***miR-181b-5p* Modulates Cell Migratory Proteins, Tissue Inhibitor of Metalloproteinase 3, and Annexin A2 During In Vitro Decidualization in a Human Endometrial Stromal Cell Line**

**Amanda Graham, BS¹, Joshua Holbert, BS¹,
and Warren B. Nothnick, PhD, HCLD¹**

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Abstract

Decidualization is essential for successful embryo implantation and is regulated by concerted actions of growth factors and hormones. More recently, microRNAs, small RNA molecules that regulate posttranscriptional gene expression, have been implicated to play a role in the decidualization process. Of these microRNAs, *miR-181b-5p* has been associated with decidualization but its precise role and targets are not well established. To address this gap in our knowledge, we assessed the expression of *miR-181b-5p*, and its target tissue inhibitor of metalloproteinase 3 (TIMP-3), during in vitro decidualization using the well-characterized human endometrial stromal cell line, t-HESC. *miR-181b-5p* expression was highest prior to decidualization and significantly decreased in response to decidualization stimulus. In contrast, TIMP-3 expression was absent prior to in vitro decidualization and increased during decidualization. Regulation of TIMP-3 expression by *miR-181b-5p* was confirmed in vitro by quantitative reverse transcription polymerase chain reaction (qRT-PCR), Western blot analysis, and 3' untranslated region reporter constructs. To identify unforeseen targets of *miR-181b-5p* during in vitro decidualization, t-HESC cells were transfected with pre-*miR-181b-5p*, and protein profiles were determined by 2-dimensional differential in-gel electrophoresis followed by matrix-assisted laser desorption-ionization time-of-flight/time-of-flight (MALDI TOF/TOF) tandem mass spectrometry. Of these proteins, several downregulated proteins associated with cell migration were identified including annexin A2, which we subsequently confirmed by qRT-PCR and Western blot analysis to be regulated by *miR-181b-5p*. In conclusion, *miR-181b-5p* is downregulated during the process of in vitro decidualization and may regulate the expression of proteins associated with cell migration including TIMP-3 and annexin A2.

Keywords

endometrium, decidualization, miRNA, *miR-181b-5p*

Introduction

MicroRNAs (miRNAs) are a class of small noncoding regulatory RNAs that regulate gene expression posttranscriptionally impacting subsequent translation of protein.^{1,2} Initially proposed to regulate translation through binding within the 3' untranslated region (UTR) of target transcripts, emerging evidence now supports the notion of posttranscriptional regulation not only at the level of the 3' UTR but also within the 5' UTR³ and/or the DNA-coding sequences (DCS) of messenger RNA (mRNA) transcripts.⁴ Posttranscriptional regulation of gene expression by miRNAs/small noncoding RNAs is essential for normal development and function of essentially all organs including the uterus.⁵⁻⁷ As such, miRNAs have emerged as critical modulators of uterine function. Our laboratory as well as those of other investigators has reported that miRNAs are regulated by estrogen and progesterone where they are proposed to fine-tune the

effects of these steroids within the uterus.⁸⁻¹⁰ Along these lines, Estella and Herrer¹¹ reported that miRNAs are required during in vitro decidualization of human endometrial stromal cells. In this report by Estella and Herrer¹¹ as well as in our previous report on estrogen regulation of uterine miRNAs in vivo,⁸ *miR-181b* was identified as one of the most significantly downregulated miRNAs. However, the potential role of *miR-181b* in decidualization

¹ Department of Molecular and Integrative Physiology, Institute for Reproductive Health and Regenerative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Corresponding Author:

Warren B. Nothnick, Department of Molecular and Integrative Physiology, Institute for Reproductive Health and Regenerative Medicine, University of Kansas Medical Center, Kansas City, KS 66160, USA.
Email: wnothnic@kumc.edu

or its regulation of key participants in the process of decidualization is currently poorly understood.

miR-181b is one of the 4 family members of highly conserved mature miRNAs, which also includes *miR-181a*, *miR-181c*, and *miR-181d*.¹² *miR-181b* is independently derived from 2 precursors: *miR-181b-1* which is located on chromosome 1 and *miR-181b-2* which is located on chromosome 9.¹³ *miR-181b-1* is derived from opposite arms of a single pre-miRNA hairpin, which are defined as *miR-181b-3p* and *miR-181b-5p*. *miR-181b-5p* is the predominant form of *miR-181b* and is now the current nomenclature for *miR-181b* (*miR-181b* will be referred to as *miR-181b-5p* from this point on in the article).

Based upon miRNA target prediction programs,^{14,15} *miR-181b-5p* is proposed to putatively regulate thousands of transcripts, many of which are of considerable interest in the context of uterine biology. For example, but not limited to, *miR-181b-5p* is predicted to target transcripts for estrogen receptor, progesterone receptor, Foxo1, and tissue inhibitor of metalloproteinase 3 (TIMP-3), all of which are proposed to play key roles in decidualization. Decidualization is essential for successful embryo implantation and occurs in response to the concerted actions of both estrogen and progesterone on the endometrium.¹⁶⁻¹⁸ The TIMP-3 has been proposed to play a role in embryo implantation and decidualization in rodents,¹⁹⁻²² humans,^{23,24} and nonhuman primates.²⁵ However, the relationship among *miR-181b-5p*, TIMP-3, and stromal cell decidualization has not been examined. The objective of this study was to determine whether *miR-181b-5p* regulates the in vitro decidualization of human endometrial stromal cells as well as the expression of the specific *miR-181b-5p* target TIMP-3. Further, using differential protein analysis, we also sought to identify novel targets of *miR-181b-5p* associated with early steps in the in vitro decidualization processes.

Materials and Methods

Endometrial Stromal Cell Culture and Transfection

To achieve experimental reproducibility and rigor, we utilized the human endometrial stromal cell line (t-HESC; obtained from the ATCC, Manassas, Virginia, and described in the study by Krikun et al²⁶) as a model system to study stromal cell decidualization in vitro. The t-HESC cells were confirmed to be mycoplasma-free using Plasmotest kits (Invivogen, San Diego, California), and cells were maintained in phenol red-free Dulbecco modified Eagle medium:nutrient mixture F-12 (DMEM:F12) supplemented with 10% charcoal-stripped fetal bovine serum (FBS), 1 mM sodium pyruvate, penicillin, streptomycin, and Normocin (100 µg/mL). For in vitro decidualization, cells were cultured in phenol red-free DMEM:F12 media containing 5% horse serum, 1 µM medroxyprogesterone acetate, 10 nM estradiol-17β, and 0.5 mM 8-bromoadenosine 3',5'-cyclic monophosphate²⁷ for 2 to 10 days with media changes every 48 hours. In vitro decidualization was confirmed by assessment of prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP1) mRNA levels by quantitative reverse transcription polymerase

chain reaction (qRT-PCR; Supplemental Figure 1). RNA or protein was isolated at day 0 (prior to in vitro decidualization), 2, 4, 6, 8, and 10 days after in vitro decidualization, as described below. The HESC cells treated with vehicle for a similar 10-day period do not decidualize, and expression of decidualization markers is not induced/does not exhibit changes in expression levels.

For assessment of *miR-181b-5p* regulation of mRNA or protein expression, cells were switched to media lacking antibiotics and FBS at 80% confluency and transfected with pre-*miR-181b-5p* mimics (pre-*miR-181b-5p*) or nontargeting mimics (pre-NT) using Lipofectamine 2000 (all materials obtained from Life Technologies, Grand Island, New York). Forty-eight hours after transfection, in vitro decidualization was induced as described above and total RNA or protein was isolated at day 0, 2, or 6 after in vitro decidualization as described below. Transfection efficiency/induction of *miR-181b-5p* expression was confirmed by qRT-PCR assessment of *miR-181b-5p* and resulted in an average increased expression ($\pm$ standard error of the mean [SEM]) above pre-NT transfected cells of 2987.3-fold ($\pm$ 164.4).

Messenger RNA and miRNA Assessment by qRT-PCR

Quantitative RT-PCR was performed as previously described.^{8,28} Briefly, total RNA was isolated using TRI reagent (Sigma Aldrich Chemical Company, St. Louis, Missouri) according to recommendations of the manufacturer. Total RNA (1 µg in 20 µL) was reverse transcribed using RT kits (Applied Biosystems, Foster City, California) following the manufacturer's protocol. Primers for *TIMP-3*, *PRL*, and annexin A2 (*ANXA2*), variants 1 to 4 (*ANXA2v1*, *v2*, *v3*, and *v4*, respectively) were designed using Primer-Blast and synthesized by Integrated DNA Technology (IDT, Coralville, Iowa). Sequences for the human *TIMP3* (NM_000362) primers were: forward, 5'-GGCACTCTGGTCTACACTATTAAGCA-3' and reverse, 5'-CATACACGCGCCCTGTCA-3', human *PRL* (NM_00948) primers were: forward, 5'-GAGCAAACCAAA-CGGCTTCT-3' and reverse, 5'-TCAGGATGAACCTGG-CTGACT-3', and human primers for each *ANXA2* variant were: *ANXA2v1* (NM_001002858): forward, 5'-CACGGC-CCAGGGTGAAAAT-3' and reverse, 5'-CATTATCCCA-TGCAGGTGCC-3', *ANXA2v2* (NM_001002857): forward, 5'-CACGGCCCAGGTTATCTTGT-3' and reverse, 5'-ATGTGTTCAACCAAGCGGGA-3', *ANXA2v3* (NM_004039): forward, 5'-ATTTGGGGACGCTCTCAGC-3' and reverse, 5'-AAGGAAGCCTTCTTCCCAGC-3', *ANXA2v4* (NM_001136015): forward, 5'-CACGGCCCAGCTTCCTT-3', and reverse, 5'-CCATATGCACTTGGGGGTGT-3'. Eukaryotic 18S ribosomal RNA endogenous control primers were obtained from Thermo Fisher Scientific (Pittsburgh, Pennsylvania). Resulting material was then used for independent qRT-PCR, which was carried out on an Applied Biosystems HT7900 Sequence Detector. To account for differences in starting material, human 18S primers and probe reagents were used for *TIMP3* and *ANXA2*, and values were expressed as fold change from the indicated control.

For *miR-181b-5p* assessment, total RNA (250 ng in 5 μ L) was reverse transcribed using RT kits (Applied Biosystems) following the manufacturer's protocol with the following modifications. Briefly, miRNAs were reverse transcribed in a single reaction using 2 μ L of each miRNA specific 5X RT primers. Resulting material was then used for independent qRT-PCR for each miRNA. To normalize for starting material, a reverse snRNA *U58* was included in the miRNA RT reactions and qRT-PCR of *U58* was performed. The qRT-PCR reactions were completed on a 7900HT Sequence Detection System (Applied Biosystems). All samples for mRNA and miRNA were run in triplicate and the average value used in subsequent calculations. The 2-delta-delta CT method was used to calculate the fold-change values among samples as previously described by our group.^{8,28} All data are displayed as the mean $\pm$ SEM.

Western Analysis

Total protein was extracted from t-HESC cells using cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton, 2.5 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, and 1 μ g/mL leupeptin) supplemented with 0.1 mg/mL PMSF (Cell Signaling Technologies, Danvers, Massachusetts). Protein concentration in each sample was determined using the DC Protein Assay (Bio-Rad Laboratories, Richmond, California). The same amount of protein (30 μ g) was subjected to 4% to 12% Bis-Tris (Invitrogen, Carlsbad, California) gel electrophoresis and electroblotted onto PVDF membranes (Invitrogen). Rabbit anti-TIMP-3 (1:300; Santa Cruz, CA), rabbit anti-ANXA2 (1:300; Abcam Inc, Cambridge, Massachusetts), and goat antirabbit secondary antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) were used. Stripping and reprobing for β -actin (Santa Cruz) was conducted to normalize TIMP-3 protein expression levels. Immunodetection was carried out using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, New Jersey).

Prolactin Media Assessment

Prolactin concentrations in t-HESC-conditioned media were assessed using the DetectX Prolactin Immunoassay kit (Arbor Assays, Ann Arbor, Michigan). Briefly, condition media was diluted 1:10 with assay diluent and assessed by EIA following the protocol provided by the manufacturer. All samples were run in duplicate, and data are presented as the mean $\pm$ SEM in pg/mL. Limit of detection and sensitivity were 13.4 pg/mL and 11.7 pg/mL, respectively, while the intra-assay coefficient of variation (CV) was <5% and inter-assay CV was <8%.

Luciferase Reporter Assays

Wild-type and mutated luciferase constructs for TIMP-3 were kindly provided by Dr. Samson T. Jacob (The Ohio State University, Columbus, Ohio)²⁹ through a material transfer agreement.

Reporter constructs (0.1 μ g) containing either the wild-type or a mutated *miR181b-5p* sequence within the human *TIMP-3*, 3' UTR were cotransfected into t-HESC cells along with a control vector containing Renilla luciferase (0.01 μ g, pRL-TK; Promega Corp, Madison, Wisconsin) plus either pre-*181b-5p* mimic oligonucleotide (*181b-5p* mimic) or NT mimic controls (30 nM final concentration of each). All cells were cultured in 24-well plates for 24 hours at 37°C, after which both firefly and Renilla luciferase activity were determined using the Dual-Luciferase reporter assay system following the protocol supplied by Promega. Mutant constructs were devoid of both of the *miR-181b-5p*-binding sites and incapable of binding with this miRNA at the 3' UTR.²⁹

Two-Dimensional Differential In-Gel Electrophoresis

To evaluate differential protein profiles, 2-dimensional differential in-gel electrophoresis (2D-DIGE) was conducted following a similar approach as previously reported²⁸ with modifications. Briefly, protein was extracted from t-HESC cells transfected with either pre-NT-miR or pre-*miR-181b-5p* mimics and cultured for 2 days with decidualization media. Protein samples were shipped to Applied Biomics (Hayward, California) for 2D-DIGE and protein identification by mass spectrometry (MS) to establish protein profiles between genotypes and subsequent protein identification. Image scans were carried out immediately following the sodium dodecyl sulfate polyacrylamide gel electrophoresis using Typhoon TRIO (GE Healthcare). Scanned images were then analyzed by Image QuantTL 8.1 software (GE Healthcare-Biosciences, Pittsburgh, Pennsylvania) and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare). The ratio change of the protein differential expression was obtained from in-gel DeCyder software analysis. Those proteins whose expression was at least 40% less in the pre-*miR-181b-5p* compared to the pre-NT miR counterparts were then subjected to isolation using Ettan Spot Picker (GE Healthcare) and subsequent MALDI-TOF (MS) and TOF/TOF (tandem MS/MS) using a 5800 mass spectrometer (AB Sciex, Framingham, MA).

For protein identification, the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer version 3.5 equipped with MASCOT search engine (Matrix Science, Boston, Massachusetts) to search the database of National Center for Biotechnology Information non-redundant. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues and with 1 missed cleavage allowed in the search parameters. Candidates with either a confidence of the protein score (CI%) or confidence of the ion score (CI%) greater than 95 were considered significant. All proteins reported in this work had a C.I.% of 100%.

Statistical Analysis

All data were analyzed using GraphPad Instat3 software. Data were analyzed using 1-way analysis of variance followed by

Figure 1. Human endometrial stromal cell line, t-HESC *miR-181b-5p* expression during in vitro decidualization. *miR-181b-5p* levels were quantitated as described in “Materials and Methods” by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalized to U58 levels. Data are displayed as the mean $\pm$ standard error of the mean (SEM) and are representative of 4 independent samples per time point per treatment ($n = 4$). Data were analyzed by 1-way analysis of variance (ANOVA) for comparison across time points and expressed as fold change from day 0 (not decidualized) values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey honest significant difference (HSD) procedure. Significance was set at $P < .05$ for all comparisons. Different letters indicate statistical significance as determined by 1-way ANOVA.

post hoc analysis using Tukey least significant difference method when appropriate or unpaired t tests. For all assessments, a P value $< .05$ was considered statistically significant.

Results

miR-181b-5p expression was highest in t-HESC cells prior to decidualization (Figure 1). In vitro decidualization resulted in a significant decrease in *miR-181b-5p* expression as early as 2 days post-decidualization and its expression remained repressed throughout the full 10-day duration of decidualization treatment (Figure 1). *TIMP-3* mRNA was expressed at all times points during in vitro decidualization with levels remaining constant during the first 4 days and then significantly increasing at days 6, 8, and 10 (Figure 2). In contrast, despite transcript expression at day 0, *TIMP-3* protein was not expressed at day 0 but increased at day 2 and remained elevated for the full 10-day period (Figure 3).

When we compared the pattern of *TIMP-3* mRNA (Figure 2) and *TIMP-3* protein (Figure 3) expression, we noticed that despite transcript expression at day 0, *TIMP-3* protein was not expressed. When we took into account the pattern of *miR-181b-5p* expression (Figure 1), we concluded that the high expression of *miR-181b-5p* at day 0 may lead to a reduction in *TIMP-3* protein expression. To determine whether *miR-181b-5p* regulates *TIMP-3* expression, we assessed *TIMP-3* mRNA and protein expression in t-HESC cells transfected with either a NT pre-miRNA or a pre-miRNA for *miR-181b-5p*. As depicted

Figure 2. Human endometrial stromal cell line, t-HESC tissue inhibitor of metalloproteinase 3 (*TIMP-3*) messenger RNA (mRNA) expression during in vitro decidualization. *TIMP-3* mRNA levels were quantitated as described in “Materials and Methods” by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalized to 18S ribosomal RNA (rRNA) levels using RNA from the same samples analyzed for *miR-181b-5p* (Figure 1). Data are displayed as the mean $\pm$ standard error of the mean (SEM) and are representative of 4 independent samples per time point per treatment ($n = 4$). Data were analyzed by 1-way analysis of variance (ANOVA) for comparison across time points and expressed as fold change from day 0 (not decidualized) values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey honest significant difference (HSD) procedure. Significance was set at $P < .05$ for all comparisons. Different letters indicate statistical significance as determined by 1-way ANOVA.

in Figure 4, pre-*miR-181b-5p* transfection (but not that of pre-NT) resulted in a decrease in *TIMP-3* mRNA expression (Figure 4A) and protein expression (Figure 4B) at day 6 of in vitro decidualization. The *miR-181b-5p*-induced changes in *TIMP-3* expression were not associated with an inhibition of in vitro decidualization as *PRL* mRNA levels (Figure 4C), as well as *PRL* concentrations in the condition media (Figure 4D) did not differ between pre-NT- and pre-*miR-181b-5p*-transfected cells.

To determine whether *TIMP-3* is regulated by *miR-181b-5p* through binding within the 3' UTR, wild-type *TIMP-3*-3' UTR and mutated *TIMP-3*-3' UTR lacking *miR-181b-5p* putative binding sites (Figure 5A) were transfected into t-HESC cells in the presence of either pre-*miR-181b-5p* mimics or NT mimics (negative control). Transfection with pre-*miR-181b-5p* mimics significantly reduced normalized luciferase activity in cell transfected with wild-type 3' *TIMP-3* constructs, while in those cells transfected with the mutant reporters, no reduction was noted (Figure 5B). Similarly, NT mimics had no effect on luciferase activity in either the wild-type or mutant reporter construct-transfected t-HESC cells (Figure 5B).

To expand our knowledge on factors that may be modulated by *miR-181b-5p* during the process of in vitro decidualization, we assessed protein profiles in pre-NT- compared to pre-*miR-181b-5p*-transfected cells at day 2 post-decidualization/transfection using 2D-DIGE. As depicted in Figure 6, more than 100

Figure 3. Human endometrial stromal cell line, t-HESC tissue inhibitor of metalloproteinase 3 (TIMP-3) protein expression increases during in vitro decidualization. **A**, TIMP-3 protein expression was analyzed by Western analysis and normalized to β -actin as described in "Materials and Methods." **B**, Data are displayed as the mean $\pm$ standard error of the mean (SEM) for TIMP-3/ β -actin for 4 independent observations ($n = 4$). Data were analyzed by 1-way analysis of variance (ANOVA) for comparison across days of in vitro decidualization and are compared to day 0 values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey honest significant difference (HSD) procedure. Significance was set at $P < .05$ for all comparisons. Different letters indicate statistical significance as determined by 1-way ANOVA

proteins were differentially expressed between pre-NT- and pre-*miR-181b-5p*-transfected cells. Of those proteins, we determined the identity of 10 of the most significantly downregulated proteins by MALDI-TOF (MS) and TOF/TOF (tandem MS/MS; Table 1). Proteins whose expression was significantly reduced (by at least 40%) in t-HESC cells transfected with pre-*miR-181b-5p* were identified as tubulin (*TUBB*), actin (*ACTG1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *ANXA2*, peptidyl-prolyl cis-trans isomerase A (*PPIA*), profilin 1 (*PFN1*), bifunctional purine biosynthesis protein (*ATIC*; 2 isoforms), polymerase and transcript release factor (*PTRF*), isocitrate dehydrogenase [NADP] cytoplasmic (*IDH1*), and L-lactate dehydrogenase A chain (*LDHA*). Of these 10 proteins, 7 were identified as potential targets of *miR-181b-5p* with potential binding sites within their 3' UTR, 5' UTR, and/or DCS (Table 2) as determined by miRNA precomputed static target predictions based on TargetScan 7.0¹⁴ and the RNA22 tool¹⁵ software.

As *ANXA2* was considered a DCS direct target and one of the most differentially regulated proteins, coupled with

its potential role in endometrial biology,²⁹⁻³² we next wanted to confirm its regulation by *miR-181b-5p*. To do so, we assessed *ANXA2* mRNA expression in t-HESC cells transfected with pre-NT or pre-*miR-181b-5p* at day 2 of in vitro decidualization as this was the time point at which we assessed protein profiles for similarly transfected cells. All 4 transcript variants of *ANXA2* were detected with *ANXA2v4* > *ANXA2v2* > *ANXA2v1* > *ANXA2v3* based upon Ct values (data not shown). As depicted in Figure 7, mRNA levels of all *ANXA2* variants except that of *ANXA2v3* were significantly reduced in the pre-*miR-181b-5p* transfected groups compared to pre-NT-transfected cells. This was not a nonspecific effect on transcription as *PRL* mRNA levels in these same cells were not significantly different between groups (data not shown).

To further support the notion that *miR-181b-5p* regulates *ANXA2* protein expression, we assessed *ANXA2* protein expression by Western blot analysis. Assessment of *ANXA2* protein levels demonstrated a minimal decrease in protein expression after 2 days of transfection in the cells transfected with *miR-181b-5p* compared to NT controls (data not shown). Possible reasons for the minimal decrease may be due to the abundance of *ANXA2* protein in the t-HESC cells such that 48 hours posttransfection of *miR-181b-5p* may not have been sufficient for the decrease in mRNA to be reflected at the protein/translational level. Therefore, we also assessed *ANXA2* protein expression in t-HESC cells 6 days posttransfection, a time point in which *miR-181b-5p* is still expressed in t-HESC cells transfected with the pre-miRNA (data not shown). As seen in Figure 8, 6 days posttransfection of *miR-181b-5p* resulted in an average, but highly consistent, 20% decrease in *ANXA2* protein expression compared to cells transfected with NT miRNA mimics ($P < .05$). Collectively, these studies demonstrate that the forced expression of *miR-181b-5p* is associated with significantly reduced levels of *ANXA2* transcript and protein expression.

Discussion

The objective of the current study was to explore the expression and relationship between *miR-181b-5p* and TIMP-3 during in vitro decidualization, as well as to identify novel targets of *miR-181b-5p* during in vitro decidualization. Among the numerous factors that have been identified as essential for this process to occur, miRNAs have recently proposed to also play an essential role in the process of decidualization and embryo implantation.³⁰⁻³⁴ In the current study, we focused on *miR-181b-5p* as this miRNA is decreased during decidualization in vitro¹¹ and during the period of embryo implantation in vivo³³ and functionally impacts embryo implantation in a mouse model.³⁴ Additional rationale came from the strong support that *miR-181b-5p* regulates TIMP-3,^{29,35-37} which itself has been proposed to play a role in decidualization.¹⁹⁻²⁵ The current study was the first to concurrently assess both *miR-181b-5p* and TIMP-3 during the process of in vitro decidualization. One of the novel observations made in this study was the modulation of TIMP-3 expression at the transcript level by

Figure 4. miR-181b-5p forced expression reduces t-HESC TIMP-3 messenger RNA (mRNA) expression but does not affect in vitro decidualization-associated prolactin expression. t-HESC cells were cultured and transfected with either a nontargeting miRNA (pre-NT) or pre-miR-181b-5p mimics, and tissue inhibitor of metalloproteinase 3 (TIMP-3) mRNA (A) and protein (B) were evaluated as described in “Materials and Methods” after 6 days of in vitro decidualization, a time when both TIMP-3 mRNA and protein are significantly elevated. To evaluate if forced expression of miR-181b-5p was associated with an alteration in decidualization, prolactin mRNA (C) and peptide released into the t-HESC conditioned media (D) were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme immunoassay (EIA), respectively, as described in “Materials and Methods.” Data are displayed as the mean $\pm$ standard error of the mean (SEM) for all end points from 3 independent observations ($n = 3$). Data were analyzed by unpaired t test comparing the 2 transfection groups. Significance was set at $P < .05$ for all comparisons, and P values are displayed within each figure. No significant differences between pre-NT and pre-miR-181b-5p transfection groups were noted in the levels of prolactin mRNA or conditioned media concentrations ($P > .05$).

miR-181b-5p prior to decidualization. miR-181b-5p expression is at peak levels prior to induction of decidualization in vitro and this coincides with the absence of TIMP-3 protein. Induction of TIMP-3 protein begins as early as day 2 post in vitro decidualization, but this increase in protein expression is not associated with an increase in TIMP-3 transcript expression. One potential explanation would be that in the presence of elevated miR-181b-5p, translation of TIMP-3 mRNA is suppressed via posttranscriptional regulation, and that during early induction of in vitro decidualization, the declining levels of miR-181b-5p allow escape of TIMP-3 mRNA from repression. At later time points (days 6–10), increased TIMP-3 protein expression is associated with an increase in TIMP-3 transcript expression and reduced miR-181b-5p expression, suggesting that TIMP-3 may be actively transcribed and translated providing robust levels of expression of this protein. We also observed that while forced expression of miR-181b-5p decreased TIMP-3 expression, it did not impact the ability of the t-HESC cells to decidualize (based

upon PRL mRNA and conditioned media concentrations). This observation may suggest that while the decrease in miR-181b-5p expression downregulates the expression of TIMP-3, this may occur as a result of, as opposed to a cause for, decidualization.

It is evident that many factors beyond TIMP-3 contribute to the process of decidualization. To identify novel targets of miR-181-5p, which may be relevant to the process of in vitro decidualization, we utilized 2D-DIGE. We identified several proteins whose expression significantly decreased as a result of miR-181b-5p overexpression, of which we focused on 10 proteins based on the criteria described in “Materials and Methods.” Of these 10 proteins, 4 have been proposed to be regulated by miR-181b-5p through binding with the 3' UTR, 1 of them at the level of the 5' UTR and 3 of them through binding within the DCS. Interestingly, 3 of the 10 proteins were not predicted to be targeted by miR-181b-5p.

From the identified 10 proteins that were determined to be regulated by miR-181b-5p, we selected ANXA2 for further

Figure 5. miR-181b-5p targets the 3' untranslated region (UTR) of tissue inhibitor of metalloproteinase 3 (TIMP-3) to regulate its expression. **A**, Sequence alignment of human *miR-181b-5p* with the 3' UTR of TIMP-3. Seed sequence of *miR-181b-5p* that corresponds to the binding sites (3499-3505 and 3573-3580) within the 3' UTR of TIMP-3 is highlighted in bold and underlined. Mutant constructs incapable of binding *miR-181b-5p* were generated by deleting both sites 1 and 2 providing a negative control. **B**, Luciferase assays were conducted as described in "Materials and Methods." Pre-*miR-181b-5p* significantly reduced luciferase activity in cells transfected with wild-type 3' UTR TIMP-3 construct but not in cells transfected with the mutated 3' UTR of TIMP-3. Pre-*miR-NT* had no effect on luciferase activity in cells transfected with either the wild-type or mutant 3' UTR TIMP-3 reporter constructs. Data are displayed as the mean $\pm$ standard error of the mean (SEM) and are representative of 4 separate experiments ($n = 4$). Different letters indicate statistically significant different means within miR between 3' UTR type while asterisk (*) indicates statistically significant different means within 3' UTR type between pre-miRs. Data were analyzed using unpaired *t* tests.

analysis based upon its proposed role in embryo implantation and pregnancy.³⁸⁻⁴⁰ Annexin A2 is a calcium-binding protein, which is associated with cell adhesion, migration, and actin rearrangement.⁴¹ The ANXA2 expression is upregulated in vivo during endometrial receptivity, and inhibition of epithelial ANXA2 expression was associated with reduced embryo adhesion in an in vitro co-culture system.³⁹ While in this same study³⁹ ANXA2 expression was noted to be most prominent in the glandular and luminal epithelium during the mid-secretory stage of the menstrual cycle, strong staining was also observed in the stromal compartment. A similar pattern of expression and proposed function for Anxa2 was also proposed in mice.⁴⁰ Thus, reduction in *miR-181b-5p* during decidualization may allow escape of ANXA2 repression by this miRNA, assuring sufficient expression for decidualization to occur.

It is interesting to note that *miR-181b-5p* is proposed to target ANXA2 by binding to a sequence within the DCS of the gene (based upon RNA22¹⁵ computations) and not the traditional 3' UTR of the gene. Overexpression of *miR-181b-5p* resulted in a statistically significant but modest (20%) reduction in protein expression, which was associated with a similar degree of suppression for ANXA2 transcript variant 1 (29%), variant 2 (25%), and variant 4 (25%) but not variant 3 (6%). Nonetheless, ANXA2 appears to be targeted by *miR-181b-5p* and this regulation may occur at the level of the DCS region of the gene.

Considering that ANXA2 is associated with actin filaments and that the pattern of expression, proposed role, and necessity of actin dynamics during decidualization,^{41,42} it is not surprising that in addition to suppressing ANXA2 expression, overexpression of *miR-181b-5p* was also associated with a reduction in other cytoskeleton components, namely actin and its interacting protein profilin-1 (PROF1)⁴³ as well as tubulin.⁴⁴ It was surprising though that none of these 3 transcripts are predicted to be targeted by *miR-181b-5p*,^{14,15} despite the detected decrease in their protein expression in cells overexpressing *miR-181b-5p*. This observation might suggest that an indirect mechanism contributed to the *miR-181b-5p*-induced downregulation of their protein expression.

With respect to a potential function of *miR-181b-5p* during in vitro decidualization, our results may suggest a regulatory role in cell proliferation/cell migration. Forced expression of *miR-181b-5p* significantly decreased TIMP-3 expression (both at the transcript and protein levels) as well as resulted in marked decreases in the expression of PROF1. Both TIMP-3 and PROF1 are proposed to regulate cell migration.⁴⁵⁻⁴⁸ It is well established that cell migration is required for normal embryo implantation and establishment of pregnancy, but when this migration is uncontrolled, pregnancy establishment is compromised.⁴⁹ As discussed earlier, TIMP-3 is proposed to play a vital role in embryo implantation,¹⁹⁻²⁵ controlling the coordination of tissue remodeling that is essential for correct establishment of pregnancy. Like TIMP-3, PROF1 may control cell migration/remodeling necessary for proper embryo implantation, but other than a single report⁵⁰ demonstrating localization of PROF1 to cells within the stroma of the villous tip, the pattern of expression of this protein during the period of embryo implantation and decidualization is largely unknown.

We are aware that this study is not without limitations. In this study, we elected to utilize the well-characterized human endometrial stromal cell line t-HESC first described by Krikun and colleagues²⁶ to avoid patient to patient variability and increase experimental rigor and reproducibility. To fully evaluate the role of *miR-181b-5p* and its target transcripts, these studies will need to be repeated using primary stromal cells. This sample size will need to be large enough and well defined to provide adequate power and experimental rigor, respectively. Further, concurrent assessment in a "control" as well as a population of women that suffer from embryo implantation/decidualization abnormalities will allow us to further determine the role of *miR-181b-5p* during normal and abnormal decidualization.

Figure 6. CyDye switch, 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis of pre-NT- and pre-miR-181b-5p-transfected t-HESC proteomes. The t-HESC cells were transfected and protein isolated as described under “Materials and Methods.” Pre-NT-transfected samples were labeled with Cy3 (green) and pre-miR-181b-5p samples with Cy5 (red). Samples were then mixed and separated on analytical 2D-DIGE. The resulting gel was scanned and the merged image is shown where red proteins represent proteins whose expression is higher in the pre-miR-181b-5p-transfected cells and green proteins represent proteins whose expression is higher in the pre-NT-transfected cells. Circled and numbered spots represent proteins that were most differentially expressed. Of these, we determined the identity of 10 proteins that are listed in Table I and indicated by the red circles. Molecular weight range is indicated on the vertical axis and isoelectric point range indicated on the horizontal axis. (The color version of this figure is available online.)

Table I. Differentially Expressed Proteins in Pre-NT-miR- and Pre-miR-181b-5p-Transfected t-HESC Cells.

Spot Number	Protein	Protein Symbol	MW	pI ^a	Fold Change ^b
36	Tubulin β -chain	TBB5	49 639	4.8	−1.53
50	Actin	ACTG	41 766	5.3	−1.54
66	Annexin A2	ANXA2	38 580	7.6	−1.64
67	Glyceraldehyde 3-phosphate dehydrogenase	G3P	36 030	8.6	−1.53
84	Peptidyl-prolyl cis-transisomerase A	PPIA	18 001	7.7	−1.71
94	Profilin-1	PROFI	15 045	8.4	−1.44
96	Bifunctional purine biosynthesis protein PURH	PUR9	64 575	6.3	−1.42
97	Bifunctional purine biosynthesis protein PURH	PUR9	64 575	6.3	−1.42
100	Polymerase I and transcript release factor	PTRF	43 450	5.5	−1.45
103	Isocitrate dehydrogenase [NADP] cytoplasmic	IDHC	46 630	6.5	−1.48
107	L-Lactate dehydrogenase A chain	LDHA	36 665	8.4	−1.47

Abbreviations: MW, molecular weight; NT, nontargeting.
^aReported isoelectric point (pI).
^bFold change reported as pre-miR-181b-5p/pre-NT-miR ratio.

Table 2. Potential miR-181b-5p Binding Sites Within the 3' UTR, 5' UTR, and/or DCS Regions of the Most Differentially Expressed Proteins in Pre-NT-miR and pre-miR-181b-5p-transfected t-HESC Cells.

Protein/Gene	3' UTR Binding ^a	5' UTR Binding ^b	DCS Binding ^b
TBB5/TUBB	Yes/yes*	No	No
ACTG/ACTG1	No/no*	No	No
ANXA2/ANXA2	No/no*	No	Yes
G3P/GAPDH	Yes/no*	No	No
PPIA/PPIA	No/no*	No	No
PROF1/PFN1	No/no*	No	No
PUR9/ATIC	No/no*	Yes	No
PTRF/PTRF	Yes/no*	No	Yes
IDH1/IDH1	Yes/yes*	No	No
LDHA/LDHA	No/no*	No	Yes

Abbreviations: DCS, DNA-coding sequence; NT, nontargeting; UTR, untranslated region.

^a3' UTR binding sites based upon TargetScan v7.0¹⁴ and RNA22¹⁵ computations. TargetScan predictions are indicated by the asterisk (*).

^b5' UTR and DCS miRNA binding based upon RNA22¹⁵ computations.

In summary, we demonstrate that prior to in vitro decidualization, t-HESC cell *miR-181b-5p* is robustly expressed as *TIMP-3* mRNA, but *TIMP-3* protein levels are low to absent. In vitro decidualization results in a significant reduction in *miR-181b-5p* expression, which is associated with an increase in *TIMP-3* protein expression. It was further revealed that *miR-181b-5p* induces a decrease in *TIMP-3* mRNA and protein expression. Lastly, *ANXA2* was identified as a novel target of *miR-181b-5p* whose expression is also decreased at the mRNA level by this miRNA, despite lacking a seed sequence for *miR-181b-5p* binding within its 3' UTR but containing a potential binding site within the DCS region. From this study, it appears that in addition to *TIMP-3*, *miR-181b-5p* also regulates the expression of cytoskeleton proteins that are essential for actin remodeling and cell migration that occur during decidualization. We conclude that the decrease in *miR-181b-5p* expression during decidualization allows for increased expression of *TIMP-3* and *ANXA2*, which then modulate cellular dynamics. What remains to be determined is whether there are alterations in *miR-181b-5p* in women who have abnormalities in endometrial decidualization and embryo implantation.

Figure 7. *miR-181b-5p* suppresses annexin A2 (*Anx2*) transcript expression in t-HESC cells. The t-HESC cells were subjected to in vitro decidualization for 2 days with concurrent transfection with either pre-nontargeting (NT) miRNA or pre-*miR-181b-5p* after which *ANXA2* transcript expression (variants 1-4) was examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described under "Materials and Methods." Data are displayed as the mean $\pm$ standard error of the mean (SEM) for 6 independent observations ($n = 6$). Data were analyzed by unpaired *t* test comparing the 2 transfection groups. Significance was set at $P < .05$ for all comparisons, and *P* values are displayed within each figure for *ANXA2v1* (A), *ANXA2v2* (B), and *ANXA2v4* (D). No significant differences between pre-NT and pre-*miR-181b-5p* transfection groups was detected for *ANXA2v3* (C; $P > .05$).

Figure 8. *miR-181b-5p* suppresses annexin A2 (ANXA2) protein expression in t-HESC cells under basal conditions. The t-HESC cells were subjected to in vitro decidualization for 2 days with concurrent transfection with either pre-nontargeting (NT) microRNA (miRNA; NT control) or pre-*miR-181b-5p* after which ANXA2 protein expression was assessed as described under “Materials and Methods” by Western blot analysis. Bar graph represents normalization of ANXA2 to β -actin levels displayed as the mean $\pm$ standard error of the mean (SEM) for 3 independent observations ($n = 3$). Data were analyzed by unpaired t test comparing the 3 transfection groups. Significance was set at $P < .05$ for all comparisons and asterisk (*) indicates statistical significance between the means. ANXA2 indicates annexin A2; t-HESC, human endometrial stromal cell line; NT, nontargeting.

Authors' Note

Amanda Graham and Joshua Holbert contributed equally to this work.

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Supplementary Material

Supplementary material is available for this article online.

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