Expression of mmu-miR-96 in the endometrium during early pregnancy and its regulatory effects on stromal cell apoptosis via Bcl2

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Received December 25, 2015; Accepted December 12, 2016

DOI: 10.3892/mmr.2017.6212

Abstract. Decidualization of endometrial stromal cells is an important feature of implantation and pregnancy. The molecular mechanism underlying decidualization remains unclear, particularly regarding the microRNA (miRNA/miR) regulation of this process. The present study revealed the temporal and spatial distribution of mmu-miR-96 in the mouse uterus during early pregnancy by reverse transcription-quantitative polymerase chain reaction and *in situ* hybridization. In addition, primary stromal cells were isolated from the mouse uterus and used to explore the role of mmu-miR-96 in decidualization. The results demonstrated that mmu-miR-96 was highly expressed in stromal cells during pregnancy, and was upregulated at implantation sites. In addition, mmu-miR-96 was strongly expressed during decidualization, which indicates that it may serve a role in the decidualization of stromal cells. Based on existing reports, mmu-miR-96 participates in apoptosis; therefore the present study investigated its effects on the apoptosis of primary endometrial stromal cells. The results indicated that overexpression of mmu-miR-96 may induce apoptosis of stromal cells. In further studies regarding the underlying mechanism, the target genes of mmu-miR-96 were screened by bioinformatics analysis, and it was confirmed that B-cell lymphoma 2, an anti-apoptotic gene, was the target of mmu-miR-96, as determined using a reporter gene assay. In conclusion, the present study suggested that mmu-miR-96 participates in the decidualization of endometrial stromal cells in mice, thereby serving a key role in pregnancy.

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Key words: mmu-miR-96, decidualization, Bcl2, apoptosis

Introduction

Embryonic implantation is a complex reproductive process involving reciprocal interactions between the blastocyst and uterus. Successful embryonic implantation is dependent on implantation of normal embryos, synchronous development of endometrial receptivity, and molecular communication between the embryo and the mother (1,2). The cellular dialogue precisely regulates endometrial decidualization, as well as the proliferation and differentiation of trophoblast cells. During development, the endometrial stromal cells are stimulated by inducing factors, and undergo proliferation and differentiation; a process referred to as decidualization. This process serves a crucial role in the establishment and maintenance of pregnancy (3-5). Recently, microRNA (miRNA/miR) regulation of endometrial gene expression during early pregnancy has received a lot of attention. Chakrabarty *et al* (6) demonstrated that mRNA expression of cyclooxygenase-2, which is critical for embryonic implantation, was post-transcriptionally regulated by mmu-miR-101a and mmu-miR-199a. Furthermore, Hu *et al* (7) demonstrated differential expression of miRNAs in the mouse uterus between implantation sites (IS) and inter-implantation sites (IIS) by miRNA microarray. Shen *et al* (8) reported that mmu-miR-200a has an important role in embryonic implantation and that phosphatase and tensin homolog is the target gene of mmu-miR-200a. However, in recent years, few studies have investigated the involvement of miRNAs in the regulation of decidualization.

miRNAs are a class of non-coding small RNA molecules, 18-24 nucleotides in length, which cause either the degradation of target mRNA, or translational inhibition through target mRNA-specific base pairing (9). They affect various biological processes, including development, cell growth, differentiation, apoptosis and maintenance of tissue identity (10). A previous study investigated the dynamic alterations in miRNA and mRNA levels during the pre-receptivity, receptivity and implantation phases, which reflect the mechanism by which miRNAs regulate their target mRNAs. Mmu-miR-96 and mmu-miR-200 were revealed to act as hub miRNAs, and were

verified to share the target gene B-cell lymphoma 2 (Bcl2), and to exert synergistic regulation (11).

Previous in house miRNA microarray results demonstrated that mouse uterine expression of mmu-miR-96 was upregulated at IS on day five of pregnancy compared with at IIS, indicating that it may participate in the regulation of implantation (11). Mmu-miR-96, which maps to chromosomal region 17, is one of the members of the miR-183 family, other members of which include microRNA-183 and microRNA-182, and has recently been found to have a role in cancer. Its expression is upregulated in various tumors, including bladder cancer (12,13), lung cancer (14), breast cancer (15), endometrioid carcinomas (16) and liver cancer (17). A previous study identified that miR-96 promoted suppression of forkhead box protein O1 (FOXO1) and may serve a key role in transitional cell carcinoma tumorigenesis by avoiding cell apoptosis (18). Furthermore, the upregulation of miR-96 has been reported to induce downregulation of the transcription factors FOXO3a and FOXO1, and thus promote cell proliferation in human breast cancer (15,19). It has also been revealed that in non-small cell lung cancer-derived cell lines, downregulation of miR-96 inhibits proliferative and invasive capacities, and promotes apoptosis (20). Therefore, the present study hypothesized that mmu-miR-96 may serve a role in decidualization by regulating the proliferation and apoptosis of endometrial stromal cells. This study aimed to investigate the target gene of mmu-miR-96. The results revealed the expression pattern and function of mmu-miR-96 in the endometrium during early pregnancy in mice. In addition, the anti-apoptotic protein Bcl2 was identified as the target gene of mmu-miR-96 in the endometrium.

Materials and methods

Animals and tissue preparation. A total of 52 female and 13 male Kunming mice (age, 6-8 weeks; weight, 25-30 g) were provided by the Laboratory Animal Center of Chongqing Medical University, [Chongqing, China; Certificate No.: SCXK (YU) 20070001]. The mice were housed in the Chongqing Medical University Animal Care Facility with *ad libitum* access to food and water under a 14 h light/10 h dark cycle, at constant temperature (22±2˚C) and humidity (50%). Ethical approval for this study was provided by the Ethics Committee of Chongqing Medical University [Certification No: SCXK (YU) 20110016]. Female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy, respectively. The presence of a vaginal plug was regarded as the first day of pregnancy (D1). The mice were sacrificed by cervical dislocation and the tissue samples of mice were obtained on pregnancy day 1, 4, 5, 6 and 7. IS and IIS tissues were collected according to a previous study (21). The artificially induced decidualization mouse model was generated according to standard criteria. Briefly, on day 4 of pseudopregnancy, 25 μl corn oil was infused into one uterine horn to induce artificial decidualization and another horn without any infusion served as the control. On day 8 of pseudopregnancy the mice were sacrificed by cervical dislocation.

In situ hybridization. The mmu-miR-96-specific probe and the negative control (scramble) were purchased from Exiqon A/S (Vedbaek, Denmark). Probe sequence: AAGCAAAAATGT GCTAGTGCCAAA. An *in situ* hybridization kit (Dingguo Biotechnology Co. Ltd., Beijing, China) was used for hybridization according to the manufacturer's protocol. Briefly, frozen endometrial tissues were sectioned (10 μm) and fixed with 4% paraformaldehyde at room temperature for 10 min. The uterine sections were treated with protease K at 37˚C for 5 min, and then incubated with prehybridization solution for 4 h at 50˚C, followed by incubation with hybridization solution including probes (40 mmol/l) overnight at 50˚C. After washing with standard saline citrate, sections were incubated with bovine serum albumin (1:100 dilution) at 37˚C for 1 h, and then with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (1:100 dilution) for 1 h. Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate was employed to indicate a positive signal and the nuclei were stained with Nuclear Fast Red. All slides were viewed directly under a microscope (BX43; Olympus Corporation, Tokyo, Japan).

Cell culture and treatment. Primary cells were isolated from the uteri of mice as previously described (22,23). The mice were sacrificed on day 4 (stromal cells) or day 8 (decidual cells) of pregnancy by cervical dislocation, and the uteri were split longitudinally. After washing with D-Hank's Balanced Salt Solution (HBSS) (Boster Systems, Inc., Pleasanton, CA, USA), uterine tissues were cut into small pieces and treated with 7.5 ml HBSS containing 1% (w/v) trypsin (Boster Systems, Inc.) and 6 mg/ml dispase (Roche Diagnostics, Indianapolis, IN, USA). The tissues were rinsed 3 times with HBSS and incubated in 2 ml HBSS containing 0.5 mg/ml collagenase I (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. The suspension was purified through a 70-μm nylon filter and centrifuged at 1,500 x *g* for 5 min. The cells were seeded in 50 ml culture flasks and cultured in phenol red-free culture medium (DMEM/Ham's F-12; 1:1; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) containing 10% charcoal-stripped fetal bovine serum (FBS) (Sigma-Aldrich; Merck Millipore). The cells were transfected with miR-96 mimic (5'-UUUGGCACUAGCACAUUUUUGCU-3' 5'AGC AAAAAUGUGCUAGUGCCAAA-3') or a negative control (5'UUUGUACUACACAAAAAGUACUG3' 5'CAGUAC UUUUGUGUAGUACAAA3') (Guangzhou RiboBio Co. Ltd., Guangzhou, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The medium was replaced with fresh DMEM/F12 medium (supplemented with 10% FBS) after transfection for 4 to 6 h, and then collected 48 h later.

In vitro decidualization. Decidualization was performed as described previously (24,25), with modification. Briefly, endometrial stromal cells isolated from the uteri of mice on day 4 of pregnancy were treated with 10 nmol/l estradiol-17β (Sigma-Aldrich; Merck Millipore) and 1 μmol/l progesterone (Sigma-Aldrich; Merck Millipore) for 96 h, whereas endometrial stromal cells not treated with E2P4 served as the control group. Culture medium was changed each day.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mouse endometrial tissues or cultured cells using TRIzol reagent

(Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RT of cDNA was conducted using the miScript II Reverse Transcription kit with miScript HiSpec Buffer (Qiagen, Inc., Valencia, CA, USA,). Briefly, RNA was mixed with miScript HiSpec Buffer, RNAse-free water and miscript Reverse Transcriptase Mix, and was incubated at 37˚C for 60 min and 95˚C for 5 min. qPCR was conducted with the miScript SYBR-Green Real Time PCR kit (Qiagen, Inc.) for miRNA detection and SYBR Premix Ex Taq. The specific primers for mmu-miR-96, U6, Bcl2, decidual/trophoblast prolactin-related protein (dtPRP) and β-actin were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table I). The qPCR master mix (15 μl) contained 7.5 μl SYBR Premix Ex Taq, 0.6 μl primers, 1.2 μl cDNA and 5.1 μl diethylpyrocarbonate-treated H_2O. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles at 95˚C for 5 sec (denaturation) and 60˚C for 30 sec, followed by 72˚C for 5 sec. Experiments were performed in triplicate. Data obtained from qPCR were analyzed using the $2^{-\Delta\Delta Cq}$ method (26).

Flow cytometric analysis. The stromal cells transfected with mmu-miR-96 mimics and negative controls were collected. At 48 h post-transfection, the cells were harvested and washed three times with PBS. Flow cytometry was used to analyze cell cycle progression and apoptosis according to a previous report (27). The experiment was repeated three times.

Western blotting. Cells were harvested for protein extraction using cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Total proteins (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween-20 (PBST) for 1 h at room temperature. Immunoblotting was performed by incubating the membranes in 5% milk-PBST overnight at 4˚C with rabbit monoclonal anti-Bcl2 (1:600; cat. no. sc-509; Santa Cruz, Biotechnology, Inc., Dallas, TX, USA). After washing three times in PBST, the membranes were incubated for 1 h with goat anti-rabbit immunoglobulin G antibody (1:1,000; cat. no. BA1055; Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Bcl2 expression was normalized to β-actin (1:1,000; cat. no. A5441; Sigma-Aldrich; Merck Millipore). The immunoreactive bands were visualized using ChemiDoc™ XRS+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and chemiluminescent reagents (cat. no. WBKLS0500; EMD Millipore). The image collection and densitometric analysis was performed using Quantity One analysis software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression levels were normalized against β-actin.

Fluorescence reporter vector analysis. Fluorescence reporter vector analysis, including generation of the mutated sequence of Bcl2, was conducted with assistance from Tianjin Saierbio Technology Incorporation (Tianjin, China). Enhanced green fluorescent protein (EGFP) cDNA was removed from the pEGFP-N2 vector (Clontech Laboratories Inc., Mountainview, CA, USA) using restriction enzymes and subcloned into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Co. Ltd.). The 3'-untranslated region fragments from Bcl2 containing the predicted mmu-miR-96 binding site were cloned into pcDNA3.1/EGFP constructs at the *Bam*HI and *Eco*RI sites. Four types of vector were constructed in the present study: i) pcDNA3/EGFP/Bcl2; ii) pcDNA3.1(+), iii) pcDNA3.1/pri-miR-96 and iv) mutant vector Mu-pcDNA3/EGFP/Bcl2. Six groups were subsequently established: i) pcDNA3/EGFP/Bcl2; ii) pcDNA3/EGFP/Bcl2 + pcDNA3.1 (+); iii) pcDNA3/EGFP/Bcl2 + pri-96; iv) Mu-pcDNA3/EGFP/Bcl2; v) Mu-pcDNA3/EGFP/Bcl2 + pcDNA3.1 (+); and vi) Mu-pcDNA3/EGFP/Bcl2 + pri-96. The Mu-pcDNA3/EGFP/Bcl2 plasmid included a mutated sequence of Bcl2 that was predicted to interact with mmu-miR-96. The mouse uterine stromal cells were co-transfected in 24-well plates with 0.5 mg EGFP reporter vector and 0.1 mg control vector containing pDsRed-C1 (Invitrogen; Thermo Fisher Scientific, Inc.). EGFP and red fluorescent protein activities were measured using a fluorospectrometer. Plasmids containing the EGFP sequence coded green fluorescence. Measuring the fluorescence intensity can be used to measure the activity of EGFP.

Digital gene expression (DGE) library preparation and sequencing. Total RNA isolated from each sample was used for DGE library preparation. The DGE libraries were established as described previously (28). Briefly, mRNA was reverse transcribed to cDNA, ligated to sequencing adapters, and the products were purified and enriched with PCR to create

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer	Sequence (5'-3')
Mmu-miR-96	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAA
	Forward	TTTGGCACTAGCACATTTTTG
	Reverse	GTGCAGGGTCCGAGGT
U6	RT primer	CGCTTCACGAATTTGCGTGTCAT
	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT
Bcl2	Forward	CGATTGTGGCAGTCCCTTA
	Reverse	CAGGATGAAGTGCTCAGGTG
dtPRP	Forward	AGCCAGAAATCACTGCCACT
	Reverse	TGATCCATGCACCCATAAAA
β-actin	Forward	TCGTGCGTGACATCAAAGAC
	Reverse	CAAGAAGGAA GGCTGGAAAA

miR-96, microRNA-96; Bcl2, B-cell lymphoma 2; dtPRP, decidual/trophoblast prolactin-related protein; RT, reverse transcription.

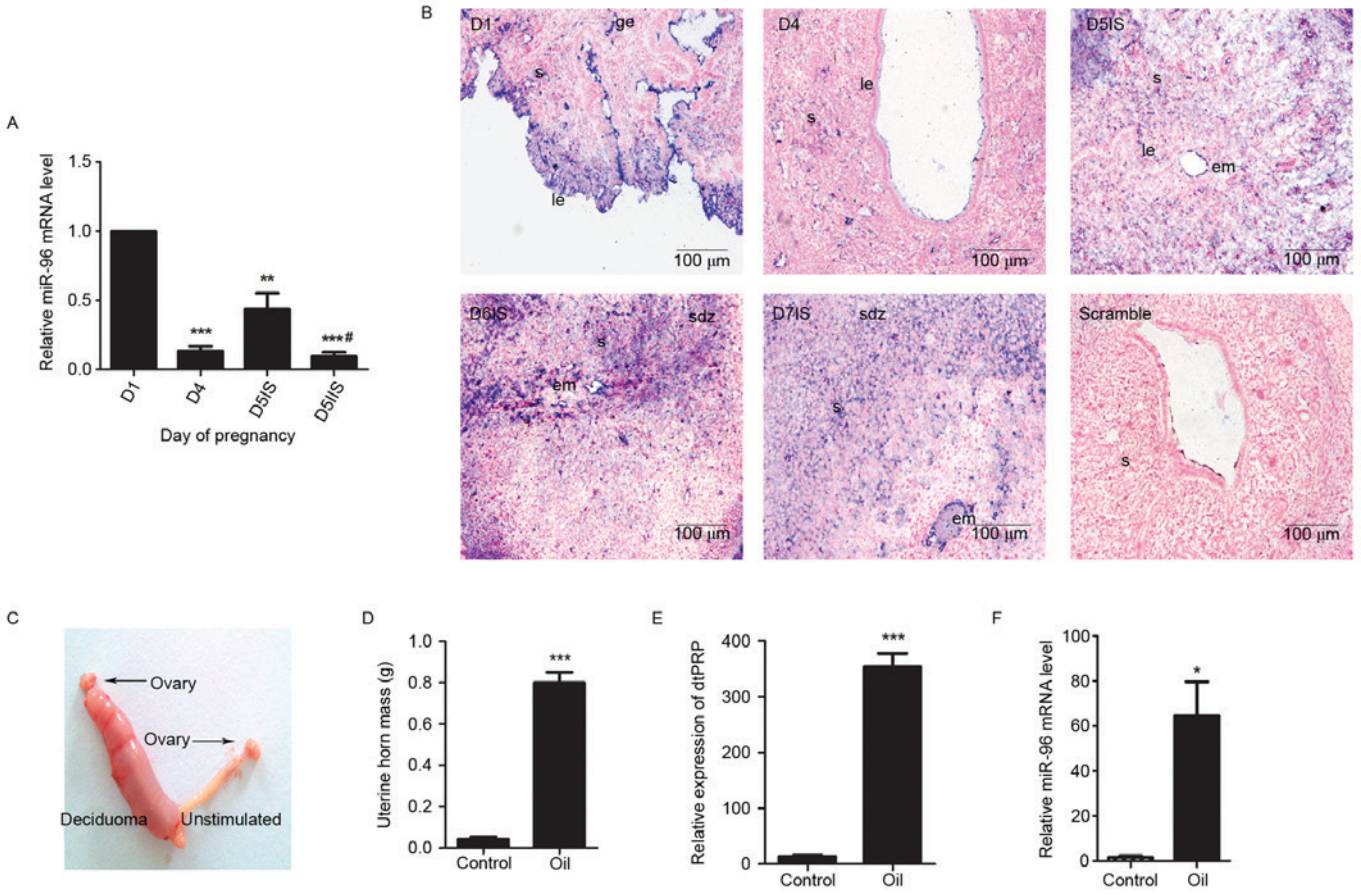

Figure 1. Expression of mmu-miR-96 in mouse endometrium. (A) mRNA expression levels of mmu-miR-96, as detected by RT-qPCR (**P<0.01, ***P<0.001, vs. D1; $^{\#}$P<0.05, vs. D5IS). (B) Location of mmu-miR-96 detected by *in situ* hybridization. Blue represents positive signals. Scrambled represents a negative control. Scale bar, 100 μm. (C) Typical uterine appearance of artificial decidualization. (D) Statistical analysis of uterine horn weight (***P<0.001). (E) Expression of dtPRP detected by RT-qPCR (***P<0.001). (F) Expression of mmu-miR-96 between the infused (oil) and non-infused (control) uterine horns. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-96, microRNA-96; IS, implantation site; IIS, inter-implantation site; dtPRP, decidual/trophoblast prolactin-related protein; le, luminal epithelium; ge, gland epithelium; s, stromal; em, embryo; sdz, secondary decidual zone.

the final cDNA libraries. Subsequently, the libraries were sequenced using the Illumina GAII system (Illumina, San Diego, CA, USA). Statistical analyses were performed, and the significantly differentially expressed genes were determined at a threshold false discovery rate (FDR) and absolute value of the log2 ratio. DGE data analysis was conducted by BGI Tech Solutions Co., Ltd. (Shenzhen, China).

Statistical analysis. The data were analyzed using SPSS13.0 (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to analyze differences between two groups, whereas the differences between numerous groups were detected using one-way analysis of variance followed by Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference. Data were expressed as the mean ± standard error of the mean of experiments repeated at least three times.

Results

Differential expression of mmu-miR-96 in mouse uterus during early pregnancy. The present study detected the temporal and spatial distribution of uterine mmu-miR-96 during early pregnancy. The results of the qPCR analysis were consistent with the miRNA array (data not shown). On day 5 of pregnancy, the expression levels of mmu-miR-96 were much higher at the IS compared with the IIS (Fig. 1A). *In situ* hybridization demonstrated that mmu-miR-96 was mainly located in the luminal epithelia on day 1 (Fig. 1B). Weak positive signals were observed in the stromal cells on day 4, whereas its expression increased in stromal cells and in the embryo on day 5 of pregnancy. Mmu-miR-96 was widely expressed in the second decidual zone (SDZ) on day 6, which was further expanded on day 7 (Fig. 1B). To explore the expression of mmu-miR-96 during decidualization, whilst avoiding influence from the blastocyst, artificial *in vivo* and *in vitro* models of decidualization using stromal cells were employed. Compared with the control horn, the oil-infused horn presented a robust deciduoma (Fig. 1C), and the weight of the deciduoma was greater than that of the control horn (Fig. 1D). Furthermore, expression of the decidualization marker dtPRP was increased in the infused horn (Fig. 1E), which confirmed the model was successfully established. Finally, mmu-miR-96 expression was much higher in the deciduoma, as compared with the control horn (Fig. 1F). These data indicated that mmu-miR-96 may participate in the transformation of stromal cells into decidual cells, or in the regulation of decidual cells to support the subsequent development of the fetus.

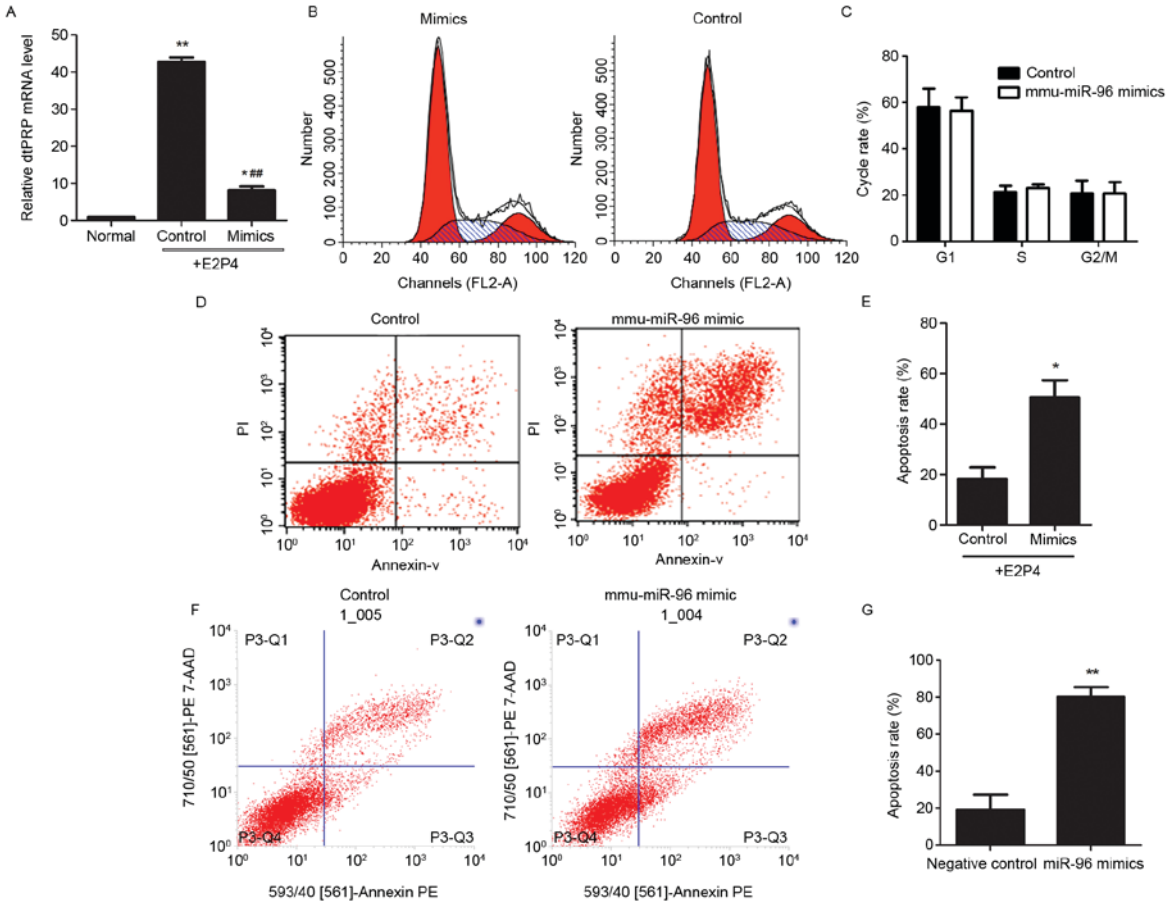

Figure 2. Effects of mmu-miR-96 on stromal cells and decidual cells. (A) Expression of dtPRP in cells transfected with mimics or controls, following hormone treatment with E2P4 detected by RT-qPCR (**P<0.01 vs. normal untransfected cells; $^{\#\#}$P<0.01 vs. control). (B) Cell cycle analysis of stromal cells detected by flow cytometry. (C) Statistical analysis of cell cycle rate. (D) Apoptotic analysis of stromal cells detected by flow cytometry. (E) Statistical analysis of the rate of apoptosis (*P<0.05). (F) Apoptotic analysis of primary decidual cells detected by flow cytometry. (G) Statistical analysis of the rate of apoptosis (**P<0.01). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-96, microRNA-96; E2P4, estradiol-17/progesterone; dtPRP, decidual/trophoblast prolactin-related protein.

Effects of mmu-miR-96 on endometrial decidualization. To identify the role of mmu-miR-96 in the endometrium during early pregnancy, further functional experiments were conducted in primary endometrial stromal cells. Compared with the control group, the mRNA expression levels of dtPRP in the hormone treatment group (E2P4) increased significantly, whereas expression of dtPRP decreased following transfection with the mmu-miR-96 mimic (Fig. 2A). Furthermore, the effects of mmu-miR-96 on the proliferation and apoptosis of stromal cells were investigated. Flow cytometric analysis indicated that mmu-miR-96 expression in stromal cells did not result in changes to the cell cycle (Fig. 2B and C), whereas the rate of apoptosis was markedly increased following overexpression of mmu-miR-96 (Fig. 2D and E). These data indicated that upregulation of mmu-miR-96 in the deciduoma may be involved in the regulation of decidual cells to maintain pregnancy. To address this, primary decidual cells were transfected with mmu-miR-96 mimics, and then the apoptotic rate was observed. Consistently, excessive mmu-miR-96 induced apoptosis in decidual cells (Fig. 2F and G). These data indicated that overexpression of mmu-miR-96 may inhibit the transformation of stromal cells into decidual cells by inducing apoptosis, and that mmu-miR-96 may serve a role in the apoptosis of decidual cells to support the subsequent development of the fetus.

Identification of the target gene of mmu-miR-96. To further determine the molecular mechanism by which mmu-miR-96 induces apoptosis, the target genes of mmu-miR-96 were predicted using TargetScan (http://www.targetscan.org/vert_71/), miRGen (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=mirgenv3%2Findex) and Pictar (www.pictar.org). Three predicted target genes, namely Bcl2, Bcl212, and Bcl213, which are associated with cell apoptosis, were of interest. From this information, combined with our recent gene expression data (data not shown) on day 5 endometrium by DGE analysis, the present study focused on Bcl2. DGE analysis demonstrated differential endometrial expression of Bcl2 between the IS and the IIS (Table II and Fig. 3A), whereas there was no significant difference in Bcl2112 and Bcl2113 expression (data not shown). To verify expression of Bcl2, the levels of Bcl2 protein in the endometrium on days 1, 4, 5, 6 and 7 of pregnancy were detected by western blotting. As shown in Fig. 3B, Bcl2 expression was lowest on day 1 and increased thereafter. On day 5, the levels of Bcl2 protein were higher at IIS compared with at IS (Fig. 3B and C). This finding suggested that the expression of mmu-miR-96 is inversely

Table II. Levels of mmu-miR-96 and Bcl2 mRNA in endometrium on day 5 detected by digital gene expression tag profiling.

	Relative expression on day 5			
Gene	IS	IIS	log2 Ratio (D5IS/D5IIS)	P-value
Mmu-miR-96	21.6031	3.0448	2.82681898	2.15E-37
Bcl2	30.05	89.4	-1.57290984047213	7.39E-33

miR-96, microRNA-96; Bcl2, B-cell lymphoma 2; IS, implantation sites; IIS, inter-implantation sites; D5, day 5.

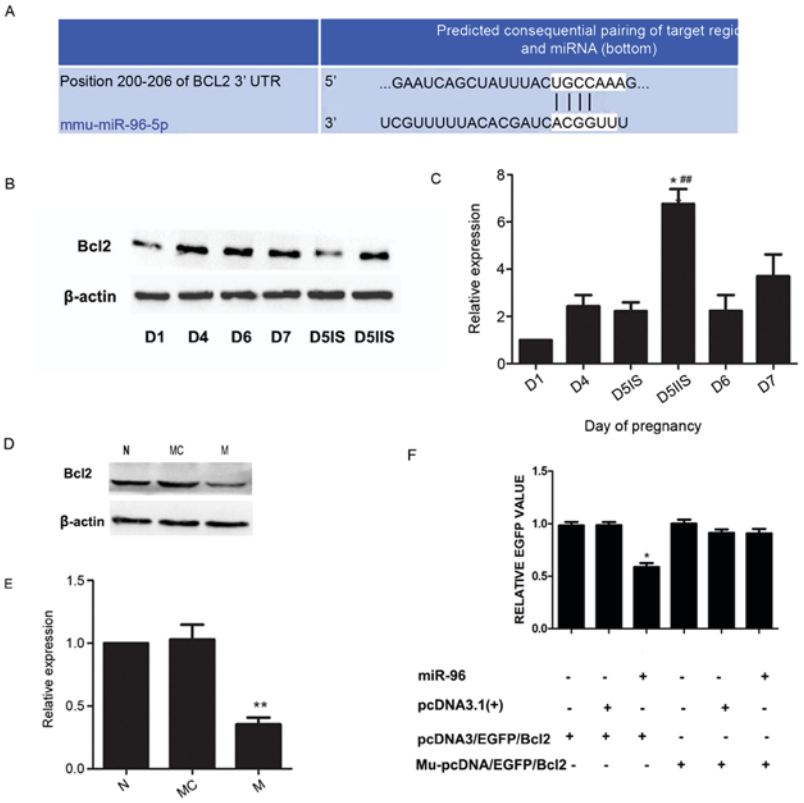

Figure 3. Bcl2 is a target gene of mmu-miR-96. (A) Predicted binding sites between mmu-miR-96 and 3'-UTR of Bcl2 retrieved by TargetScan. (B) Protein levels of Bcl2 in mouse endometrium detected by western blot analysis. β-actin was used as a loading control. (C) Relative protein expression levels of Bcl2 in mouse endometrium, from the western blot analysis shown in B (*P<0.05 vs. D1; $^{\#\#}$P<0.01 vs. D5IS). (D) Expression of Bcl2 in stromal cells post-transfection with mmu-miR-96 mimics. (E) Quantification of relative expression (**P<0.01). (F) EGFP fluorescence reporter vector analysis (*P<0.05). UTR, untranslated region; Bcl2, B-cell lymphoma 2; IS, implantation site; IIS, inter-implantation site; N, untreated cells; MC, negative control of mimics; M, mimics; EGFP, enhanced green fluorescent protein.

correlated with Bcl2 protein levels. Levels of Bcl2 expression were also assessed in the mmu-miR-96 mimic-transfected endometrial stromal cells. The results revealed that Bcl2 protein levels were reduced when mmu-miR-96 was overexpressed in stromal cells (Fig. 3D and E). Finally, a fluorescent reporter gene assay was performed (Fig. 3F). The expression levels of EGFP were lowest in pcDNA3/EGFP/Bcl2 and mmu-miR-96 transfected cells, indicating that mmu-miR-96 significantly reduced EGFP expression compared with the control, whereas the EGFP expression remained unaffected in the Mu-pcDNA3/EGFP/Bcl2 transfected cells. This result confirmed that mmu-miR-96 targets the 3'-UTR of Bcl2 mRNA in stromal cells. These results suggested that mmu-miR-96 regulates expression of Bcl2 in stromal cells and Bcl2 is a target gene of mmu-miR-96. The data indicated that mmu-miR-96 may participate in decidualization by regulating endogenous Bcl2.

Discussion

Following implantation of the blastocyst, the endometrium thickens and the stromal cells differentiate; their morphological transformation from fibroblast cells to larger, multinuclear cells is associated with increasing proliferation and degradation, and this series of changes in the endometrium is called the decidual reaction (29,30). A previous study provided the miRNA signature of human endometrial stromal cells (hESC) during the decidualization process *in vitro* and analyzed the role of the enzyme Dicer during this process (31). Research also illustrated that miR-181a

serves an important role in the decidualization of human endometrial stromal cells by inhibiting kruppel-like factor 12 (KLF12) (32). These studies suggested that miRNAs participate in the regulation of decidualization. The present study demonstrated that mmu-miR-96 is also involved in decidualization by regulating cell apoptosis, and potentially affecting embryo implantation.

miR-96 is generally considered to be an oncogene due to its ability to promote cell proliferation and inhibit cell apoptosis. The present study revealed that mmu-miR-96 expression at IS was higher than at IIS, and was mainly located in stromal cells. Mmu-miR-96 was widely expressed in the SDZ on day 6, which was further expanded on day 7. The rate of apoptosis was markedly increased following overexpression of mmu-miR-96; excessive mmu-miR-96 was able to induce apoptosis of decidual cells. These results suggested that it may be involved in the decidualization of stromal cells or in the maintenance of decidual cells.

Endometrial stromal cells undergo extensive proliferation prior to implantation, followed by differentiation and transformation into decidual cells following implantation. It has previously been reported that miR-96 regulates FOXO1 expression in decidualized hESCs (31). To further investigate whether mmu-miR-96 is associated with endometrial decidualization, artificial decidualization was performed *in vivo* and *in vitro*, and the results indicated that mmu-miR-96 expression was increased in decidual cells compared with untreated groups. It was therefore hypothesized that mmu-miR-96 may affect the process of decidualization through a specific mechanism. In order to investigate this hypothesis, the biological functions of mmu-miR-96 were examined at the cellular level. Upregulation of mmu-miR-96 promoted stromal and decidual cell apoptosis, which is a vital molecular event for stromal cell decidualization, and which is a key factor for successful implantation. Boeddeker and Hess (33) indicated that apoptosis served a major role in the female reproductive tract with regards to different requirements throughout the menstrual cycle, including decidualization and implantation, or menstrual shedding of the non-pregnant endometrium. Therefore, increased apoptosis of stromal cells may have an effect on decidualization and implantation.

mmu-miR-96 may function by reverse regulating its target genes. Chen *et al* (11) revealed a regulatory relationship between miRNAs and mRNAs, and discovered that the miRNAs mmu-miR-96 and mmu-miR-200b target the anti-apoptotic protein Bcl2, as well as Kruppel-like factor 13. Correia-da-Silva *et al* (34) investigated the spatial and temporal pattern of expression of the Bcl2 family members in uterine tissues at the IS, from the post-implantation period to parturition, and demonstrated that the apoptotic mitochondrion-dependent pathway is involved in decidual regression during pregnancy progression. Joswig *et al* (35) confirmed the presence of Bcl2-associated X protein and Bcl2 in mouse decidua, and indicated that both were absent in the uterine epithelium. These findings suggested that Bcl2 has an important role in this process. The present study used computational analysis to predict potential mmu-miR-96 target sequences and found three potential anti-apoptosis-related gene targets: Bcl2, Bcl2112 and Bcl2113. The results demonstrated that Bcl2 expression was higher at IIS compared with at IS on day 5 of pregnancy through DGE analysis, and this result is inverse to the expression pattern of mmu-miR-96 on the same day of pregnancy. In addition, Bcl2 protein expression was inhibited following the upregulation of mmu-miR-96. Finally, a Bcl2-fluorescent reporter assay was used to further confirm that Bcl2 is indeed a target gene of mmu-miR-96.

In conclusion, the present study indicated that mmu-miR-96 affects the apoptosis of stromal cells and decidual cells via regulation of Bcl2, a process that is important in the establishment and maintenance of pregnancy. The mechanism of mmu-miR-96 participation in embryonic implantation should be investigated in greater detail in future studies.

Acknowledgments

The present study was supported by the National Natural Science Foundation of China (grant no. 31501207) and by the Science and Technology Commission of Chongqing, Chongqing, China [grant no. SCXK (YU) 2012-0001].

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