efficiency.
Figure 2 Embryo implantation is impaired by transgenic expression of miR-181. (A) The real-time RT–PCR analysis showed that miR-181 RNA levels were indeed higher in the uterus of miR-181-transgenic mice than that of control mice during pregnancy. (B) Wild-type and miR-181-transgenic mice were superovulated before they were individually housed with a fertile male mouse. Zygotes were then collected from oviducts and cultured in vitro. The embryo development was evaluated by counting the numbers of 2-cell, 4-cell, and morula embryos. (C) Uteri from wild-type and miR-181-transgenic mice were collected on Day 8 of pregnancy. The deciduas were then weighed. (D) Uteri from miR-181-transgenic and control mice in the evening of Day 5 of pregnancy were collected and stained with the blue dye. Representative images are shown. (E) Uteri from miR-181-transgenic and control mice on Day 7 of pregnancy were collected. Pictures of these uteri were taken and representative images are shown. (F) Uteri from wild-type and miR-181-transgenic and control mice on Day 7 of pregnancy were collected. The number of implanted embryos was counted. Data are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
that allowed to obtain clean and consistent data. Either miR-181a or miR-181b was able to reduce luciferase expression from wild-type but not mutant reporter plasmid (Figure 4B and C). Furthermore, in Jurkat cells that efficiently express LIF protein, treatment with miR-181a/b inhibitors resulted in elevated LIF protein levels, whereas miR-181a/b mimics showed the opposite effect (Figure 4D). It has been reported that p53-deficient mice exhibit implantation failure due to the reduction of LIF expression.

To exclude the possibility that miR-181a/b regulated LIF expression through p53, Jurkat cells expressing control shRNA or p53 shRNA were transfected with miR-181a/b mimics, followed by western blot analysis to examine LIF expression levels. The results showed that miR-181a/b was able to decrease LIF expression in both p53 knockdown and control cells (Figure 4E), indicating that the regulation of LIF expression by miR-181a/b is independent of p53.

To further confirm that miR-181a/b inhibited embryo implantation through directly targeting LIF, we utilized the miR-181a/b mutant mimics in which the seed region paired with LIF 3′UTR was mutated (UUA to CCC) (Figure 4F). The miR-181a/b mutant mimics packed by nanoparticles were repeatedly administrated into mice on Day 2 and 3 of pregnancy. The results showed that embryo implantation was not affected by miR-181a/b mutant mimics (Figure 4F), although miR-181a/b mimics exhibited inhibitory effect on embryo implantation (Figure 1D). Taken together, these data indicate that miR-181 is able to downregulate LIF expression, therefore inhibiting the embryo implantation process.

**miR-181 is transcrionally regulated by Emx2**

To investigate the mechanism by which miR-181a/b was regulated during early pregnancy, we first inspected the genomic sequence upstream of genes coding for miR-181a1/b1 and miR-181a2/b2 by using the genomatix suite of sequence analysis tools (MatInspector). Our sequence analysis revealed the presence of a putative Emx2-binding site in both miR-181a1/b1 and miR-181a2/b2 promoter region (Figure 5A). Emx2 is located outside of Hox cluster and plays an important role in nerves system and embryo development (Kastury et al., 1994). In human uterus, the expression of Emx2 is downregulated at the time of endometrial remodeling and blastocyst implantation (Daftary and Taylor, 2004). Overexpression of Emx2 in mice reduces litter size, while downregulation of Emx2 is necessary for embryo implantation (Taylor and Fei, 2005), indicating the critical role of Emx2 in the regulation of embryo implantation. The Chromatin immunoprecipitation (ChiP) assays verified the association of endogenous Emx2 and the chromatin fragments corresponding to the Emx2-binding elements in miR-181a1/b1 and miR-181a2/b2 promoters (Figure 5B). In addition, we evaluated whether the Emx2-binding elements within the miR-181a1/b1 and miR-181a2/b2 promoter region confer Emx2-dependent transcriptional activation. DNA fragments containing either wild-type or mutant Emx2-binding element were inserted into the promoter region of a firefly luciferase reporter plasmid. Emx2 was able to induce luciferase expression from wild-type but not mutant reporter plasmid in MCF7 cells (Figure 5C and D). Furthermore, ectopic expression of Emx2 increased, whereas knockdown of Emx2 decreased levels of both primary and mature forms of miR-181a/b (Supplementary Figure S4A and B) in MCF7 cells. Administration of Emx2 siRNA to mice also decreased miR-181a/b levels in their uteri (Figure 5E). Collectively, these data demonstrate that miR-181a/b is transcriptionally regulated by Emx2. More importantly, similar to the expression pattern of miR-181a/b during pregnancy (Figure 1B), uterine Emx2 levels were decreased on Day 4 of pregnancy, but recovered rapidly on Day 7 of pregnancy (Figure 5F). These results suggest that the Emx2–miR-181a axis plays an inhibitory role in regulating embryo implantation.

To further determine the functional correlation among Emx2, miR-181, and LIF during early pregnancy, we performed immunohistochemistry and *in situ* hybridization experiments that examined localization and expression of LIF, miR-181a/b, and Emx2 in uteri of WT and miR-181a/b-transgenic mice from Day 0 to 5 of pregnancy. Consistent with previous reports (Bhatt et al., 1991; Yang et al., 1995), LIF was expressed in the luminal epithelium on Day 1 and glandular epithelium on Day 4 of pregnancy (Supplementary Figure S4A). miR-181 and Emx2 were expressed in both luminal epithelium and glandular epithelium of both pregnant and non-pregnant mice (Supplementary Figure S4B and C). As expected, miR-181 and Emx2 were indeed decreased in the gland on Day 4 of pregnancy (Supplementary Figure S4B and C). The quantitative results from real-time RT–PCR analysis consistently showed that LIF expression was gradually increased, whereas miR-181a/b and Emx2 levels were concurrently decreased in mouse uterus from Day 1 to 4 of pregnancy (Supplementary Figure S4D). Taken together, these data indicate the importance of the Emx2–miR-181a/b–LIF axis in the regulation of embryo implantation.

**Discussion**

The current study identifies an important function of miR-181 in the regulation of embryo implantation, in which LIF functions as the downstream mediator of miR-181 in mice. Decreased expression of miR-181 on Day 4 of pregnancy is essential for the onset of embryo implantation. Either transient or prolonged transgene expression of miR-181 results in the impaired ability of embryos to implant, which can be rescued by exogenous LIF injection.

Embryo implantation is a critical step for the establishment of a normal pregnancy. In rodents and humans, the onset of implantation is marked by the close interaction between the trophoblast and the apical surface of the luminal epithelium. The control of embryo implantation is primarily maternal with estrogen and progesterone regulating the expression of various cytokines and transcriptional factors. For instance, in the mouse, uterine expression of LIF is shown to be upregulated by estrogen and LIF can replace

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**Table 1 miR-181-transgenic mice exhibit smaller litter size and lower pregnancy rate compared with control mice.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Litter size</th>
<th>Pregnancy rate (%)</th>
<th>Number of breeding pairs</th>
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<tbody>
<tr>
<td>WT</td>
<td>8.8 ± 0.86</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>miR-181a</td>
<td>4.1 ± 1.04***</td>
<td>86.7</td>
<td>15</td>
</tr>
<tr>
<td>miR-181b</td>
<td>4.2 ± 0.5B***</td>
<td>85.7</td>
<td>14</td>
</tr>
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***p < 0.001.
miR-181 inhibits embryo implantation

Figure 3 miR-181-impaired embryo implantation can be rescued by LIF. (A) Real-time RT–PCR analysis of the expression of LIF in mouse uterus on Day 0, 4, 6, and 7 of pregnancy. Female proestrus mice were served as non-pregnant controls. (B and C) miR-181a or miR-181b mimics were repeatedly administrated into mice on Day 2 and 3 of pregnancy. Uteri were collected on Day 4 of pregnancy and analyzed for RNA (B) and protein (C) levels of LIF. Blots were quantified by using the Gel-pro analyzer software. (D) LIF protein levels in the uteri of miR-181-transgenic and control mice on Day 4 of pregnancy were analyzed by western blot analysis. Representative blots of three biological replicates are shown. Blots were quantified by using the Gel-pro analyzer software. (E) LIF was injected on Day 4 of pregnancy after repeated administrations of miR-181a or miR-181b mimics into mice on Day 2 and 3 of pregnancy. Uteri were collected on Day 7 of pregnancy. Pictures of these uteri were taken and representative images are shown (n=5). The number of implanted embryos was counted. (F) The miR-181-transgenic mice were injected with LIF on Day 4 of pregnancy. Uteri were collected on Day 7 of pregnancy. Pictures of these uteri were taken and representative images are shown. The number of implanted embryos was counted. Results are represented as means ± SD from three, if not otherwise specified, independent experiments. **P < 0.01, ***P < 0.001.

Kidnatory estrogen for the onset of embryo implantation (Bhatt et al., 1991; Yang et al., 1996; Chen et al., 2000). These findings suggest a close link between estrogen and the burst LIF expression in the endometrial epithelium at the time of embryo implantation. However, the underlying mechanisms of estrogen-regulated LIF expression remain elusive. In this study, we find that miR-181 directly targets LIF and downregulates LIF expression. Since miR-181a has been shown to be suppressed by estrogen (Maillot et al., 2009), our data indicate miR-181 as a central node that mediates estrogen-induced LIF expression. We also show that miR-181 expression is controlled by the transcriptional factor Emx2. Given the previous finding that Emx2 is
Figure 4 LIF is a direct target of miR-181. (A) Illustration of base pairing between miR-181 and the 3′ UTR of LIF. Substitution of AAU with CCC for the mutant reporter construct is also shown. (B) Schematic illustration of pSi-CHECK-based luciferase reporter constructs used for examining the effect of miR-181 on the 3′ UTR of LIF. (C) miR-181a or miR-181b mimics were transfected into H1299 cells together with the indicated reporter constructs. Twenty-four hours after transfection, reporter activity was measured and plotted after normalization to Renilla luciferase activity. (D) Jurkat cells were treated with miR-181 mimics and miR-181 inhibitors as indicated. Thirty-six hours later, cell lysates were analyzed for LIF protein expression by western blot. Representative blots of three biological replicates are shown. Blots were quantified by using the Gel-pro analyzer software. (E) Jurkat cells expressing control or p53-specific shRNA were transfected with miR-181a/b mimics. Thirty-six hours later, cell lysates were analyzed by western blot to evaluate LIF protein levels. Representative blots are shown. (F) miR-181a or miR-181b mutant mimics were repeatedly administered into mice on Day 2 and 3 of pregnancy. Uteri were collected on Day 7 of pregnancy. Pictures of these uteri were taken and representative images are shown. The number of implanted embryos was counted. Data are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
downregulated by estrogen (Troy et al., 2003), it is conceivable that in response to the elevated levels of estrogen, the transcription factor Emx2 is suppressed, leading to the decreased expression of miR-181. This Emx2-mediated downregulation of miR-181 relieves its inhibitory effect on LIF, thereafter increasing expression levels of LIF to facilitate embryo implantation (Figure 5G).

miRNAs belong to a class of small non-coding RNAs that regulate numerous biological processes by paring with the 3′UTR of mRNAs and inhibiting their translation (Bartel, 2009). Increasing evidence suggests that miRNAs have a regulatory role in embryo implantation. Our finding that decreased miRNA-181 expression is required for the onset of embryo implantation reinforces the important functions of miRNAs in regulating this process. However, we should mention that the impaired embryo implantation phenotype is obtained in the transgenic mice systemically expressing miR-181a/b. Therefore, further study using the transgenic mice with uterus-specific expression of miR-181 is needed to confirm the important function of miR-181 in the regulation of embryo implantation.

Implantation failure is considered as the most common cause of pregnancy loss after in vitro fertilization (IVF) and embryo transfer (Kay et al., 2006). Sufficient uterine expression of LIF is essential for

**Figure 5** miR-181 expression is regulated by Emx2. (A) Putative Emx2 consensus binding elements located 3410–3393 bp upstream of pre-miR-181a1/b1 and 3550–3440 bp upstream of pre-miR-181a2/b2. (B) Lysates from MCF-7 cells were subjected to ChIP assay with anti-Emx2 antibody. ChIP products were examined by real-time RT–PCR analysis. (C) pGL3-basic-based wild-type and mutant reporter constructs used for luciferase assays to examine the transcriptional activity of the Emx2-binding elements. (D) Emx2 transactivates Emx2 consensus binding elements in miR-181 gene. MCF-7 cells were cotransfected with the indicated reporter plasmids together with GFP-Emx2 or GFP control. Twenty-four hours after transfection, reporter activity was measured and plotted after normalization to Renilla luciferase activity. (E) Emx2-specific or control siRNA packed with nanoparticles was intraperitoneally administered into female proestrus mice. Twenty-four hours later, uteri were collected. miR-181 and Emx2 expression levels in these uteri were analyzed by real-time RT–PCR. (F) Protein levels of Emx2 in mouse uterus on Day 0, 4, and 7 of pregnancy were determined by western blot analysis. Female proestrus mice were used as non-pregnant controls. Representative blots of three biological replicates are shown. Blots were quantified by using the Gel-pro analyzer software. Data are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (G) A proposed model illustrating the role of the Emx2–miR-181–LIF pathway in the regulation of mouse embryo implantation. In this model, Emx2-mediated miR-181 expression directly targets LIF and downregulates LIF expression, thereafter inhibiting embryo implantation.
embryo implantation, while deregulated LIF production has been reported in infertile women (Hambartsoumian, 1998; Vogeis and Salamonsen, 1999). A recent study reported the differential expressions of miRNAs between RIF-IVF (repeated implantation failure—in vitro fertilization) patients and fertile women (Revel et al., 2011), implicating the possible role of miRNAs in the regulation of human embryo implantation. Given the strict regulation of LIF expression by miR-181, it would be important to conduct a future investigation for whether upregulation of miR-181 is associated with human embryo implantation defects.

**Material and methods**

**Cell culture and reagents**

To isolate mouse endometrial epithelium cells, the uterus of mouse on Day 4 of pregnancy was cut lengthwise into 1–2 mm pieces and subjected to enzymatic digestion using collagenase I (Invitrogen). After enzymatic digestion for 30 min at 37°C, cells were dispersed through a 150 μm sieve. The cells were then collected by centrifugation at 500 g for 10 min. Jurkat, EL4, MCF7, and HeLa cells by Oligofectamine (Invitrogen) according to the manufacturer’s instruction.

**miRNA mimics and inhibitors**

miR-181 mimics and inhibitors were purchased from GenePharma Company. These miRNA mimics and inhibitors were transfected into Jurkat, EL4, MCF7, and HeLa cells by Oligofectamine (Invitrogen) according to the manufacturer’s instruction.

**ChIP**

MCF7 cells expressing GFP-Emx2 or GFP control protein were cross-linked with 1% formaldehyde for 15 min. ChIP assays were performed by using anti-GFP and the ChIP kit (Millipore) according to the manufacturer’s instructions. Anti-rabbit IgG (Promega) were used as controls. The bound DNA fragments were subjected to PCR using the following primer pairs: miR-181a1 bs 5′-CTTTCTTCTTCTAGTGAGAACATGTC-3′ and 5′-CTCCAGCTGCGACTATTACCTC-3′; miR-181a2 bs 5′-GAGAGATTCCATATGCGGTTTTGATGAT-3′ and 5′-GACATATTTGGAATTAAGTGCCTCAC-3′. PCR products were separated by gel electrophoresis on a 2% agarose gel.

**Real-time RT–PCR**

Total RNA was isolated by Trizol (Invitrogen). One microgram of total RNA was used to synthesize cDNA using PrimerScript™ RT Reagent Kit (TakaRa, DRR037A) according to the manufacturer’s instruction. The reverse transcription primers were as follows:

- miR-181a, 5′-GTCTTATCCAGTGCGGTCTGGAAGTGCGAACCTTCA-3′; miR-181b, 5′-GTCGATACGACACACTC-3′.

Real-time PCR was performed using SYBR premix EX Taq (TakaRa) and ROX and analyzed with Stratagene MX3000p (Agilent Technologies). Real-time PCR primer sequences were as follows: U6, 5′-CGTTTCAGATTTGGTCTCAT-3′; pri-miR-181a1, 5′-GGTTCCTCAGGTACATCAACGC-3′ and 5′-GTTGACGTATGGTGACATACCAAGGTCT-3′; pri-miR-181a2, 5′-CCATTGACATCAACGCCTGGC-3′ and 5′-CCGACCGTTGTGACTGCTCTTGG-3′; pri-miR-181b1, 5′-GACGACATTAGTGCAATCACC-3′ and 5′-GACGAGCTGCAATCACC-3′; pri-miR-181b2, 5′-GACGACATTGGAATCGTCTGTGGTG-3′ and 5′-GACGACATTGGAATCGTCTGTGGTG-3′; pri-miR-181b3, 5′-GACGACATTGGAATCGTCTGTGGTG-3′ and 5′-GACGACATTGGAATCGTCTGTGGTG-3′.

**Luciferase reporter assay**

To investigate whether miR-181a/b targets LIF, LIF-3′UTR and LIF-3′UTR-mut were constructed into psi-CHECK2 reporter plasmid (Promega). These plasmids were transfected into H1299 cells together with miR-181a/b mimics or miR-181a/b inhibitors as indicated. Firefly and Renilla luciferase activities were analyzed by Dual-Luciferase Reporter Assay System according to the manufacturer’s instruction (Promega). To determine the effect of Emx2 on miR-181a/b promoter, the miR-181a/b promoter containing the putative Emx2-binding element was cloned into pGL3-based reporter plasmid. pGL3-miR-181a/b promoter was then transfected into MCF7 cells together with the indicated plasmids. Firefly and Renilla luciferase activities were measured. The relative luciferase activity was calculated by normalizing the firefly luciferase activity to Renilla luciferase activity.

**Immunohistochemistry**

Mouse tissues were dissected into pieces, fixed with 4% paraformaldehyde (PFA), and embedded in paraffin wax. The paraffin-embedded sections were heat in 10 mM sodium citrate buffer (pH 6.0) at 100°C for 10 min, followed by incubation in PBS containing 3% H2O2 and 0.1% Triton X-100 for 30 min. The sections were then blocked with 10% normal donkey serum (Jackson ImmunoResearch Labs, Inc.) for 1 h at room temperature, treated with anti-LIF or anti-EMX2 antibody overnight at 4°C, and incubated with biotinylated secondary antibody (Abcam) for 2 h at room temperature. The sections were eventually developed with a DAB kit.

**In situ hybridization**

To investigate the distribution of miRNA-181 in the uterus, in situ hybridization was performed with Digoxin-labelled DNA oligonucleotides (LNAs) complementary to the mature miRNAs. The probe sequences were as follows: miRNA-181a, 5′-ACCTGAGCTGACATAATTGIC-3′ and miRNA-181b, 5′-ACCCACGACAGCAAAGTTAGGT-3′. In situ hybridization in mouse tissues were performed essentially as described by Obernosterer et al. (2007).

**Mouse experiments**

All mouse experiments were carried out in accordance with the
guidelines from the Institutional Animal Care and Use Committee. C57BL/6 mice were used for breeding assays. To evaluate the effect of transgenic overexpression of miR-181a/b on embryo implantation, miR-181a/b-transgenic male and female mice were placed in cages for breeding, and female mice were checked every morning for copulation plugs and signs of parturition. The day on which the plug was observed was considered to be Day 1 of pregnancy. Uteri were collected on Day 7 of pregnancy and the number of implanted embryos was counted. Pregnancy rate was calculated as the ratio of the number of pregnant female mice to the number of female mice housed with male mice. To overexpress miR-181a or miR-181b in mouse uterus, nanoparticles packed with miR-181a or miR-181b were repeatedly administered to female mice by intraperitoneal injection on Day 2 and 3 of pregnancy. LIF (Millipore) was injected to mice as previously described (Chen et al., 2000). Uteri were collected on Day 7 of pregnancy and the number of implanted embryos was counted.

**Estrous cycle monitoring**

To determine the estrous cycle phases in mice, vaginal smears were examined. Vaginal smears were taken by using pipette tips to instill 10 μl phosphate-buffered saline (PBS) into the mouse vagina. The aspirated PBS and vaginal secretion were applied to glass slides. Smears were dried and stained with 0.1% toluidine blue solution for 5 sec, followed by the investigation under a light microscope.

**Male mouse vasectomy**

C57BL/6 male mice were anesthetized with intraperitoneal injection of 1% barbitral sodium. The fur on the lower abdomen was shaved and the skin was sprayed with 70% ethanol. Mice were then vasectomized via an abdominal surgical approach. Briefly, a 1-cm wide incision was made through the skin and the abdominal body wall. The vasa deferentia were identified and the loop of each vas deferens was removed using cautery. The skin and the body wall were sutured, and the mice were allowed to recover for at least 2 weeks before mating.

**Embryo collection and in vitro culture**

WT, miR-181a-, and miR-181b-transgenic female mice were superovulated by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) (10 IU per mouse) followed by injection of human chorionic gonadotropin (HCG) (10 IU per mouse) at 46–48 h later. Female mice were then individually housed with a fertile male mouse, and sacrificed in the following morning upon detection of a vaginal plug. Oviducts were isolated and transferred to a drop of M2 medium containing 1 mg/ml hyaluronidase to dissociate the cumulus mass. Zygotes were collected and washed with KSOM medium (Millipore) to remove excess hyaluronidase. Zygotes were then placed into drops of KSOM medium under light mineral oil (SAGE) and cultured at 37°C, 5% CO2 in a humidified atmosphere to examine the embryo development.

**Blastula flush**

WT, miR-181a-, and miR-181b-transgenic female mice were superovulated before isolating uteri of these mice on Day 4 of pregnancy. The blastulas were then collected by flushing them from the horn of uterus with 1× PBS buffer, and counted under a light microscope.

**Embryo transfer**

Pseudopregnant miR-181a/b-transgenic and WT female mice were served as recipients for WT embryos on Day 2 post coitus. Pseudopregnancy was induced by mating recipients with vasectomized WT male mice. The oviduct was then exposed through a paramo- lumbar incision before transferring cultured 2-cell embryos (20 embryos per recipient) into the oviduct of each recipient. Female mice were allowed to recover and were sacrificed on Day 9 post coitus to examine the number of implanted embryos.

**Generation of miR-181-transgenic mice**

miR-181a and miR-181b DNAs were amplified using Platinum Pfx DNA polymerase (Invitrogen), and then cloned into the pCAG plasmid. pCAG-miR-181a and pCAG-miR-181b plasmids were linearized by I-CEUI and separately injected into the zygote pronucleus, which was then transplanted into the oviducts of the surrogate female mice. Ten days after the pups were born, mouse-tale genotyping was performed by PCR analysis to confirm transgenic mice carrying the correct gene of interest using the following primer pairs: miR-181a, forward 5′-GGCACCTTTGAAATGTAAT-3′ and reverse 5′-CTGTGTCAAGAAGAATCGG-3′; miR-181b, forward 5′-GGCACCTTTGAAATGTAAT-3′ and reverse 5′-CTGTGTCAAGAAGAATCGG-3′.

**Statistical analysis**

Statistical analysis was carried out in Microsoft Excel and GraphPad Prism to assess differences between experimental groups. Statistical significance was analyzed by Student’s t-test and expressed as P-value. P-values lower than 0.05 were considered to be statistically significant.

**Supplementary material**

Supplementary material is available at Journal of Molecular Cell Biology online.

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