


Endometrial miR-200c is Altered During Transformation into Cancerous States and Targets the Expression of ZEBs, VEGFA, FLT1, IKK β , KLF9, and FBLN5

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Abstract

A number of microRNAs (miRNAs), including miR-200 family, are aberrantly expressed in endometriosis and endometrial cancer. Here we assessed the expression and functional aspects of miR-200c in endometrial tissues (N = 52) from normal endometrial biopsies (N = 15), endometrial tissues including those exposed to hormonal therapies (N = 20), and grade I-III endometrial cancer (N = 17). miR-200c expression was elevated in normal endometrial biopsies from mid- and late-luteal phase, and in endometrial tumors as compared to endometrial tissues from peri- and postmenopausal period ($P < .05$) and its pattern of temporal expression displayed an inverse relationship with the expression of ZEBs. The expression of E-cadherin (CDH1) varied, but expressed at low levels, specifically in endometrial tissues and endometrial tumors. The endometrial expression of ZEBs and CDH1 in patients who were exposed to Depo-Provera and gonadotropin-releasing hormone agonist GnRHa displayed a trend toward lower expression as compared to proliferative phase; however, treatment of Ishikawa cells with 17 β -estradiol, progesterone, and medroxy progesterone acetate had modest effects on the expression of miR-200c and ZEBs without affecting CDH1 expression. Gain of function of miR-200c in Ishikawa cells repressed ZEBs, as well as VEGFA, FLT1, IKK β , and KLF9 expression at transcriptional and translational levels through direct interaction with their respective 3' untranslated regions and increased the rate of their proliferation. These results indicated that endometrial miR-200c expression undergoes dynamic changes during transition from normal into cancerous states; possibly influenced by hormonal milieu and by targeting the expression of specific genes with key regulatory functions in cellular transformation, inflammation, and angiogenesis may influence these events during normal and disease progression.

Keywords

miR-200c, expression, regulation, endometrium, menstrual cycle, cancer

Introduction

MicroRNAs (miRNAs) are a member of family of noncoding small RNAs of ~22 nucleotide (nt) in length and through binding to miR response elements (MREs) within 3' untranslated regions (3'UTRs) of their target genes, posttranscriptionally represses their expression.¹⁻⁴ Through this mechanism, miRNAs regulate many normal cellular activities ranging from cell growth and differentiation, apoptosis, metabolic activities, angiogenesis, and tissue turnover. Conversely, aberrant expression of a number of miRNAs has been closely associated with various human diseases, more specifically events related to cellular transformation, tumorigenesis, inflammation, and tissue fibrosis.¹⁻⁶ In the uterus, conditional inactivation of Dicer, which is required for miRNA processing, resulted in altered expression of a large number of miRNAs, as well as histological abnormalities associated with reduction in size of oviducts and uterine horns, where direct blastocyst transfer did not result in pregnancy.⁷⁻⁹ In human endometrium, several studies, including next generation sequencing have

identified the expression of a large number of miRNAs, some of which showed altered expression in endometriosis and endometrial cancer.¹⁰⁻¹⁷ Among those altered in endometriosis and endometrial cancer, include the members of miR-200 family: miR-200c/141 and miR-200b/200a/429 clusters.^{11,13,15,18-22}

The expression and function of miR-200 family has been well documented in various tissues and cells, where they target the expression of many genes, including ZEBs, the transcription factors that regulate cellular transformation, more specifically

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epithelial-to-mesenchymal transition (EMT) during cancer development and progression through repression of adhesion molecules such as E-cadherin.^{2,5,23} In addition to well-established regulatory function of miR-200 on the expression of *ZEB1* and *ZEB2*, a few studies have also validated *FLT1*, *ETS1*, *SUZ12*, *PDGF*, *TGFB2*, and *conexin43* as direct targets of different miR-200 family members, implicating their functions in angiogenesis and tissue turnover.²⁴⁻³¹

Endometrium during normal menstrual cycle and benign and cancerous states undergoes extensive cellular and molecular changes, including events regulated by miR-200 target genes. However, evidence suggests that miRNAs expression and regulatory function of their target genes occurs in cell- and tissue-specific manners.^{32,33} As such and due to limited information regarding miRNAs regulatory functions in the endometrium, we investigated the expression, regulation, and function of miR-200c and few genes targeted by miR-200 in normal endometrial biopsies and in endometrial tissues obtained from patients undergoing hysterectomy including those who received hormonal therapy, pre- and postmenopausal states, and those with endometrial cancer at different stages of the disease. Using Ishikawa cells as an in vitro model we also assessed the hormonal regulation of miR-200c and either confirmed or validated specific genes targeted by gain of function of miR-200c.

Materials and Methods

Tissue Collection

Endometrial biopsies (N = 15) were obtained from women with previously documented fertility who were requesting permanent surgical sterilization (tubal ligation) under informed consent approved by the University Florida Institutional Review Board prior. These patients were not taking any hormone therapy for the last 3 months prior to collection of the biopsies and based on histological evaluation and the last menstrual period they were from mid- (N = 2) and late-proliferative (N = 1), and early- (ES; N = 5), mid- (MS; N = 3), and late (LS; N = 4)-secretory phases of the menstrual cycle. Similarly, endometrial tissues (N = 20) were also collected from women scheduled to undergo gynecological surgery due to pelvic pain, symptomatic leiomyomas, and uterine prolapse. These patients ranged in age from 23 to 67 years (median = 42.5) and based on endometrial histology and last menstrual period they were from mid-late proliferative (N = 3) and early-mid secretory (N = 5) phases of the menstrual cycle. The tissues also included inactive endometrium from perimenopausal (N = 3) and postmenopausal women (N = 2), and patients who were exposed to gonadotropin-releasing hormone agonist (GnRHa; N = 2) and Depo-Provera (N = 5). Endometrial tissues from women diagnosed with endometrial cancer (N = 17) were obtained from the University of Florida Tissue Bank and based on histological typing conducted according to surgical staging (International Federation of Gynecology and Obstetrics [FIGO])

were from grade I to III. The patients age ranged from 40 to 90 years (median = 65). All the endometrial tissues were collected at the University of Florida affiliated Shands Hospital with prior approval from the Institutional Review Board. After collection the endometrial tissue samples were snap frozen and kept in liquid nitrogen until further analysis.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from a small portion of the endometrial tissues using Trizol reagent, and their quantity and quality was assessed using ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Two μg or 10 ng of total RNA (for miRNA) was reverse transcribed using random primers for gene expression or specific stem-loop primers for miRNA expression (Applied Biosystems Foster City, California). Real-time polymerase chain reaction (PCR) was carried out using TaqMan reagents. Reactions were incubated for 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minutes at 60°C, and level of messenger RNAs (mRNAs) and miRNAs expression was determined using Applied Biosystems 7300 Detection System with 18S rRNA and *RNU6B* used for normalization, respectively. All reactions were run in triplicate and relative expression was analyzed with the comparative cycle threshold method using $2^{-\Delta\Delta\text{CT}}$ method according to the manufacturer's guidelines.

Cell Culturing and Transfection

Ishikawa cells were cultured in Dulbecco modified Eagle medium (DMEM)/F12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) of antibiotic and antimycotic solution. All the supplies for cell culture were purchased from Sigma-Aldrich (St Louis, Missouri), Invitrogen (Carlsbad, California), and Fisher Scientific (Pittsburgh, Pennsylvania). The cells were seeded at various densities in 6-well plates or 10 cm petri dishes and cultured in antibiotic-/antimycotic-free media until reaching 70% confluence and then transfected with 50 nmol/L of pre-miR negative control (NC) or pre-miR-200c (Ambion/Applied Biosystems) using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. After transfection, the cells were cultured for an additional 24 and 48 hours, respectively, and total RNA and protein were isolated and subjected to real-time PCR and Western blot analysis.

Ovarian Steroid Treatments

Ishikawa cells were cultured in 6-well plates as above for 24 hours, washed, and incubated in phenol red-free media with charcoal-stripped FBS for additional 24 hours and then treated with 17 β -estradiol (E2), progesterone (P4), or medroxy progesterone acetate (MPA; Sigma) at 10⁻⁸ mol/L concentration for 6 and 24 hours. Total RNA was isolated

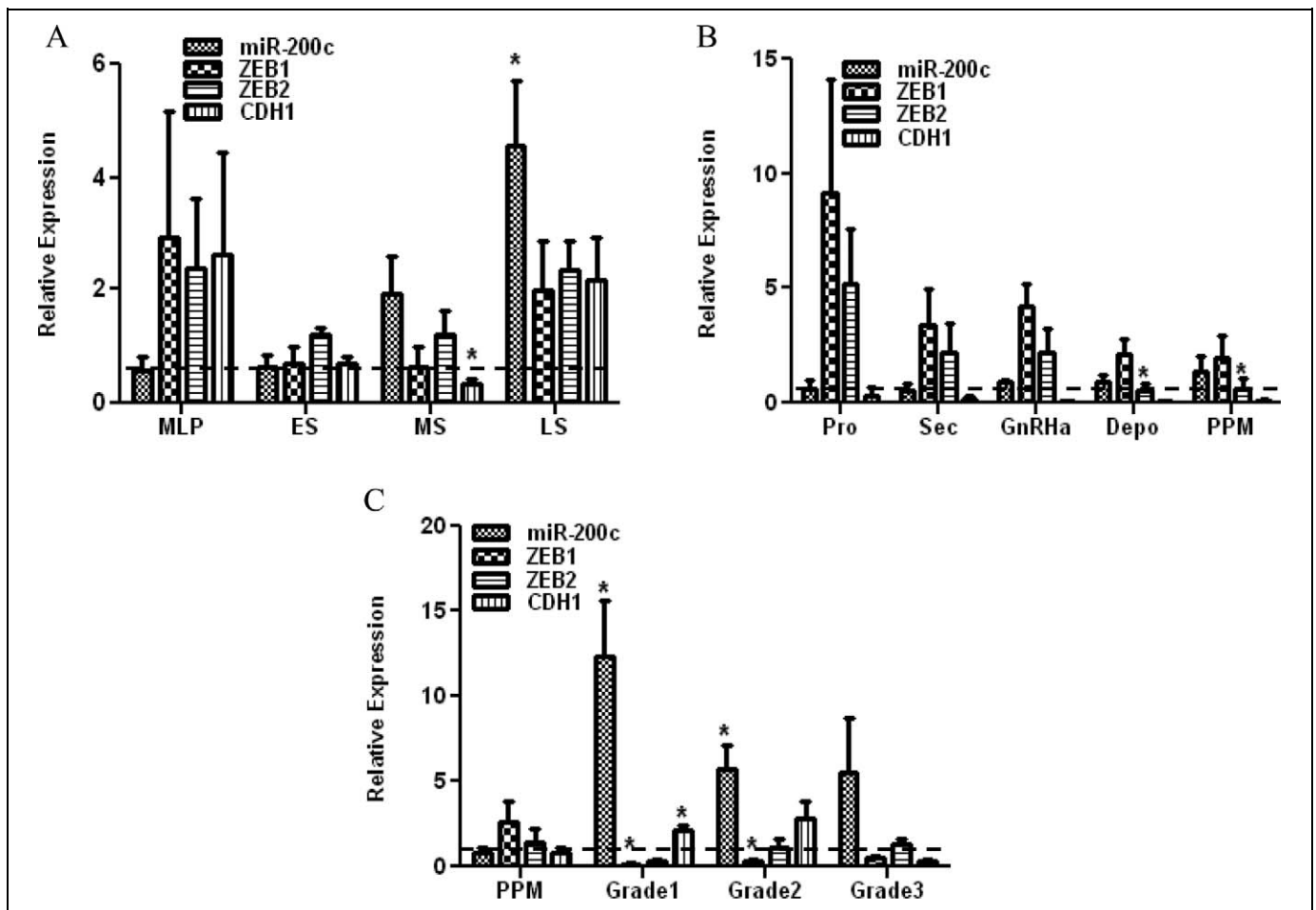


Figure 1. Relative expression of miR-200c, *ZEB1*, and *ZEB2*, in endometrial biopsies (A) from mid-late proliferative phase (MLP), early- (ES), mid- (MS), and late (LS)-secretory phase of the menstrual cycle and endometrial tissues (B) from proliferative (Pro) and secretory (Sec) phases of the menstrual cycle, peri- and postmenopausal period (PPM), women exposed to GnRH agonist (GnRH) and Depo-Provera (Depo), and grade I, II, and III endometrial cancer (C). The results are presented as mean \pm standard error of the mean (SEM) and analyzed using analysis of variance (ANOVA) or nonparametric student *t* test (* $P < .05$ as compared to levels in mid-late proliferative phase [MLP] in Figure A, proliferative phase [Pro] in Figure B and peri- and postmenopausal period [PPM] in Figure C).

and subjected to quantitative (Q)-RT-PCR as described above.

Luciferase Reporter Assay

Ishikawa cells were seeded in 6-well plates and transfected with pre-miR200c or NC, as described above. After 24 hours, the medium was changed and the cells were transfected with dual luciferase reporter plasmid containing 3'UTR of *ZEB1*, *ZEB2*, *VEGFA*, *FBLN5*, *KLF9* (Genecopoeia, Rockville, Maryland), *IKK β* (gift from Dr Gil Mor³⁴), and *NF κ B* reporter plasmid containing *NF κ B* response element (Signosis Inc, Sunnyvale, California) using Lipofectamine 2000 (Invitrogen). For *IKK β* 3'UTR reporter assay and *NF κ B* reporter assay, the cells were co-transfected with either *IKK β* 3'UTR reporter plasmid or *NF κ B* reporter plasmid along with pRL-TK plasmid (Promega, Madison, Wisconsin) encoding Renilla luciferase (0.2 μ g/well) as a control for assessing the transfection efficiency. Firefly and

Renilla luciferase activities were measured after 48 hours using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction, with some modification for measuring *NF κ B* reporter assay and *IKK β* 3'UTR luciferase activity, according to recommendation from GeneCopoeia. Firefly luciferase activity was normalized to Renilla luciferase activity and the level of induction was reported as the mean \pm standard error of the mean (SEM) and compared with a ratio in cells transfected with pre-miR negative control (NC). Each experiment was done in triplicates.

Western Blot Analysis

Total protein was isolated from Ishikawa cells and following centrifugation, supernatants were collected and protein concentrations were determined by standard method (Pierce BCA Protein Assay Kit, Fisher Scientific). Aliquots of 30 μ g of total protein were subjected to Western blot analysis

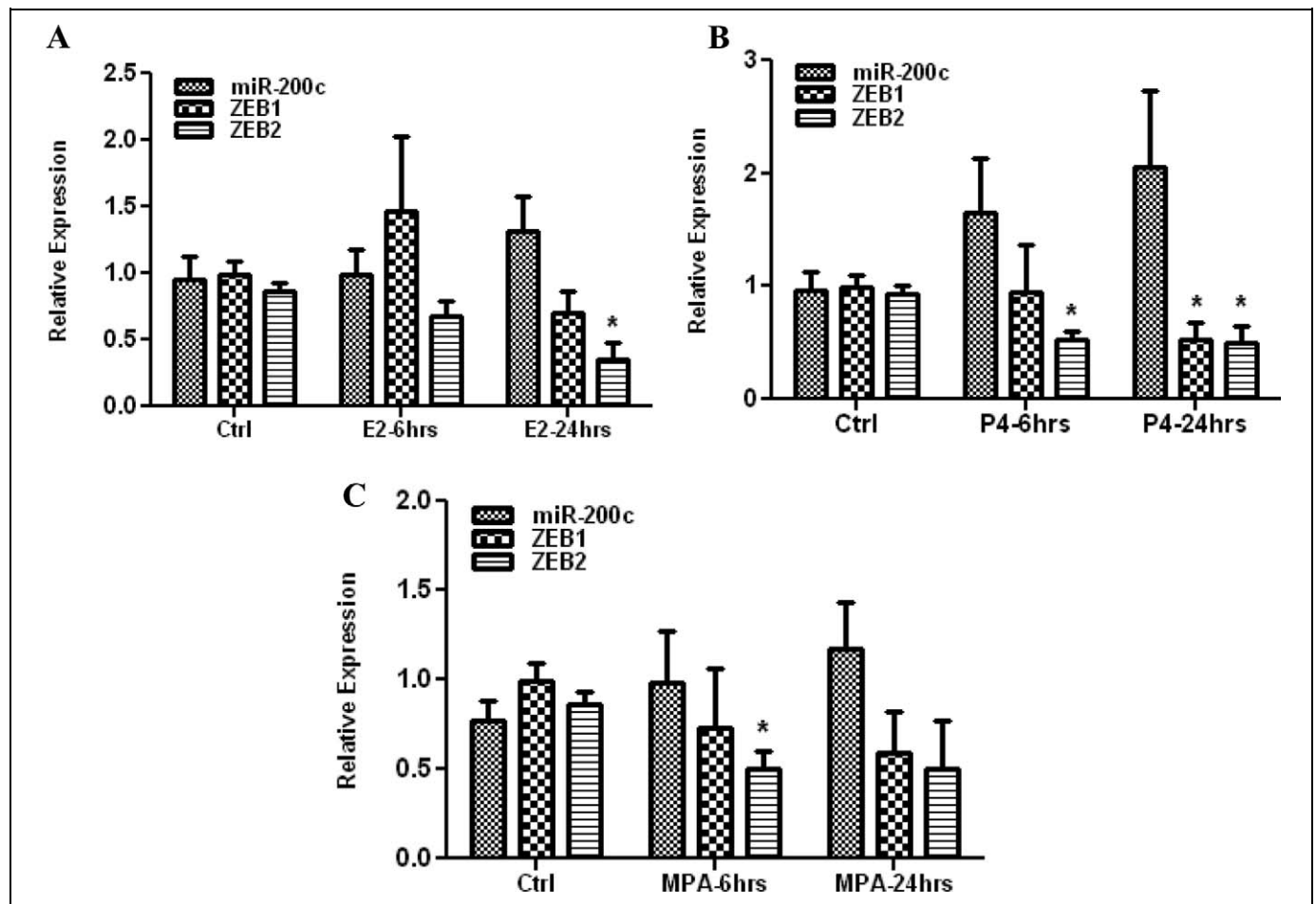


Figure 2. Relative expression of miR-200c, ZEB1, and ZEB2 in Ishikawa cells treated with 17- β Estradiol (E2) (A), progesterone (P4) (B), or medroxy progesterone acetate (MPA) (C) for 6 and 24 hours as compared to untreated control (Ctrl). The results are presented as mean \pm standard error of the mean (SEM) and analyzed by analysis of variance (ANOVA) and nonparametric student *t* test (**P* < .05 from their respective Ctrl).

and transferred to polyvinylidene difluoride (PVDF) membrane, and then the membranes were probed with primary antibodies (dilution:1:200) generated against ZEB1, ZEB2, VEGFA, FLT1, FBLN5, TIMP2, IKK β , and KLF9 (Cell Signaling Technology, Danvers, Massachusetts; Abcam Inc, San Francisco, California; Santa Cruz Biotechnology, Santa Cruz, California). α -tubulin (1:2000) was used for normalization and loading control. The membranes were exposed to horseradish peroxidase-labeled secondary antiserum and immunoreactive proteins were detected with the ECL Western Blotting Substrate (Fisher Scientific).

Cell Proliferation Assay

Ishikawa cells were transfected with pre-miR-200c or pre-miR NC and the rate of their proliferation was determined after 1 to 3 days posttransfection, using CellTiter 96 Cell Proliferation Assay (Promega), according to manufacturer's instruction. The rate of proliferation was calculated independently for days 1, 2, and 3 for pre-miR-200c transfected cells and compared to the same day control NC, respectively.

Statistical Analysis

All in vitro experiments were performed in 3 sets of independent assays in duplicates. Where appropriate, the results were reported as mean \pm SEM and statistically analyzed using nonparametric Student *t* test for comparisons between 2 groups or analysis of variance (ANOVA) followed by post hoc pairwise multiple comparisons among several groups. Pearson correlation coefficient was used to assess correlation between miR-200c and ZEBs expression. *P* values of <.05 were considered significant. GraphPad Prism 4.0 software was used for all data analysis.

Results

The Expression of miR-200c is Altered During Endometrial Transition into Disease States

We first determined the expression of miR-200c in endometrial tissues (N = 52) consisting of biopsies obtained from normal menstrual cycle, endometrial tissue from untreated

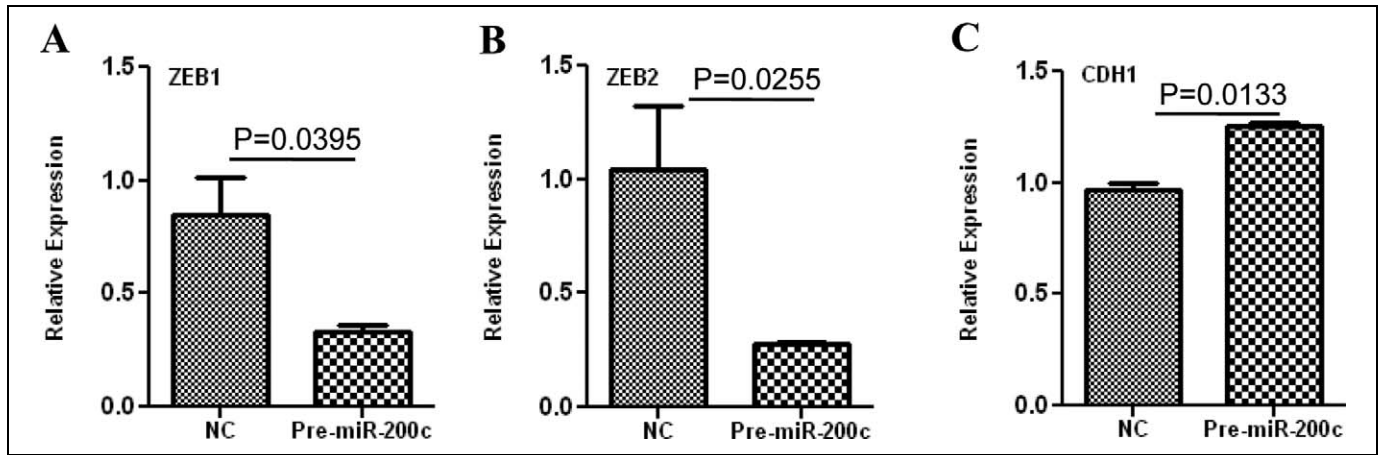


Figure 3. Relative expression of *ZEB1* (A), *ZEB2* (B), and *CDH1* (C), in Ishikawa cells transfected with pre-miR-200c or pre-miR negative control (NC) determined by real-time polymerase chain reaction (PCR). The results are presented as mean \pm standard error of the mean (SEM) and analyzed using nonparametric student t test with *P* values indicated by corresponding lines.

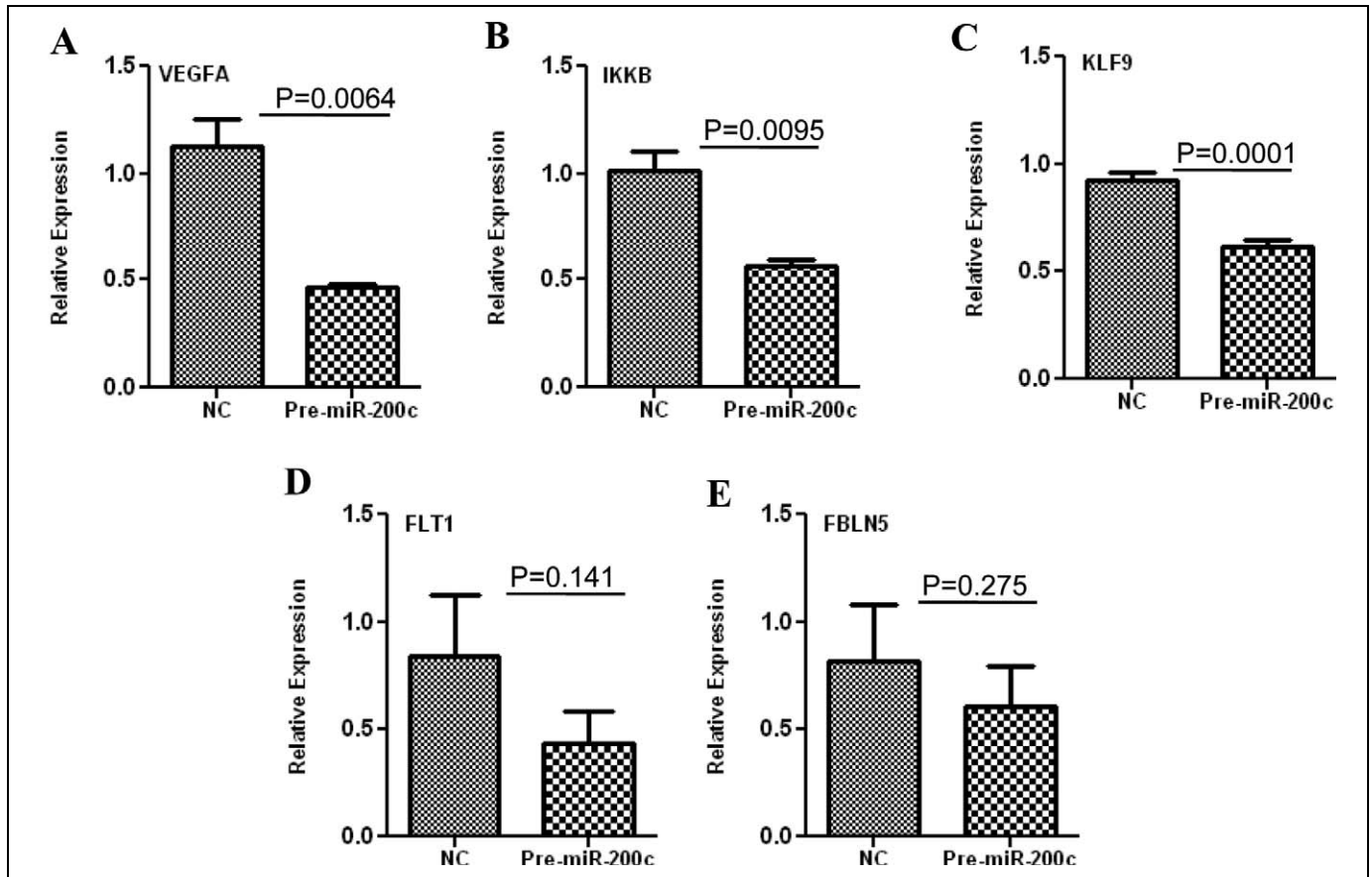


Figure 4. Relative expression of *VEGFA* (A), *IKKβ* (B), *KLF9* (C), *FLT1* (D) and *FBLN5* (E) in Ishikawa cells transfected with pre-miR-200c and pre-miR negative control (NC) determined by real-time polymerase chain reaction (PCR). The results are presented as mean \pm SEM and analyzed using nonparametric unpaired student t-test with *p* values indicated by corresponding lines.

and patients exposed to different hormonal therapies, and grade I-III endometrial cancer. The relative expression of miR-200c varied among these cohorts with endometrial biopsies and was higher in mid-secretory (MS; $P = .052$) and late-secretory (LS) phase (P

$= .050$), as compared to mid-late proliferative (MLP; Figure 1A). The relative expression of miR-200c did not significantly change (ANOVA) in endometrial tissues from secretory (Sec) phases of the menstrual cycle, peri- and postmenopausal period (PPM) with

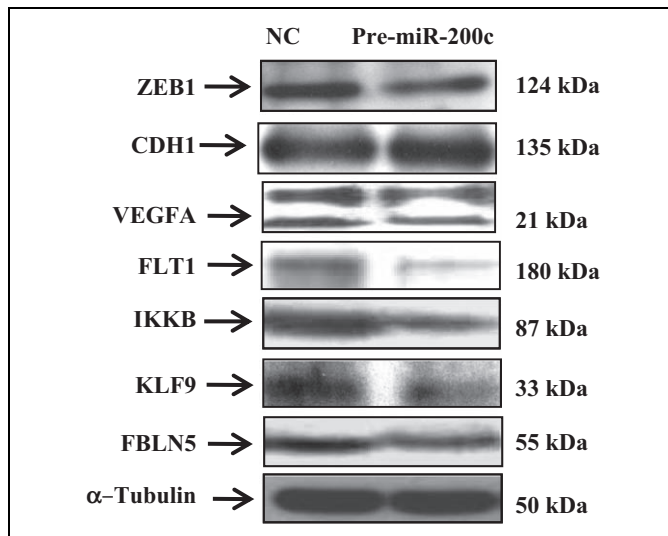


Figure 5. Western blot analysis of *ZEB1*, *CDH1*, *VEGFA*, *IKKβ*, *KLF9*, *FLT1* and *FBLN5* in Ishikawa cells transfected with pre-miR-200c and pre-miR negative control (NC) with α -Tubulin serving as loading control.

inactive endometrium, or women who were exposed to GnRHa and Depo-Provera (Depo; Figure 1B), when compared to the proliferative (Pro) phase. However, the level of miR-200c expression was significantly elevated in grade I and II endometrial tumors as compared to PPM period ($P = .0079$, $P = .0376$; Figure 1C). We next examined the expression of *ZEB1* and *ZEB2* and their downstream target genes, E-cadherin (*CDH1*), in these tissues and found variable level of expression in endometrial biopsies, which displayed a trend toward lower levels in ES and MS (Figure 1A). In endometrial tissues, the highest level of *ZEB1* and *ZEB2* was detected during proliferative phase, with a gradual reduction in tissues from secretory phase, GnRHa, Depo, and PPM groups (Figure 1B). In endometrial tumors from grade I and II, the level of *ZEB1*, but not *ZEB2*, was significantly lower ($P = .007$, $P = .005$) with increased *CDH1* levels ($P = .0317$, $P = .0734$), as compared to tissues from PPM (Figure 1C). Although there was no statistically significant correlation between miR-200c and *ZEBs* expression among these tissues, the pattern of miR-200c expression, specifically in endometrial tissues, and endometrial tumors, was inversely related to *ZEBs*, specifically *ZEB1* expression (Figure 1B and C).

Ovarian Steroids had Variable Effects on the Expression of miR-200c and Target Genes

We next assessed the influence of ovarian steroids on miR-200c, *ZEBs*, and *CDH1* expression using Ishikawa cell as an in vitro model. As shown in Figure 2, treatments with E2, P4, and MPA had variable effects on the expression of miR-200c, which tend to increase with time in all the cases; however, comparisons did not reach statistical significance. The expression of *ZEBs* was repressed by E2, P4, and MPA in a time-dependent manner, with E2 inhibition of *ZEB2* after 6 hours, P4 inhibition of *ZEB1* and *ZEB2* after

24 hours, and MPA inhibition of *ZEB2* after 6 hours ($P < .05$; Figure 2). There was no significant difference between P4 and MPA effects on miR-200c and *ZEBs* expression. In all the cases, there was no significant change in *CDH1* expression (data not shown) which was expressed at very high levels.

miR-200c Targets the Expression of *ZEBs*, *VEGFA*, *FLT1*, *IKKβ*, *KLF9*, and *FBLN5* and Alters Cellular Proliferation

ZEBs are well established as direct targets of miR-200 family, including miR-200c; however, miRNAs expression and regulatory function of their target genes has been shown to occur in cell- and tissue-specific manners.^{32,33} As such using Ishikawa cells, we also found that gain of function of miR-200c repressed the expression of *ZEB1* and *ZEB2* at mRNA levels (Figure 3A and B), and *ZEB1* at protein level (Figure 5); however, *ZEB2* expression was too low and could not be detected by Western blot analysis. Overexpression of miR-200c in Ishikawa cells was also accompanied by elevated expression of *CDH1* at both mRNA (Figure 3C) and protein levels (Figure 5). Gain of function of miR-200c repressed *ZEBs* expression through direct interaction with their respective 3'UTR as determined by luciferase reporter assay (Figure 6A and B). In addition to *ZEBs*, miR-200c is predicted to target the expression of *VEGFA*, *FLT1*, *IKKβ*, *KLF9*, *FBLN5*, and *TIMP2* (Table 1). As such we extended our investigation and further demonstrated that gain of function of miR-200c also repressed the expression of *VEGFA*, *IKKβ*, and *KLF9* at mRNA (Figure 4A-C) and protein (Figure 5) levels and reduced the expression of *FLT1* and *FBLN5* at protein (Figure 5), but not at mRNA levels (Figure 4D and E) without affecting *TIMP2* expression either at mRNA or protein level (Supplementary Figure 1). Gain of function of miR-200c repressed *VEGFA*, *IKKβ*, and *KLF9* expression through direct interaction with their respective 3'UTRs (Figure 6C-E), but not with *FBLN5* 3'UTR (Supplementary Figure 1) as determined by luciferase reporter assay. Since miR-200c directly regulated the expression of *IKKβ*, a key cofactor in *NFκβ* activation, we examined and found that gain of function of miR-200c significantly decreased *NFκβ* activity in Ishikawa cells (Figure 7). In addition, gain of function of miR-200c increased the rate of Ishikawa cell proliferation during the 3 days of incubation as compared to pre-miR negative control (NC) transfected cells (Figure 8).

Discussion

Using a large cohort of endometrial tissues from normal, benign, and cancerous conditions our results indicated that the endometrial miR-200c expression undergoes dynamic changes that reflected possible influence of hormonal milieu and its correlation with disease progression. The endometrial expression of miR-200c to some extent inversely correlated with the expression of *ZEBs*, a well-established target of miR-200 family, however the pattern of their expression did not reflect such regulatory interactions, specifically in endometrial cancer,

Table 1. The miR-200c seed sequence and its complementary binding site on respective 3' untranslated region (UTR) of its predicated target genes (represented in bold letters)

Position 369-375 of ZEB1 3'UTR hsa-miR-200c	5'AUUGUUUUUAUCUUUA CAGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 454-461 of ZEB2 3'UTR hsa-miR-200c	5'CGCACUACAAUGCAU CAGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 901-907 of IKBKB 3'UTR hsa-miR-200c	5'UCUCACAGCAUCUAG CAGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 1298-1304 of VEGFA 3'UTR hsa-miR-200c	5'AGUAGGGUUUUUUUU CAGUAUUC ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 68-74 of FLT1 3'UTR hsa-miR-200c	5'GAAACUAGCUUUUGCC CAGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 3041-3047 of KLF9 3'UTR hsa-miR-200c	5'AGUUUUUAAUUUUUGUA CAGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 2447-2452 of TIMP2 3'UTR hsa-miR-200c	5'AAAUCUUUGCUUGAU AGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...
Position 222-227 of FBLN5 3'UTR hsa-miR-200c	5'GACACAGUUAUCAAAA AGUAUUA ... 3'AGGUAGUAAUGGGCC GUCAUAAU ...

since loss of miR-200 family and overexpression of ZEBs accompanied by loss of *CDHI* has been closely associated with cellular transformation and tumorigenesis in various tissues. In this context, a previous study reported the presence of immunoreactive *ZEB1* only in endometrial stromal cells which increased during the secretory phase³⁵ and in low-grade endometrial cancer with aberrant expression in epithelial-derived tumor.³⁶ Real-time PCR analysis in our study indicated a trend toward lower expression of *ZEBs* in normal and benign endometrial tissues during the secretory phase and all grades of endometrial cancer, whereas our results with miR-200c expression in endometrial cancer is supported by two recent reports showing elevated levels of all miR-200 family, including miR-200c in all stages of endometrial cancer.^{22,37} Since miRNA regulatory functions have been reported to be tissue and cell specific,^{32,33} differences in miR-200 and *ZEBs* expression in normal, benign and cancer states may reflect such specificity, not only in regulating the specific gene/genes at given stage, but also in differences in expression within other tissues. Although transcriptional decay of miRNA target genes may not correspond to the level of miRNA expression, correlating translational repression of a given target genes may provide some possibility of establishing a functional relationship between miRNA and their target genes.¹

Using Ishikawa cells with epithelial characteristics as an in vitro model, we found low levels of *ZEBs*, specifically *ZEB2* and high level of *CDHI* expression, corresponding to *ZEB1* levels reported in endometrial epithelial cell layer.³⁸ However, overexpressing or blocking miR-200c which targets *ZEBs* expression did not cause any noticeable changes in Ishikawa cell morphology, possibly due to high basal expression of *CDHI*. With regard to the hormonal influence, ovarian steroids modestly altered the expression of miR-200c and *ZEBs*, with P4 and MPA increasing miR-200c expression while inhibiting *ZEBs*, which reflected the patterns seen at tissue level corresponding to natural hormonal milieu. Treatment of isolated

human myometrial smooth muscle cells or ovariectomized mice with P4 or estrogen has been shown to increase the expression of *ZEB1*³⁵ whereas P4 inhibited miR-200b and miR-429 expression in T47D, breast cancer cell line.³¹ Several studies have demonstrated differential influence of ovarian steroids on miRNAs expression in several cell lines, including endometrial cancer cells, through a mechanism possibly involving transcriptional regulation of their processing enzymes.³⁹

There is considerable evidence in support of miR-200 family regulation of *ZEBs* in several cell types from different origins.^{31,35,40-44} We also confirmed that *ZEBs* are direct targets of miR-200c in Ishikawa cells. The importance of endometrial expression and regulation of *ZEBs* may be related to their regulatory influence on the expression of number of genes functionally involved in cell cycle progression, differentiation, and cellular transformation, specifically EMT during tumorigenesis.⁴⁵⁻⁴⁷ Endometrium during normal menstrual cycle, progression into pre- and postmenopausal states, endometriosis, and endometrial cancer, undergoes significant morphological changes involving many cellular events regulated by miR-200 target genes. We demonstrated that miR-200c either directly or through indirect mechanism regulates the expression of *VEGFA*, *FLT1*, *IKK β* , *KLF9*, and *FBLN5*, without affecting *TIMP2* expression. This finding may be biologically significant due to a wide range of cellular activities regulated by these genes; *KLF9* serves as transcription factor, *VEGF*, *FLT1*, and *FBLN5* are angiogenic factors; *IKK β* , is an inflammatory mediator; *FBLN5* supply the extracellular matrix protein essential for elastic fiber assembly and vasculogenesis; and *TIMP2* is physiological inhibitor of *MMPs* known to regulate inflammation, matrix degradation, and cellular invasion. Although our finding with miR-200c regulation of *KLF9* is the first of such validation, others reported *VEGFA*, *FLT1*, *ETS1*, *SUZ12*, *PDGF*, *TGF- β 2*, and *conexin43*, as well as Notch pathway components, such as Jagged1 (*JAG1*) and mastermind-like

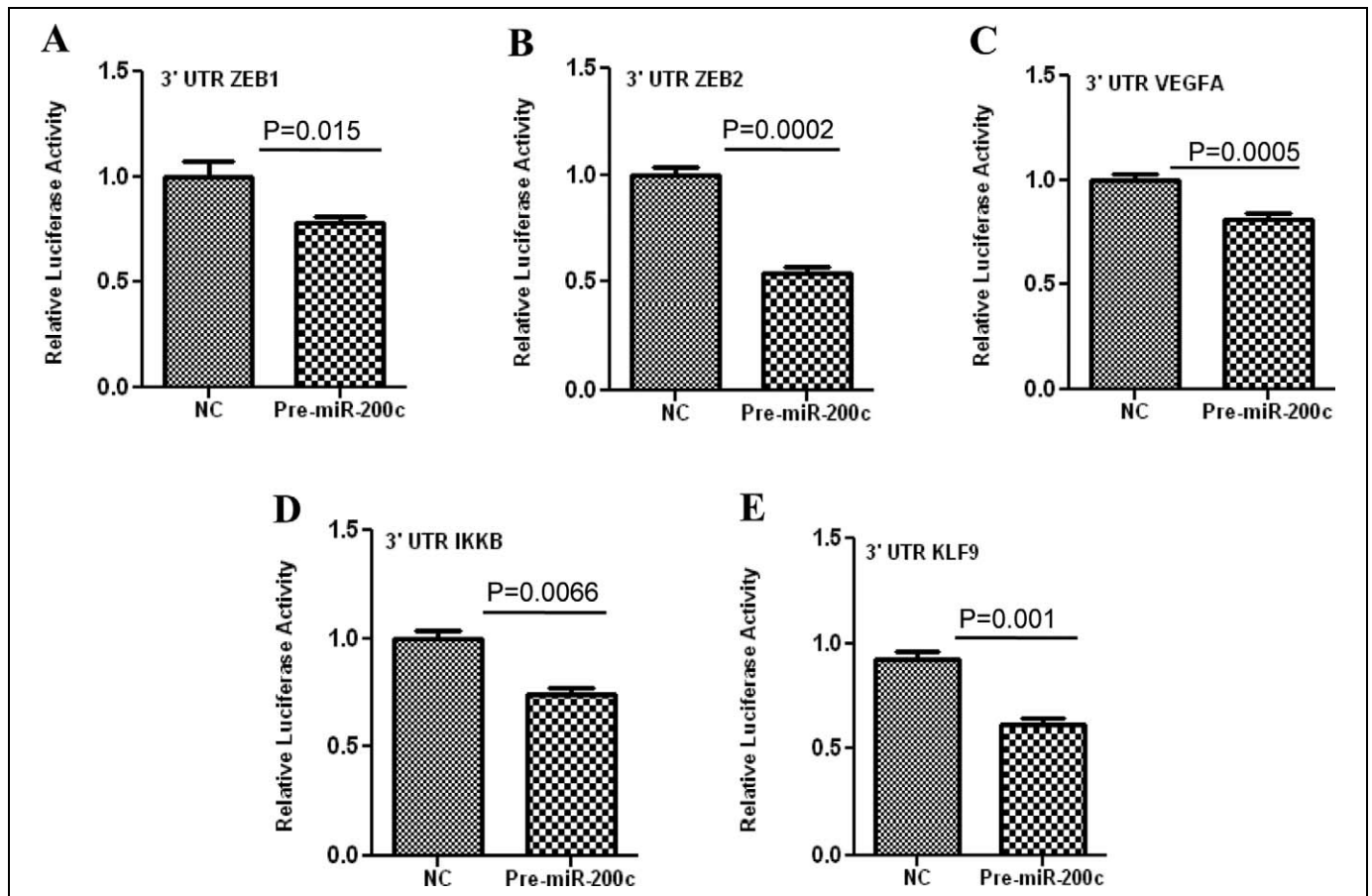


Figure 6. Firefly luciferase assay with *pZEX-MT01* or *pGL3-Luc* constructs carrying a 3' untranslated region (3' UTR) fragment of *ZEB1* (A), *ZEB2* (B), *VEGFA* (C), *IKKβ* (D), and *KLF9* (E), respectively. Ishikawa cells were co-transfected with firefly luciferase reporters, Renilla luciferase transfection control plasmid (in case of *IKKβ*), pre-miR-200c, or pre-miR negative control (NC). The ratio of firefly: Renilla was determined and reported as relative luciferase activity as compared to empty vector and analyzed using non-parametric student t-test with p values indicated by corresponding lines.

coactivators, *MAML2* and *MAML3*, as direct targets of different member of miR-200 family.^{24-30,41}

Because of the lack of adequate endometrial biopsies and endometrial cancer tissues, we could not determine the coexpression of *VEGFA*, *FLT1*, *IKKβ*, and *KLF9* in the same tissues; however, several studies reported their expression in human endometrium with altered or aberrant expression in endometriosis and endometrial cancer.⁴⁸⁻⁵⁸ The biological significance of *VEGFA* and their receptors (*FLT1* and *VEGFR1*), in various activities, including angiogenesis, is well established, including in normal endometrium throughout the menstrual cycle, dysfunctional uterine bleeding, and endometrial repair and endometrial cancer.^{53,54,57} Additionally, *IKKβ* by serving as a cofactor for activation of *NFκβ* pathway plays a key role in regulating the expression of many proinflammatory mediators, including interleukin 8.^{48,59} Several miRNAs have been predicted to target *IKKβ* and in addition to our finding with miR-200c a recent report also demonstrated *IKKβ* as a direct target of miR-199a.^{34,59} Our results also suggest that miR-200c via downregulation of *IKKβ* may regulate *NFκβ* activation, which has been shown to upregulate Snail expression and induce EMT.⁶⁰ *KLF9* serves as a key

regulator of genes functionally involved in cellular proliferation, differentiation, apoptosis, and tumorigenesis whose expression, and to some extent function, has been well documented in the uterus with altered expression in endometriosis and endometrial cancer.^{56,61,62} We found that *KLF9* is a direct target of miR-200c, and since gain of function of miR-200c increased the rate of Ishikawa cell proliferation, we speculate that this increase in the cell proliferation is mediated through *KLF9* repression, similar to the results reported earlier using *KLF9* siRNA approach.⁶² Since several other miRNAs are predicted to target the expression of these genes, further investigation is needed for establishing the complex regulatory interaction among these genes and miRNAs that target their expression.

In summary, using a cohort of normal, benign, and cancerous endometrial tissues we found a dynamic temporal pattern of miR-200c expression and confirmed and/or validated a number of genes, including *ZEBs*, *VEGFA*, *FLT1*, *IKKβ*, and *KLF9* and *FBN5*, as direct and/or indirect targets of miR-200c regulatory function. Considering the importance of these gene products in various cellular activities ranging from cell growth, differentiation, inflammation, angiogenesis, cellular transformation, and

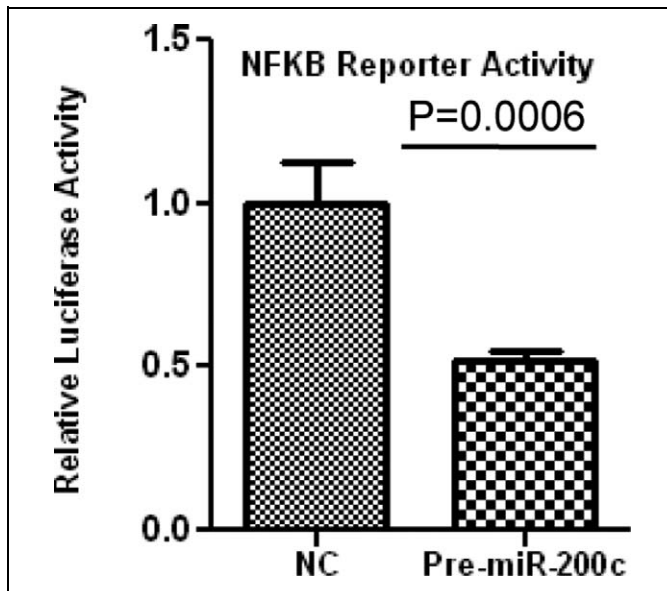


Figure 7. Firefly luciferase assay with pTF-Luc reporter constructs carrying *Nfkβ* response element to determine *Nfkβ* activation following pre-miR-200c transfection. Ishikawa cells were co-transfected with firefly luciferase reporter, Renilla luciferase transfection control plasmid, pre-miR-200c, or pre-miR negative control (NC). The ratio of firefly–Renilla was determined and reported as relative luciferase activity and analyzed using nonparametric unpaired student *t* test and *P* values is indicated by corresponding line.

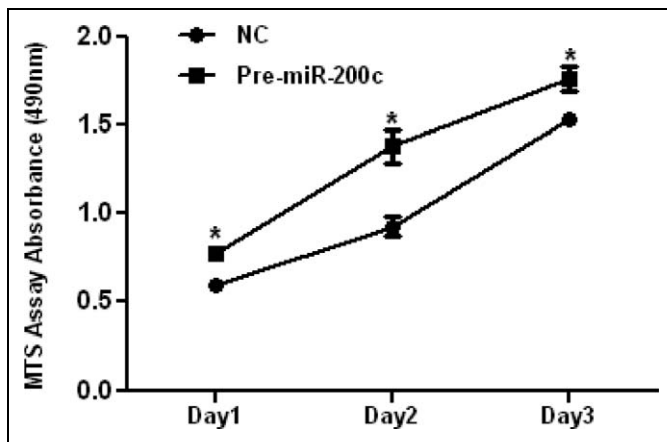


Figure 8. The effect of gain of function of miR-200c on Ishikawa cell proliferation. The cells were transfected with pre-miR-200c or pre-miR negative control (NC) and cell proliferation was determined using MTS assay on indicated days. The results are shown as mean \pm standard error of the mean (SEM) and analyzed using nonparametric student *t* test (**P* < .05 as independently compared to corresponding negative control for each day).

tissue turnover that led either to cellular transformation and tumorigenesis or tissue fibrosis, miR-200 family including miR-200c could play a key role in regulating these processes in normal endometrium and in disorders such as endometriosis, abnormal uterine bleeding, and endometrial cancer (Figure 9). While the goal of this study was to better understand the

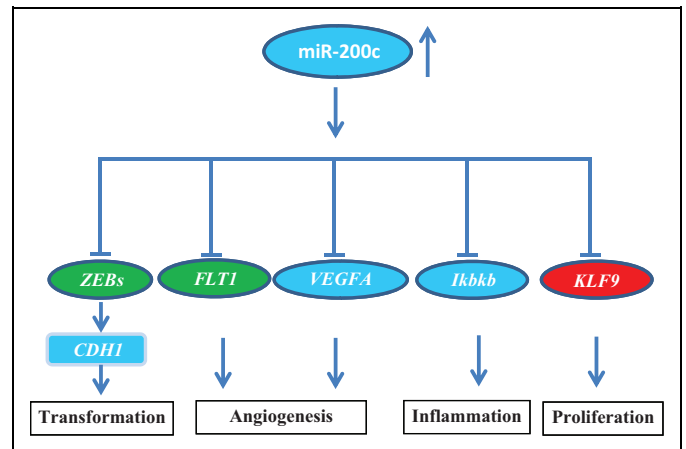


Figure 9. Schematic presentation of miR-200c and specific genes either directly and/or indirectly regulated by miR-200c in endometrium. Through such regulatory functions altered expression miR-200c could influence cell proliferation, migration, inflammation, and tissue turnover, events with central roles in normal cellular activities as well as in the outcome of disorders such as endometriosis and endometrial cancer and associated symptoms.

regulatory function of miR-200c in endometrial tissue, future investigation is required to address the role of miR-200c and other members of this family in endometrium and its diseased states such as endometriosis and endometrial cancer.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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