MICRORNA EXPRESSION AND REGULATION IN MOUSE UTERUS DURING EMBRYO IMPLANTATION

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MicroRNAs (miRNAs) are 21-24 nucleotide non-coding RNAs and found in diverse organisms. Although hundreds of miRNAs have been cloned or predicted, only very few miRNAs have been functionally characterized. Embryo implantation is a crucial step in mammalian reproduction. Many genes have been shown to be significantly changed in mouse uterus during embryo implantation. However, miRNA expression profile in mouse uterus between implantation sites and inter-implantation sites are still unknown. In this study, miRNA microarray was used to examine differential expression of miRNAs in mouse uterus between implantation sites and inter-implantation sites. Compared to inter-implantation sites, there were 8 up-regulated miRNAs at implantation sites, which were confirmed by both Northern blot and in situ hybridization. miR-21 was highly expressed in the subluminal stromal cells at implantation sites on day 5 of pregnancy. Since miR-21 was not detected in mouse uterus during pseudopregnancy and under delayed implantation, miR-21 expression at implantation sites was regulated by active blastocysts. Furthermore, we showed that Reck was the target gene of miR-21. Our data suggest that miR-21 may play a key role during embryo implantation.

Introduction

The implantation of the blastocyst into the maternal uterus is a crucial step in mammalian reproduction and involves a reciprocal interaction between the blastocyst and receptive uterus (1). Many factors are involved in embryo implantation through endocrine, paracrine, autocrine, and/or juxtacrine actions (2). Impaired uterine receptivity is one of the major reasons for the failure of assisted reproductive techniques (3). Defects in implantation and trophoblast invasion are presently considered the major challenges for the successful establishment of pregnancy (4). Although many specific factors have been identified during the implantation period, the molecular mechanism of embryo implantation is still unknown.

MicroRNAs (miRNAs) are 21-24 nucleotide non-coding RNAs and found in diverse organisms from plants to humans (5,6). In animals, mature miRNAs are incorporated into the RNA inducing silencing complex (RISC), and target specific messenger RNAs via imperfect base pairing for translational repression or mRNA cleavage (7). Dicer is a cytoplasmic multi-domain RNase III enzyme that processes the pre-miRNA to produce mature miRNAs. Dicer-deficient embryos showed a retarded phenotype and died between days 12.5 and 14.5 of gestation due to angiogenesis defects (8). The growing oocytes from the mice with a specific deletion of Dicer failed to progress through the first cell division, probably because of disorganized spindle formation (9).

It is currently estimated that miRNAs account for approximately 1% of predicted genes in higher eukaryotic genomes and that up to 30% of genes might be regulated by miRNAs. Although...
hundreds of miRNAs have been cloned and/or predicted, only very few miRNAs have been functionally characterized and the general functions of miRNAs are not globally studied (10). Through the analysis of mRNA expression by microarray and SAGE, many genes are shown to be significantly changed in mouse uterus between implantation sites and inter-implantation sites (11,12). However, miRNA expression profile in mouse uterus between implantation sites and inter-implantation sites is still unknown. Locked nucleic acid (LNA)-modified capture probes enable high affinity hybridizations and can discriminate between single nucleotide differences in closely related miRNA family members (13). LNA-modified microarrays were used to distinguish miRNA expression patterns in mouse uterus at implantation sites in this study. The expression and regulation of target genes of differentially expressed miRNAs were also investigated.

MATERIALS AND METHODS

Animal treatments

Mature mice (Kunming White outbred strain) were maintained in a controlled environment with a 14-h light/10-h dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Xiamen University. Female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1 is the day of vaginal plug). The implantation sites on day 5 of pregnancy were identified by intravenous injection of 0.1 ml of 1% Chicago blue (Sigma). The implantation sites and inter-implantation sites on day 5 of pregnancy were collected and stored at -80 °C ready for miRNA microarray analysis.

For delayed implantation, pregnant mice were ovariecctomized under ether anesthesia at 0830-0900 h on day 4 of pregnancy. Delayed implantation was maintained from days 5-7 by injecting progesterone (1 mg/mouse, Sigma). Estradiol-17β (25 ng/mouse, Sigma) was given to progesterone-primed delayed implantation mice to initiate delayed implantation. The mice were sacrificed to collect uteri 24 h after estrogen treatment. Delayed implantation was confirmed by flushing the blastocysts from one horn of the uterus.

Treatments with steroid hormones were started 2 weeks after ovariectomy. The ovariecctomized mice were treated with an injection of estradiol-17β (100 ng/mouse) or progesterone (1 mg/mouse) for 24 h. All steroids were dissolved in sesame oil and injected subcutaneously. Controls received the vehicle only (0.1 ml/mouse).

miRNA microarray

Small RNAs were isolated from mouse uteri at implantation sites and inter-implantation sites using PureLink™ miRNA Isolation Kit (Invitrogen) and labeled with Cy3 dye using miRCURY™ LNA Array labeling kit (Exiqon, Denmark) as per the manufacturer’s instructions. The Cy3-labeled RNA molecules were hybridized with miRCURY™ LNA Arrays (Exiqon, Denmark), consisting of control probes, mismatch probes, and 427 capture probes, perfectly matched probes for all human, mouse and rat miRNAs as registered and annotated in the miRBase release 7.1 at The Wellcome Trust Sanger Institute. Gene Pix 4000B scanner and GenePix Pro 6.0 software (Axon Instruments, Union city, CA) were used to scan images. Each group was hybridized with three miRCURY™ LNA Arrays in triplicate with independent samples for implantation or inter-implantation sites, respectively.

The median intensities for each feature and the corresponding background were calculated. After the median intensity of the background was subtracted from the median intensity of the feature, the net intensity values were normalized to per-chip median values. As a result, each chip had a median value of 400. These normalized intensity values were then used to obtain geometric means of each miRNA. Arithmetic means and standard errors for two groups in triplicate were calculated and fold changes were measured. Each miRNA signal was transformed to logarithm base 2 and 2-sample t-test was conducted. miRNAs with a significant value of 0.05 or lower and fold change value of 2 or higher were listed and supposed to be differentially expressed between implantation sites and inter-implantation sites. Statistical analysis was conducted by MATLAB 7.0 (The MathWorks, 2005).
In situ hybridization

Uteri were cut into 4-7 mm pieces, flash frozen in liquid nitrogen and stored at -80 °C. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxysilane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. In situ hybridization was performed as previously described (14). All the Digoxigenin-labeled LNA probes for each miRNA were purchased from Exiqon, Denmark. The hybridization temperature for each probe was 21 °C below the Tm (calculated melting temperature of the miRCURY™ detection probe). Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). All of the sections were counterstained with 1% methyl green. The positive signal was visualized as a dark brown color.

For performing in situ hybridization of Reck mRNA expression, total RNAs from mouse uterus were reverse-transcribed and amplified with the corresponding primers for mouse Reck (5'-ACTATGTGCTGGGATGTTACGG and 5'-GATGACGGGAGACTGCTGGA, Accession No. NM_016678). The amplified fragment was cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega, Madison, WI) and verified by sequencing. The recombinant plasmid was amplified with the primers for T7 and SP6 to prepare templates for labeling sense or antisense probes. Digoxigenin-labeled Reck antisense or sense cRNA probe was transcribed in vitro using DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). In situ hybridization was performed as above-mentioned and previously described (14).

Northern blot

Small RNAs were isolated from mouse uteri using the mirVana™ miRNA Isolation Kit (Ambion, Am1560). One microgram of small RNAs was resolved on 15% acrylamide/8 M urea gel, transferred onto nylon membranes and UV cross-linked. Digoxigenin-labeled LNA probe for each miRNA was purchased from Exiqon, Denmark. Hybridization was performed in the hybridization solution (BioDev, MK161-2, Beijing, China) at 42 °C for 16 h. The membranes were washed in 2×SSC/0.1% SDS at 42 °C twice 5 min each and in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20) at room temperature for 5 min. After blocked in 1% Blocking reagent (Roche) for 30 min, the membranes were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase for 30 min. Following washing in washing buffer and detection buffer (0.1 Tris, 0.1 M NaCl, pH 9.5), the membranes were incubated in CDP-Star (Roche), exposed and developed. U6 RNA was used as loading control for normalization.

Dual-luciferase activity assay

For luciferase reporter experiments, the 3'-UTR segment of mouse Reck predicted to interact with miR-21 was amplified by PCR from mouse cDNA and inserted into the pGL3 control vector (Promega), using the Fse I and XbaI site immediately downstream from the stop codon of luciferase. Primers used for amplifying 3'-UTR of mouse Reck were 5'-CAGCTTAGATGTGCTGACTTCTTTGATA and 5'-ACTGGCCGGCTATTGCTATATTTCCAGAGGGTA(Accession No. NM_016678), in which Fse I and XbaI sites were underlined, respectively. The recombinant pGL3 was designated as Reck-pGL3. pRL-TK containing Renilla luciferase was co-transfected with Reck-pGL3 for data normalization.

Mouse 3T3 cells were pre-plated in 24-well tissue culture plates at a concentration of 3 × 10^4 cells per well and cultured in DMEM with 10% FBS. The next day 3T3 cells were transfected with 40 ng of pRL-TK and 200 ng of Reck-pGL3 vector. These 3T3 cells were co-transfected with miR-21 precursor (pre-miR-21), the negative control of Pre-miR™ miRNA Precursor, miR-21 inhibitor (anti-miR-21) or Anti-miR™ Negative Control (Ambion), respectively. All the transfections were performed with Lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Invitrogen). Cell lysate was collected and assayed 30 h after transfection. Firefly and Renilla luciferase activities were measured using a Dual Luciferase Reporter Assay System (Promega) and each transfected well was assayed in triplicate.
**Real time RT-PCR**

Total RNAs were extracted from mouse uteri or cultured cells with TRIZOL reagent, digested with DNase I and reverse-transcribed into cDNA with PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaLa, Dalian, China). There were 0.5 μg total RNAs, 2 μl PrimeScript Buffer, 25 pmol Oligo dT Primer, 50pmol Random 6 mers, and 0.5 μl PrimeScript RT Enzyme Mix I in 10 μl volume. Reverse transcription was performed at 37 °C for 15 min and 85 °C for 5 sec. Real-time PCR was performed using a SYBR Premix Ex Taq™ kit (TaKaLa DRR041S) on the Rotor-Gene 3000A system (Corbett Research, Mortlake, VIC, Australia) for 95 °C for 10 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 34 sec. All reactions were run in triplicate. Primers used for real-time RT-PCR were listed in Table 1. Mouse rPL7 gene was amplified as a reference gene for normalization.

**Primary culture of uterine stromal cells**

Uterine horns from estrous mice were cleaned of fat tissues, slit longitudinally, and washed thoroughly in Hanks’ balanced salt solution (HBSS, sigma) without Ca²⁺/Mg²⁺ and phenol red but containing 100 U/ml penicillin (Gibco-BRL) and 100 μg/ml streptomycin (Gibco-BRL). Tissues were then placed in 5 ml of fresh medium (HBSS with antibiotics) containing 6 mg/ml dispase (Gibco-BRL) and 10 mg/ml trypsin (Sigma), and incubated in sequence for 1 h at 4 °C, 1 h at room temperature, and then 10 min at 37°C. Following the digestion steps, tissues were shook several times to dislodge the sheet of luminal epithelial cells. After the supernatant containing epithelial cells was discarded, the tissues remaining after the digestion were washed three times in fresh medium and digested in HBSS containing 0.15 mg/ml collagenase (Gibco-BRL) at 37°C for 30 min. Following digestion and shaking, the digested cells (mainly for stromal cells) were passed through a 70-μm filter to eliminate epithelial sheets and centrifuged. The cell pellets were washed twice with HBSS and re-suspended in phenol red-free Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS (Gibco-BRL) and antibiotics. Cells were plated at 4×10⁵ cells per 25 square cm dish. After an initial culture for 1 h, the medium was changed to remove free-floating cells and replaced with DMEM containing 10% FBS.

**Reck over-expression**

When cultured uterine stromal cells reached 80% confluent, transfection was performed with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions. Cells were transfected with RECK over-expression vector pCMVβ-RECK (kindly provided by Dr Hansoo Kim, Seoul National University, Korea) in 250 μl of Opti-MEM I Reduced Serum Medium without serum. pcDNA3.1(+) was used for control. Medium was changed 6 h later. Cells were cultured in DMEM containing 10% FBS for 24 h and then in serum-free DMEM for additional 24 h. RECK over-expression was confirmed by RT-PCR and Western blot. Conditioned medium was collected and subjected to gelatin zymography.

**Western blot**

Cells were collected in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.25% sodium deoxycholate) and briefly sonicated to shear DNA and reduce sample viscosity. Protein concentration was measured by BCA Reagent kit (Applygen, Beijing, China). Samples were run on a 8% PAGE gel and transferred onto nitrocellulose membranes. After blocked in 5% nonfat dry milk in TPBS (0.1% Tween 20 in PBS) for 1 h, the membranes were incubated with monoclonal anti-human RECK (1:500, MBL International Corporation, Woburn, MA) overnight at 4 °C. After three washes in 5% milk/TPBS, the membranes were incubated in goat anti-mouse antibody conjugated with horseradish peroxidase for 1 h followed by two washes in 5% milk/TPBS, followed by two washes in 5% milk/TPBS, TPBS and PBS 5 min each, respectively. The signals were developed in ECL Chemiluminescent kit (Amer sham Pharmacia Biotech, Arlington Heights, IL).

**Gelatin zymography**

After conditioned medium was collected, cell
number was determined by counting using hemocytometer. Conditioned medium from an equal number of cells was separated on 10% acrylamide gels containing 0.1% gelatin (Sigma). The gels were incubated in 2.5% Triton X-100 solution at room temperature with gentle agitation to remove SDS and were soaked in reaction buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 10 mM CaCl₂, and 0.5 mM ZnCl₂) at 37°C overnight. Then the gels were stained with 0.1% Coomassie brilliant blue in 30% methanol and 10% acetic acid for 1 h, and destained in the same solution without Coomassie brilliant blue. Gelatinolytic activity of Mmp-2 and Mmp-9 was visualized as clear bands against a dark background of stained gelatin.

RESULTS

Microarray analysis of miRNA expression at implantation sites

In order to examine differential expression of miRNAs in mouse uterus between implantation sites and inter-implantation sites, miRNA microarray analysis was performed using miRCURY LNA microarray from Exiqon containing 427 miRNAs, of which 249 are mouse miRNAs. Scatter plot and hierarchical clustering analysis were shown in Figure 1. Compared to inter-implantation sites, 13 miRNAs were up-regulated at least 2 fold, and 2 miRNAs were down-regulated at least 2 fold at implantation sites in mouse uterus. The up-regulated genes were miR-143, miR-298, let-7a, let-7c, let-7d, let-7b, miR-21, miR-20a, let-7g, miR-26a, let-7i, let-7e and let-7f. Two down-regulated genes were miR-290-5p and miR-292-5p (Table 2).

Confirmation of miRNA microarray data at implantation sites on day 5

In order to confirm results from miRNA microarray and examine tissue distribution of miRNA expression, 8 miRNAs from the up-regulated ones were chosen for Northern blot analysis. Compared to inter-implantation sites, all of the 8 miRNAs were up-regulated at implantation sites, including miR-143, miR-21, miR-20a, miR-26a, let-7a, let-7b, let-7c and let-7d (Fig. 2A).

In our study, two bands at the location of mature miR-143 were clearly detected in mouse uterus by Northern blot, either at implantation sites or inter-implantation sites. In order to know the sizes of two bands of miR-143, we chemically synthesized different lengths of artificial miR-143 with different modifications, including mature miR-143 (21 nt), mature miR-143+U (22 nt) and mature miR-143+UU (23 nt). These standards and small RNAs from implantation sites were run on the same gel and hybridized with miR-143 probe. Compared to these standards, the upper band in our Northern blot should be 21 nt and the lower band 20 nt (Fig. 2B).

We also performed Northern blot to confirm the expression of miR-298, miR-290-5p and miR-292-5p. However, there were no detectable mature forms for miR-298, miR-290-5p and miR-292-5p (Fig. 2C). The precursor forms for miR-290-5p and miR-292-5p were strongly detected at both implantation sites and inter-implantation sites. A strong band sized between precursor and mature forms was detected for miR-298. Because there were no detectable mature forms, miR-298, miR-290-5p and miR-292-5p were not used for further analysis.

In order to further confirm results from miRNA microarray and examine tissue distribution of miRNA expression, 8 miRNAs from the up-regulated ones were also chosen for in situ hybridization in the uterus on day 5 of pregnancy (Fig. 3). miR-21 was not expressed at inter-implantation sites, but was strongly expressed in the sub-luminal stroma surrounding the implanting blastocyst at implantation sites. Compared to inter-implantation sites, the expression levels of miR-143, miR-20a, miR-26a, let-7a, let-7b, let-7c and let-7d were slightly higher in the sub-luminal stroma at implantation sites.

miR-21 expression and regulation in mouse uterus during early pregnancy

Since miR-21 was shown to be highly expressed at implantation site by both Northern blot and in situ hybridization, miR-21 was chosen for further analysis. In situ hybridization was performed to examine miR-21 expression and regulation in mouse uterus during early pregnancy. miR-21 expression was not detected in mouse uterus from
days 1 to 4 of pregnancy. On day 5 of pregnancy, although miR-21 expression was not detected at inter-implantation sites, miR-21 was strongly expressed in the subluminal stroma underlying the implanting blastocyst at implantation site. When 5'-DIG labeled miR-21 probe was replaced with 5'-DIG labeled Scramble-miR probe (Exiqon), there was no detectable signal in day 5 mouse uterus at implantation site. From days 6 to 8 of pregnancy, miR-21 expression was also highly detected in the decidua (Fig. 4).

To see whether miR-21 expression was dependent upon the presence of embryos, the uterus on day 5 of pseudopregnancy was performed for in situ hybridization analysis. miR-21 expression was not detected in the uterus on day 5 of pseudopregnancy (Fig. 4), suggesting that miR-21 expression was dependent upon the presence of embryos.

In order to examine whether miR-21 expression was dependent upon the active status of embryos, delayed implantation model was used for in situ hybridization analysis (Fig. 4). Under delayed implantation, miR-21 expression was not detected in the uterus. After delayed implantation was terminated by estrogen treatment and embryos implanted, miR-21 expression was strongly seen in the subluminal stroma at implantation site, suggesting that miR-21 expression was dependent upon the presence of active blastocysts.

To examine whether miR-21 expression was regulated by steroid hormones, Northern blot was performed (Fig. 5). In the ovariectomized mouse uterus, a high level of miR-21 expression was detected. Progesterone slightly down-regulated miR-21 expression, while miR-21 expression was significantly reduced by estradiol-17β treatment.

Relationship between miR-21 and Tmem49

In this study, we showed that miR-21 was highly expressed at implantation sites than at inter-implantation sites. Because miR-21 gene is located in the 3'-UTR region of Tmem49, real-time RT-PCR was performed to check Tmem49 expression level with a pair of primers located in the 3'-UTR region of Tmem49 to evaluate miR-21 expression (Fig. 6). On day 5 of pregnancy, Tmem49 expression at implantation sites was significantly higher than that at inter-implantation sites, suggesting that Tmem49 and miR-21 were co-expressed.

Prediction and confirmation of target genes of miR-21

We predicted the target genes for 13 up-regulated miRNAs using both miRanda and TargetScan algorithms. There were total 611 genes predicted by both algorithms. Functional classification of these target genes was assigned according to Gene Ontology Consortium. These target genes were mainly responsible for signal transducer activity, transporter activity, kinase activity, transcription regulator activity and enzyme regulator activity. Because there were only 2 down-regulated miRNAs at implantation sites, the functional classification of the target genes for the down-regulated miRNAs was not performed.

Since miR-21 was shown to be highly expressed at implantation site by both Northern blot and in situ hybridization, target genes of miR-21 was predicted for further analysis. It is difficult to judge which of the algorithms produces the most reliable and/or sensitive target predictions. It was shown that TargetScan and PicTar algorithms produce similar overall sets of predicted target sites (15). miRanda is also a good algorithm in the miRBase Target database to predict target genes (16). Therefore, target genes for miR-21 were predicted by both TargetScan and miRanda algorithms.

There were 775 target genes for miR-21 predicted by miRanda 3.0 and 186 target genes for miR-21 predicted by TargetScan 4.0. In total, 42 genes were predicted by both algorithms. Reck was predicted to be the putative target gene of miR-21 by both softwares with high scores. We aligned the 3'-UTR segment of Reck gene containing miR-21 putative binding site from dog to human. The “seed” sequence which played a crucial role in miRNA:mRNA interaction was conserved in all aligned sequences (Fig. 7A). Thermodynamic analysis indicated this interaction was very strong (Fig. 7B).

In order to confirm that Reck is indeed the target
gene of miR-21, the 3'-UTR segment of mouse Reck predicted to interact with miR-21 was amplified by PCR from mouse cDNA and inserted into the downstream of luciferase reporter gene in the pGL3 control vector for dual-luciferase assay (Fig. 7B).

In mouse 3T3 cells, both miR-21 precursor and mature miR-21 were detected by Northern blot analysis (Fig. 7C). 3T3 cells were co-transfected with Reck-pGL3 and pRL-TK containing Renilla luciferase. Compared to Pre-miR negative control, the luciferase activity was significantly decreased by miR-21 precursor. Furthermore, the luciferase activity was significantly up-regulated by miR-21 inhibitor compared to anti-miR negative control (Fig. 7D). Mutation was also performed to further confirm the binding site for miR-21. The seed sequence AGCTTAT for miR-21 in the 3'-UTR of mouse Reck gene was single-base mutated into AGGTTAT by PCR as described previously (17). When 3T3 cells were co-transfected with Reck-pGL3 plasmid containing the normal binding site and miR-21 precursor, the luciferase activity was low. However, the luciferase activity was significantly up-regulated after 3T3 cells were transfected with Reck-pGL3-Mutant plasmid containing the mutated binding site and miR-21 precursor (Fig. 7E). These results showed that Reck should be the target gene of miR-21.

**miR-21 regulation on Reck expression**

Although Reck was identified as a target gene for miR-21, it was unknown whether miR-21 could regulate endogenous Reck expression. Based on a brief screening on RECK expression in human uterine cell lines, we found that RECK protein was strongly detected in Hela cells, but weakly in Ishikawa cells. Because RECK protein was at a high level in Hela cells, Hela cells were transfected with miR-21 precursor to see whether miR-21 could regulate endogenous RECK. Compared to Pre-miR control, the level of RECK protein in Hela cells was significantly down-regulated by miR-21 precursor (Fig. 8A). Additionally, Ishikawa cells were transfected with miR-21 inhibitor to see whether miR-21 could regulate RECK expression since RECK protein was at a low level in Ishikawa cells. Compared to anti-miR control, the level of RECK protein in Ishikawa cells was significantly up-regulated by miR-21 inhibitor. Based on these data, endogenous RECK protein expression was indeed regulated by miR-21 (Fig. 8B).

**Reck expression at implantation site and its relationship with Mmp-2 and Mmp-9**

After Reck was shown to be the target gene of miR-21, real-time RT-PCR was performed to examine whether there was an opposite pattern between Reck and miR-21 expression in mouse uterus on day 5 of pregnancy. Compared to implantation sites, Reck expression level was significantly higher at inter-implantation sites (Fig. 9A), which was opposite to miR-21 expression pattern.

We then performed in situ hybridization to examine the localization of Reck mRNA expression in mouse uterus on day 5 of pregnancy. Reck mRNA expression was mainly localized in the luminal epithelium and slightly in the subluminal stroma (Fig. 9B).

Since Reck was shown to be able of down-regulating Mmp-2 and Mmp-9 activity (18), the expression level of both Mmp-2 and Mmp-9 was also examined by real-time RT-PCR. Compared to implantation sites, the expression level of both Mmp-2 and Mmp-9 was significantly lower at inter-implantation sites, which was in contrast to Reck expression pattern (Fig. 10A).

In order to further show whether Reck could regulate Mmp-2 and Mmp-9 activity in uterine stromal cells, RECK over-expression and gelatin zymography were performed. After cultured uterine stromal cells were transfected with RECK-expressing vector pCMVβ-RECK, RECK expression was confirmed by RT-PCR and Western blot. After RECK was over-expressed in mouse uterine stromal cells, Mmp-9 activity was significantly down-regulated, but Mmp-2 activity was not affected (Fig. 10B), suggesting that Reck mainly inhibited Mmp-9 activity in uterine stromal cells.

**DISCUSSION**
Confirmation and comparison of miRNA microarray data

Compared to inter-implantation sites, there were 13 miRNAs up-regulated at least 2 fold and 2 down-regulated at least 2 fold at implantation sites. There were 8 up-regulated miRNAs confirmed by both Northern blot and in situ hybridization. Up to now, there was only one miRNA microarray study on mouse uterus. They compared day 4 uterus with day 1 uterus. Compared to day 1 uterus, there were 32 miRNAs significantly up-regulated at least 1.5 fold and 5 down-regulated at least 1.5 fold in day 4 uterus (19). Both let-7b and miR-20a were shown to be significantly up-regulated by both them and us. There were more miRNAs significantly changed in their data. This may come from both the big difference between day 1 and day 4, and 1.5 fold limit used in their study. In human endometrium, let-7a, let-7b, let-7c, let-7d, let-7f, let-7i, miR-21 and miR-26a were also highly expressed. Compared to ectopic endometrium, both miR-21 and miR-26a were highly expressed in endometrium without endometriosis (20). In our study, miR-21 expression was 2.35 fold higher at implantation sites than inter-implantation sites. In a miRNA microarray analysis in human uterus, miRNA-21 expression in human leiomyoma was significantly higher than that in matched myometrial tissue, which was also confirmed by real-time RT-PCR (21).

In our study, two bands were clearly shown at the location of mature miR-143. According to our standards, these two bands should be 20 nt and 21 nt, respectively. The prevailing modifications were caused by A-to-I editing (identified as A-to-G changes) and 3'-terminal A and U additions. The A-to-G transitions occurred at 4-fold lower frequency in mouse than in human (0.5% in mouse and 2.2% overall in human)(22). A careful examination of northern blot results in some published reports also reveals multiple bands for one miRNA (23,24). In C. elegans, miR-84 shows 3 clearly visible bands in northern blots, indicating that miR-84 has 3 main variations (25). Based on large-scale sequencing for both mouse and human small RNA libraries, 3 kinds of 3'-end modification were identified for mature miR-143, mainly for 22 nt (A->G and A->U) and 23 nt (G->U) (22), which were longer than 20 and 21 nt bands of miR-143 in our study. These polymorphisms may be produced from 3'-end deletion. In miR-142-3p, both 3'-end deletion and 3'-end extensions were identified (23). In our study, whether the polymorphisms occur at 3'-end or 5'-end is still unknown. The changes in the 5'-end seed sequence can affect target selection. However, the biological significance of the 3'-end polymorphism in miR-143 is not clear.

We also checked the expression of miR-298, mir-290-5p and miR-292-5p by Northern blot. However, there were no detectable mature forms for miR-298, mir-290-5p and miR-292-5p. By Northern blot, the precursor forms for miR-290-5p and miR-292-5p were detected, but miR-298 precursor was not detected. Michael et al. (26) used Northern blots to show that the precursors to miR-143 and miR-145 are expressed in colorectal tissues and tumors, but the mature miRNA was detectable only in the normal colorectal tissue. Beuvink et al (27) reported that mir-214, mir-185 and mir-146 were not detected by RT-PCR although these miRNAs were scored as positive on their MOE-ER array, suggesting non-specific hybridization of other cellular RNAs to the array probes. These indicated that miRNA microarray data should be explained with cautions and validated by an independent method.

miR-21 regulation and function

In this study, miR-21 was strongly expressed in subluminal stromal cells at implantation sites, but not in inter-implantation sites. Furthermore, miR-21 was also not detected in mouse uterus during pseudopregnancy and under delayed implantation, suggesting that miR-21 expression at implantation sites was regulated by active blastocysts.

Because miR-21 gene is located in the 3'-UTR region of Tmem49, a pair of primers was designed at the 3'-UTR region of Tmem49 to examine Tmem49 expression by real-time RT-PCR. We showed that Tmem49 expression was significantly higher at implantation sites than that at inter-implantation sites, which was similar to miR-21 expression pattern. The co-expression of both Tmem49 and miR-21 indicated that miR-21
should be co-transcribed with Tmem49. Some miRNAs are within the introns of host genes and are usually coordinately expressed with their host gene mRNA (28). Numerous strongly correlated expression profiles were observed between miRNAs and their host genes, providing evidence that the miRNAs are processed from the same primary transcripts as their host genes (28,29).

miRNAs are thought to function as both tumor suppressors and oncogenes (30). miR-21 is widespread over-expression in diverse tumors including those derived from breast, colon, lung, pancreas, stomach and prostate (31). Enhanced miR-21 expression by transfection with precursor miR-21 in cultured human hepatocellular cancer cells increased tumor cell proliferation, migration, and invasion. Moreover, an increase in cell migration was observed in normal human hepatocytes transfected with miR-21 precursor (32). Suppression of miR-21 can inhibit tumor growth (24). The dysregulated expression of let-7 family, miR-21, miR-23b, miR-29b, and miR-197 in human uterine leiomyomas was strongly associated with tumor sizes and races (33). In human ovarian carcinomas, the levels of miR-21, miR-203, and miR-205 were up-modulated compared with normal tissues (34). Compared to normal cervical samples, miR-21 was also highly expressed in human cervical cancer samples (35). In the ovariecotomized mouse uterus, miR-21 expression was slightly reduced by progesterone and significantly down-regulated by estrogen. In cultured human glandular epithelial cells and stromal cells, miR-21 expression was also down-regulated by estrogen. In contrast to miR-21, Reck expression at implantation sites was significantly lower than that at inter-implantation sites. Reese et al (11) also reported that Reck was down-regulated at implantation sites. The inverse expression pattern between Reck and miR-21 also suggest that Reck should be regulated by miR-21. In our study, miR-21 and Reck were co-expressed in the subluminal stromal cells at implantation sites although they were expressed in a reverse pattern. In general, targets are co-expressed at relatively low or undetectable levels in the same tissues as the miRNAs predicted to regulate them (41). In many cases, target mRNA levels diminish but do not disappear (42,43). Moderate down-regulation at the mRNA level, possibly in combination with translational repression, may be sufficient to maintain tissue or developmental gene expression or sharpen target expression domains (38,39). The inhibition of miR-21 by either estrogen or progesterone suggests that the action of estrogen and progesterone in the uterus may be fine-tuned or sharpened through miR-21.

**miR-21 targets Reck at implantation site**

In animals, imperfect complementarity with miRNAs makes computational prediction of their targets particularly challenging (5). In addition, only a small number of predicted targets have been experimentally validated (40). In our study, Reck was predicted to be the target gene of miR-21 by both miRanda and Targetscan. In the luciferase assay transfected with the recombinant vector bearing putative miR-21 binding site in 3′-UTR region of Reck, the luciferase activity was shown to be down-regulated by miR-21 precursor and up-regulated by miR-21 inhibitor, suggesting that Reck should be the target gene of miR-21. Our mutation study also confirmed that the binding site in the 3′-UTR of Reck gene was specific for miR-21. In cultured human uterine cell lines, we also showed that the high level of endogenous RECK protein was down-regulated by miR-21 in Hela cells, while the low level of endogenous RECK protein was up-regulated by miR-21 inhibitor in Ishikawa cells. These data showed that RECK was not only the target gene of miR-21, but endogenous RECK protein level was also regulated by miR-21.
patterns. Moreover, a delay or inability to degrade selected miRNA targets may be advantageous to sequester repressed transcripts for the possibility of re-activation (41).

The key action of Reck is to inhibit Mmp-2, Mmp-9 and Mtp-1 involved in breakdown of the extracellular matrix and angiogenesis. Additionally, Reck has a significant effect on tumorigenesis by limiting angiogenesis and invasion of tumors through the extracellular matrix (18). Reck-deficient mice die around E10.5 with defects in collagen fibrils, the basal lamina, and vascular development. The phenotype in Reck-deficient mice could be partially suppressed by Mmp-2 null mutation (44). The glycosylation of RECK Asn297 residue was involved in the suppression of Mmp-9 secretion and Asn352 residue was necessary to inhibit Mmp-2 activation (45). In our results from real-time RT-PCR, the level of both Mmp-2 and Mmp-9 expression in mouse uterus at implantation sites was significantly higher than that at inter-implantation sites, while Reck was down-regulated at implantation sites. However, in cultured uterine stromal cells, Reck over-expression only significantly down-regulated Mmp-9 activity, but had no effects on Mmp-2 activity, suggesting that Reck may mainly inhibit Mmp-9 activity in mouse uterus.

Mmp-9 mRNA is expressed in the subluminal stromal cells surrounding the implanting blastocyst on day 5, and later in trophoblast giant cells on day 8 of pregnancy (46). The predominant Mmp produced by cultured blastocysts was shown to be Mmp-9. The neutralizing antibody for Mmp-9 can inhibit blastocyst invasiveness through degradation of the maternal extracellular matrix by trophoblast cells (47,48). These data suggest that Mmp-9 should play a key role in blastocyst invasiveness. In addition to Mmp-9, a significant inverse correlation between RECK-expression and tumor angiogenesis was documented in non-small cell lung cancer, indicating that RECK could suppress the angiogenesis induced by VEGF (49).

The possible relationship between miR-21 and Stat3

Additionally, two Stat3 binding sites were found in the upstream enhancer of miR-21 and are highly conserved in vertebrates. miR-21 induction by IL-6 was strictly Stat3 dependent (50). Leukemia inhibitor factor (Lif) is essential for embryo implantation. Female mice lacking Lif produce normal blastocysts, but implantation fails (51). Lif has been shown to cause activation and nuclear translocation of Stat3 in mouse uterine epithelium (52). The inhibition of Stat3 activation in the endometrium can prevent implantation (53). It is possible that Lif can induce miR-21 expression through Stat3 activation. Interestingly, Stat3 was predicted to be the target gene of miR-21 by TargetScan. Once miR-21 was induced by Stat3, miR-21 could down-regulate Stat3 via negative feedback loop through mRNA cleavage and translation repression. Lif was shown to stimulate the production of Mmp-9 and urokinase-type plasminogen activator during trophoblast outgrowth of blastocysts for 3 days in culture, suggesting that Lif is a key regulator of Mmp-9 and other proteinases involved directly or indirectly in the implantation process (54). However, whether Lif can regulate miR-21 and Mmp-9 through Stat3 still remains to be determined.

In conclusion, miR-21 expression was strongly localized in the subluminal stromal cells at implantation sites and regulated by active blastocysts. Reck was shown to be the target gene of miR-21 and might play a key role during embryo implantation mainly through inhibiting Mmp-9 activity.

REFERENCES


**FOOTNOTES**

This work was supported by National Basic Research Program of China (2006CB504005 and 2006CB944006) and Chinese National Natural Science Foundation grants 30570198 and 30770244.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Accession number</th>
<th>Size and location</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>GCCTTCCGTGTCTTCAACCACCC TGCCTGCTTCAACCACCC</td>
<td>NM_008084</td>
<td>102 bp (734-835)</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Mmp2</td>
<td>AGGAGCTCTATGGGCCCTCC TCTGTGTGCTCAGATCTCCG</td>
<td>NM_008610</td>
<td>97 bp (1325-1421)</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>Mmp9</td>
<td>CCCAAGACCTGAAAACCTCC TTCTCTCCCATCATCTGGGC</td>
<td>NM_013599</td>
<td>101 bp (100-200)</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>Reck</td>
<td>CTCCACGAGCTCTCCCGTCAT GTTGTGGGGTGTAGGCTCTA</td>
<td>NM_016678</td>
<td>120 bp (2814-2933)</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>RECK</td>
<td>CGCGTGGCAGTGGTACTCTCTAC AGGAGCGAAGGACACTTGACA</td>
<td>NM_021111</td>
<td>102 bp (2424-2525)</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>rPL7</td>
<td>GCAGATGTACCCGACTGAGATTC ACCTTTGGGGTGTAGGCTCTA</td>
<td>M29016</td>
<td>129 bp (276-404)</td>
<td>Real time PCR</td>
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<tr>
<td>Tmem49</td>
<td>TCAGTCCACTCCACGTCATTTCTT GGTATCATGAAGGACAGGTCTT</td>
<td>NM_029478</td>
<td>81 bp (1516-1596)</td>
<td>Real time PCR</td>
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Table 2  List of the miRNAs with at least 2-fold changes at implantation sites

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold (IM/Inter-IM)</th>
<th>nonpaired t test</th>
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</thead>
<tbody>
<tr>
<td>miR-143</td>
<td>4.37</td>
<td>0.005</td>
</tr>
<tr>
<td>miR-298</td>
<td>3.52</td>
<td>0.005</td>
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<tr>
<td>let-7a</td>
<td>2.78</td>
<td>0.018</td>
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<tr>
<td>let-7c</td>
<td>2.71</td>
<td>0.019</td>
</tr>
<tr>
<td>let-7d</td>
<td>2.68</td>
<td>0.015</td>
</tr>
<tr>
<td>let-7b</td>
<td>2.57</td>
<td>0.015</td>
</tr>
<tr>
<td>miR-21</td>
<td>2.35</td>
<td>0.009</td>
</tr>
<tr>
<td>miR-20a</td>
<td>2.30</td>
<td>0.011</td>
</tr>
<tr>
<td>let-7g</td>
<td>2.26</td>
<td>0.019</td>
</tr>
<tr>
<td>miR-26a</td>
<td>2.24</td>
<td>0.018</td>
</tr>
<tr>
<td>let-7i</td>
<td>2.21</td>
<td>0.016</td>
</tr>
<tr>
<td>let-7e</td>
<td>2.13</td>
<td>0.006</td>
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<tr>
<td>let-7f</td>
<td>2.02</td>
<td>0.030</td>
</tr>
<tr>
<td>miR-290-5p</td>
<td>-3.23</td>
<td>0.003</td>
</tr>
<tr>
<td>miR-292-5p</td>
<td>-3.45</td>
<td>0.003</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1 Clustering analysis of miRNA microarray data. (A) Scatter plot represents miRNAs significantly changed at least 2 fold at implantation sites in day 5 pregnant mouse uterus. Dash lines indicate a 2-fold difference in the level of gene expression from the mean. Red indicates expression level greater than 2 fold at implantation site, and green represents expression level more than 2 fold at inter-implantation site. (B) Hierarchical clustering analysis of significantly changed miRNAs. Background-subtracted values were log-transformed, centered relative to the median, and gene-to-gene normalized. Relative changes in their expression level were indicated by a color code. Red indicates that the level of gene expression is higher than median, and green indicates that the level is lower than median.

Figure 2 Northern blot confirmation of miRNA microarray data. (A) Northern blot analysis for 8 miRNAs from the up-regulated ones in mouse uteri at both implantation sites (IM) and inter-implantation sites (Inter-IM). After the density of each band was normalized with its corresponding U6 density, the ratio between IM and I-IM was shown at the right side. (B) miR-143 editing. Different lengths of miR-143 with different modifications were chemically synthesized, including mature miR-143 (21 nt), mature miR-143+U (22 nt) and mature miR-143+UU (23 nt). These standards and small RNAs from day 5 mouse uterus at implantation sites were run on the same gel and hybridized with miR-143 probe. (C) Northern blot analysis of miR-298, miR-290-5p and miR-292-5p expression at both implantation sites and inter-implantation sites in day 5 pregnant mouse uterus. Small RNAs from implantation sites were run and hybridized with miR-21 probe to show the locations of both precursor and mature miR-21. There were no detectable mature forms for miR-298, miR-290-5p and miR-292-5p.

Figure 3 In situ hybridization confirmation for 8 miRNAs significantly up-regulated at implantation sites. Compared to inter-implantation sites, these miRNAs were highly expressed in the subluminal stroma at implantation sites. Bar=220 μm

Figure 4 In situ hybridization of miR-21 expression in mouse uteri. miR-21 expression during early pregnancy was shown on days 1 (D1), 2 (D2), 3 (D3), 4 (D4), 5 (I for implantation site, and NI for inter-implantation site), 6 (D6), and 8 (D8). miR-21 expression in day 5 pseudopregnant uterus was also shown (PD5). miR-21 expression was not detected under delayed implantation (Delay), but was strongly seen in the stromal cells after activation by estrogen treatment (Activation). When 5'-DIG labeled miR-21 probe was replaced with 5'-DIG labeled Scramble-miR probe, there was no detectable signal in day 5 mouse uterus at implantation site [D5(control)]. *: implanting blastocyst. Bar=100 μm

Figure 5 Northern blot analysis of miR-21 expression in the ovariectomized mouse uteri. Ovariectomized mice were treated with sesame oil (for control), progesterone or estradiol-17β for 24 h. After the density of each band was normalized with its corresponding U6 density, the ratio among treatments was shown in the middle. miR-21 expression was down-regulated by either progesterone or estrogen.

Figure 6 Real-time RT-PCR of Tmem49 expression. Tmem 49 expression was normalized with rPL7.

Figure 7 The prediction and confirmation of miR-21 target. (A) Cross-species comparison of miR-21 binding sites in the 3′-UTR region of Reck. (B) Schematic diagram for constructing miR-21 binding site into pGL3 control vector. Seed sequences were marked in yellow. (C) Northern blot confirmation of miR-21 expression in mouse 3T3 cells. (D) Confirmation of the target gene of miR-21. The 3′-UTR segment of mouse Reck predicted to interact with miR-21 was amplified by PCR from mouse cDNA and inserted into the downstream of luciferase reporter gene in the pGL3 control vector for dual-luciferase assay. pRL-TK containing Renilla luciferase was co-transfected with Reck-pGL3 for data normalization. These transfected 3T3 cells were co-transfected with miR-21 precursor (pre-miR-21), the negative control of Pre-miR miRNA Precursor, miR-21 inhibitor (anti-miR-21) or Anti-miR Negative Control, respectively. Cell lysate was collected and assayed for dual luciferase activities 30 h after transfection.
activity was not affected by Pre-miR negative control, but significantly decreased by miR-21 precursor. Furthermore, the luciferase activity was not affected by anti-miR negative control, but was significantly up-regulated by miR-21 inhibitor. (E) Mutation analysis of miR-21 binding site. When the binding site (AGCTTAT) was single-base mutated into AGGTTAT (mutated G in red color), the luciferase activity was significantly increased after mouse 3T3 cells were co-transfected with miR-21 precursor.

Figure 8 miR-21 regulation on Reck expression. (A) Hela cells were treated with pre-miR control or miR-21 precursor for 48 h. Compared to pre-miR control, RECK protein expression was significantly inhibited by miR-21 precursor. After the density of each band was normalized with its corresponding GAPDH density, the ratio between pre-miR control and miR-21 precursor was shown in the middle. (B) Ishikawa cells were treated with anti-miR control or miR-21 inhibitor for 48 h. Compared to anti-miR control RECK protein expression was significantly up-regulated by miR-21 inhibitor. After the density of each band was normalized with its corresponding GAPDH density, the ratio between anti-miR control and miR-21 inhibitor was shown in the middle.

Figure 9 Reck expression in mouse uterus. (A) Real-time RT-PCR of Reck expression in day 5 pregnant mouse uteri at implantation sites and inter-implantation sites. rPL7 was used to normalize the expression level of each gene. (B) In situ hybridization of Reck mRNA expression in the longitudinal section of mouse uterus on day 5 of pregnancy. Reck mRNA expression was mainly localized in the luminal epithelium and slightly in the subluminal stroma. Arrowhead, implantation site; arrow, inter-implantation site. Bar=60 μm.

Figure 10 Reck regulation on Mmps. (A) Real-time RT-PCR of Mmp-2 and Mmp-9 mRNA expression in day 5 pregnant mouse uteri at implantation sites and inter-implantation sites. rPL7 was used to normalize the expression level of each gene. (B) RECK over-expression and gelatin zymography. Uterine stromal cells were transfected with Reck over-expression vector pCMVβ-RECK. RECK over-expression was confirmed by RT-PCR and Western blot. Conditioned medium was collected and subjected to gelatin zymography for Mmp-2 and Mmp-9 activity 24 h after RECK over-expression in serum-free DMEM.
Figure 1

(A) Log2 value of implantation site signals vs. log2 value of inter-implantation site signals.

(B) Heatmap of expression levels of various miRNAs across different samples.
Figure 2

(A) | IM | I-IM | Ratio (IM/I-IM)
---|----|------|----------------
miR-143 | grayscale image | grayscale image | 2.70
miR-21 | grayscale image | grayscale image | 2.08
miR-20a | grayscale image | grayscale image | 1.59
miR-26a | grayscale image | grayscale image | 2.08
let-7a | grayscale image | grayscale image | 1.82
let-7b | grayscale image | grayscale image | 1.56
let-7c | grayscale image | grayscale image | 1.92
let-7d | grayscale image | grayscale image | 1.22
U6 | grayscale image | grayscale image | grayscale image

(B) | Synthetic standards (nt) | Bases
---|------------------|------
| 21 | 22 | 23 |
---|----|----|----|
| grayscale image | 23 | 22 | 21 |

(C) | miR-21 | miR-298 | miR-290 | miR-292
---|-------|-------|-------|-------
| grayscale image | grayscale image | grayscale image | grayscale image | grayscale image

Precursor | Mature | U6
---|-------|-----
| grayscale image | grayscale image | grayscale image
Figure 3

Inter-implantation site

Implantation site

miR-143

miR-21

miR-20a

miR-26a

let-7a

let-7b

let-7c

let-7d
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>Oil</th>
<th>Progesterone</th>
<th>Estrogen</th>
</tr>
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<tbody>
<tr>
<td>miR-21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>1</td>
<td>0.59</td>
<td>0.25</td>
</tr>
<tr>
<td>U6</td>
<td></td>
<td></td>
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</table>
Figure 6

Tmop expression level over rPL7

Implantation site  Inter-implantation site

*
Figure 7

(A) miR-21

(B) pGL3 control vector

(C) 3T3 cells

(D) Relative luciferase activity

(E) Relative luciferase activity

Human
Chimpanzee
Rhesus
Dog
Mouse
Rat

conservative seed binding site

Reck mRNA
GUUUUGACGUUUGAAAUAAGCUA
mR-21
AGUUGUAGUCAGAC - UAUUCUGAU

ΔG: -19.07 kcal/mole
Figure 8

(A) Pre-miR control  miR-21 precursor

RECK
Ratio  1  0.56
GAPDH

(B) Anti-miR control  miR-21 inhibitor

RECK
Ratio  1  2.49
GAPDH
Figure 9

(A) 

Reck expression over rpl7

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

Implantation site

Inter-implantation site

*B

(B) 

In situ hybridization of Reck mRNA in day 5 uterus
Figure 10

(A) Mmp expression level over rPL7

(B) Comparison of Control and pCMVβ-RECK conditions for RECK mRNA, Gapdh mRNA, RECK protein, Gapdh protein, Mmp-9 activity, and Mmp-2 activity.
MicroRNA expression and regulation in mouse uterus during embryo implantation
Shi-Jun Hu, Gang Ren, Ji-Long Liu, Zhen-Ao Zhao, Yong-Sheng Yu, Ren-Wei Su,
Xing-Hong Ma, Hua Ni, Wei Lei and Zeng-Ming Yang

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