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Research Article

MicroRNAs associated with adenomyosis promote endometrial epithelial cell migration via MMP-2 and MMP-9 upregulation†

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Abstract

Adenomyosis is a common gynecological disorder characterized by the presence of endometrial tissue in the myometrium, causing chronic pelvic pain and abnormal bleeding. Although dysregulated microRNAs (miRNAs) in stromal cells of adenomyosis patients have been implicated in disease-associated extracellular matrix (ECM) remodeling, their role in regulating endometrial epithelial cell (EEC) behavior remains poorly understood. This study investigated the effect of selected adenomyosis- and endometriosis-associated miRNAs on EEC migration and matrix metalloproteinase (MMP) expression. Ishikawa cells, a human endometrial epithelium-like cell line, were transfected with mimics and inhibitors of Let-7b, miR-451a, miR-125b, miR-7 and miR-150. Cell migration was assessed using wound healing assays. MMP-2 and MMP-9 mRNA expression were quantified by quantitative real-time polymerase chain reaction, while protein production and secretion were evaluated by enzyme-linked immunosorbent assay. Transfection with Let-7b-5p inhibitor and miR-451a, miR-125b-1 and miR-150 mimics significantly enhanced cell migration and led to increased MMP-2 and MMP-9 mRNA expression, intracellular protein levels and secretion. These findings suggest that these miRNAs promote EEC migration and ECM remodeling through MMP upregulation, pointing to a potential mechanism for lesion formation in adenomyosis. Future research should seek to validate these findings in primary EECs and explore the therapeutic potential of targeting miRNA-MMP pathways for adenomyosis treatment.

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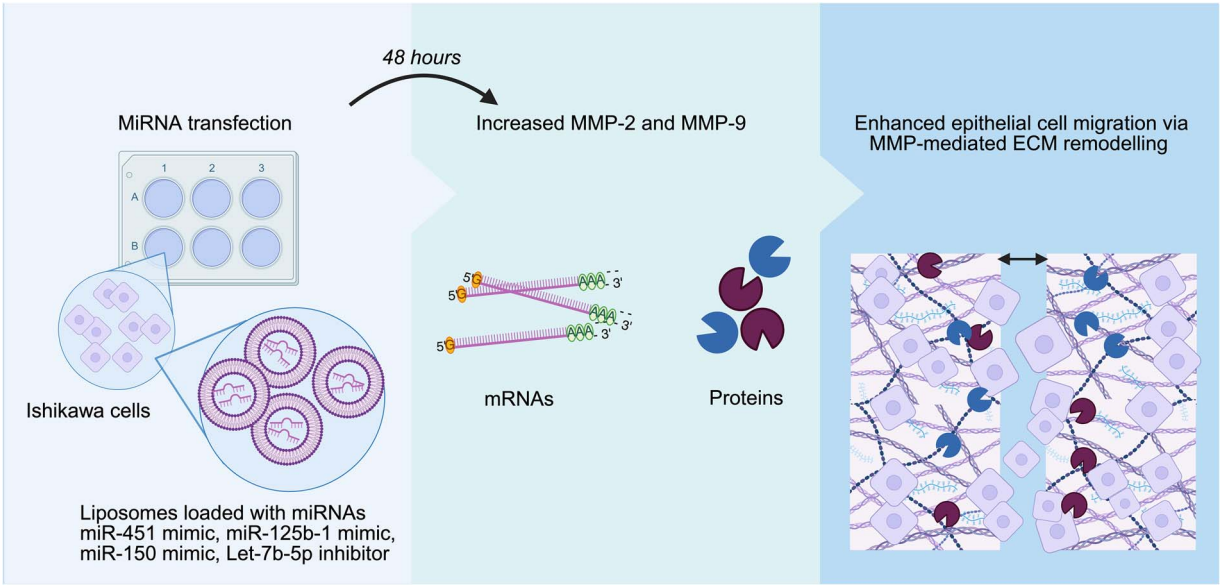
Graphical Abstract



MiRNA-induced MMP expression promotes epithelial cell migration.
MMP: matrix metalloproteinase; ECM: extracellular matrix.

Key words: micrornas, endometrial epithelial cells, metalloproteinases, cell migration, ishikawa cells, adenomyosis, ecm remodeling

Introduction

Adenomyosis is an estrogen-dependent gynecological disorder that significantly impacts women's reproductive health and quality of life [1]. It is histologically characterized by irregularly shaped islands of endometrium-like glands and stroma within the myometrium, often surrounded by smooth muscle hyperplasia and hypertrophy [2, 3, 4]. Its insidious clinical presentation (chronic pelvic pain, abnormal uterine bleeding and infertility) and common association with endometriosis make adenomyosis somewhat challenging to diagnose [5, 6]. Despite its prevalence, ranging from 20% to 35% [7], the molecular mechanisms underlying adenomyosis pathogenesis have not yet been elucidated [8, 9].

Emerging evidence suggests that eutopic endometrial cells undergo molecular changes that contribute to lesion formation [10]. Among their key regulators are microRNAs (miRNAs), a type of small endogenous non-coding RNA that modulates both physiology and pathology by suppressing target gene expression post-transcriptionally [11]. Transcriptomic studies have identified a number of differentially expressed miRNAs associated with adenomyosis pathogenesis [12, 13, 14], implicating them in aberrant extracellular matrix (ECM) remodeling and lesion development. MiRNAs including miR-451a, Let-7b, miR-125b, miR-7 and miR-150 have been found to be dysregulated in stromal cells from adenomyosis subjects and detectable in blood from endometriosis patients [15, 16, 17, 18], pointing to possible shared molecular features. However, their direct functional impact on endometrial epithelial cell (EEC) behavior remains unknown.

Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, are key mediators of ECM degradation and essential to tissue remodeling and cell migration [19, 20]. Several studies have reported increased stromal MMP expression in eutopic endometrium from adenomyosis patients compared to healthy individuals [21, 22, 23]. While MMP dysregulation has been studied in various reproductive pathologies, very little is known about the regulatory relationship between disease-related miRNAs and epithelium-derived MMPs in adenomyosis.

This study investigated the regulatory effects of adenomyosis- and endometriosis-associated miRNAs on MMP-2 and MMP-9 expression in Ishikawa cells, a well-established model of endometrial epithelium-like cells. The model circumvents the limitations of primary EEC availability [24] and ensures experimental consistency. By integrating transcript, protein and functional enzymatic activity assessments, we aimed to shed light on the contribution of EEC-derived MMPs to the migratory phenotype observed in adenomyotic glands. Our findings provide novel insights into how miRNA dysregulation may promote lesion formation through enhanced ECM degradation, highlighting potential miRNA-based therapeutic strategies for patients suffering from this chronic condition.

Materials and methods

Ishikawa cell culture

Ishikawa cells (epithelium-like cell line) were cultured in 0.22 μm filtrated minimum essential medium 1× (MEM) with 10% heat-inactivated fetal bovine serum (HI FBS) and 1% antibiotic-antimicotic 100× (anti-anti) (Thermo Fisher Scientific, Waltham, MA, USA).

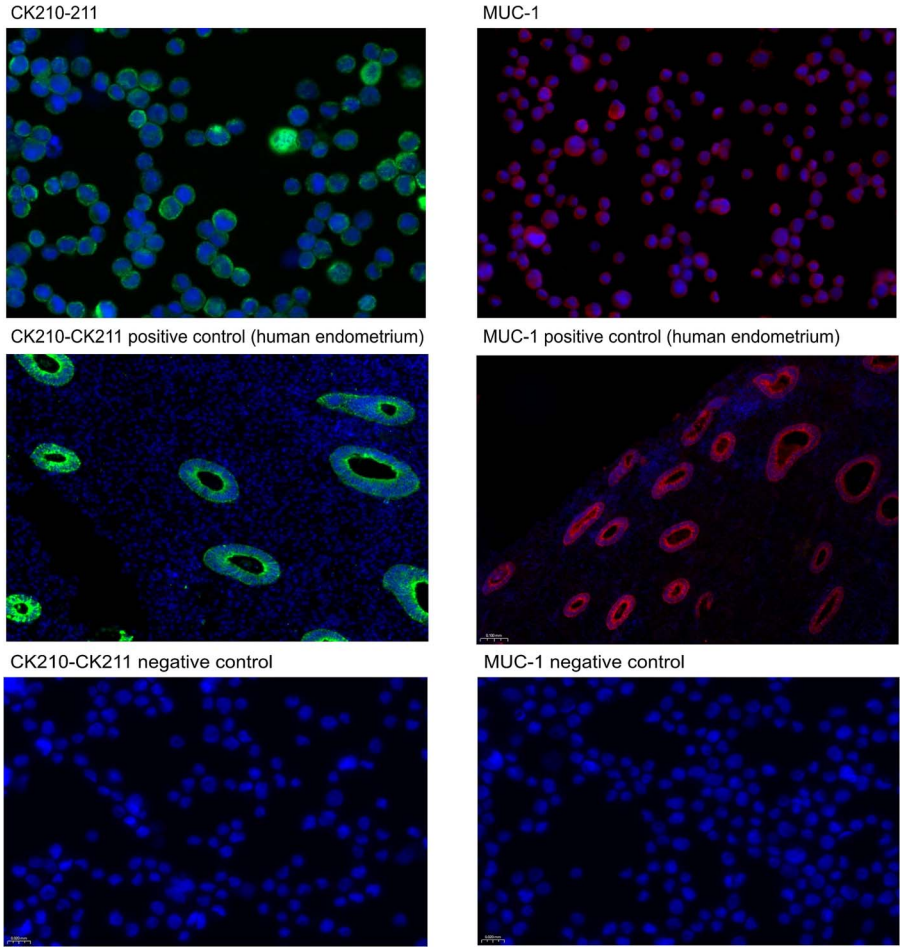


Figure 1. Ishikawa cell phenotyping prior to use. Representative images of CK210–211 and MUC-1 immunocytochemistry-concentrated Ishikawa cells. After fixation in 4% paraformaldehyde, 300 μl resuspended Ishikawa cells (5×10^4 – 5×10^5 cells/ml) were aggregated on slides using the Shandon Cytospin 2 for 10 min at 0.033 g, then stained with mouse anti-human CK210–211 and MUC-1 antibodies. Culture purity was 92% (91.8 ± 1.79). Magnification 40×. Universal negative mouse cocktail was utilized instead of the primary antibody for negative control (magnification 40×). Human endometrium served as positive controls (magnification 20×).

The Ishikawa cell phenotype was tested by immunocytochemistry. After fixation in 4% paraformaldehyde for 20 min, 300 μl resuspended cells (5×10^4 to 5×10^5 cells/ml) were aggregated on slides using the Shandon Cytospin 2 (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 0.033 g, then stained with mouse anti-human MUC-1 antibody (ready-to-use, Sanbio, Uden, the Netherlands) and mouse anti-human CK210–211 antibody (ready-to-use, Sanbio, Uden, the Netherlands). Universal negative mouse cocktail (ready-to-use, Dako, Carpinteria, CA, USA) was utilized instead of the primary antibody for negative controls (NCs). Human endometrium served as positive controls. Culture purity was 92% (91.8 ± 1.79) (Figure 1).

Transfection of miRNAs

Ishikawa cells were transfected with mimics, inhibitors and respective controls of miRNAs 451a, 7, Let-7b, 125b-1 and 150. Mimics are mature miRNA sequences that result in functional miRNAs, while inhibitors are small nucleotide sequences that bind to functional miRNAs and inhibit their action. For these experiments, all miRNAs (1 μmol) with cartridge purification were obtained from the W.M. Keck Oligonucleotide Synthesis Facility (Yale University, New Haven, CT, USA). NCs are the scrambled sequences that do not exert any effect on the target genes of a mature miRNA. Both mimics (SMC-2003) and inhibitors (SMC-2103) (20 nmol) were obtained from Bioneer Inc. CA, USA. Liposomal transfection was done in duplicate. Briefly, 6×10^5 Ishikawa cells were placed in 2 mL/well 0.22 of μm filtrated MEM with 10% HI FBS without anti-anti. After 24 h, the medium was removed and the cells were transfected with miRNAs and NC (50 nmol) dissolved in 2 mL Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I (1x) (Thermo Fisher Scientific, Waltham, MA, USA) without HI FBS or anti-anti. Each miRNA was transfected inside two wells in identical conditions. After 24 h, transfection medium was replaced with 0.22 μm filtrated MEM with 10% HI FBS and 1% anti-anti. Supernatants were collected from both wells 48 h post-treatment for enzyme-linked immunosorbent assay (ELISA). Cells were detached from one well and counted, setting aside 300,000 cells per insert for the wound healing assay. The remaining cells were lysed with 350 μl RNA lysis buffer supplemented with 1% β-mercaptoethanol for RNA extraction and further quantitative real-time polymerase chain reaction (qRT-PCR). Transfection efficiency of Ishikawa cells was assessed by qRT-PCR as previously described [15, 29] and found to range from 65% to 75% (Supplementary Figure S1).

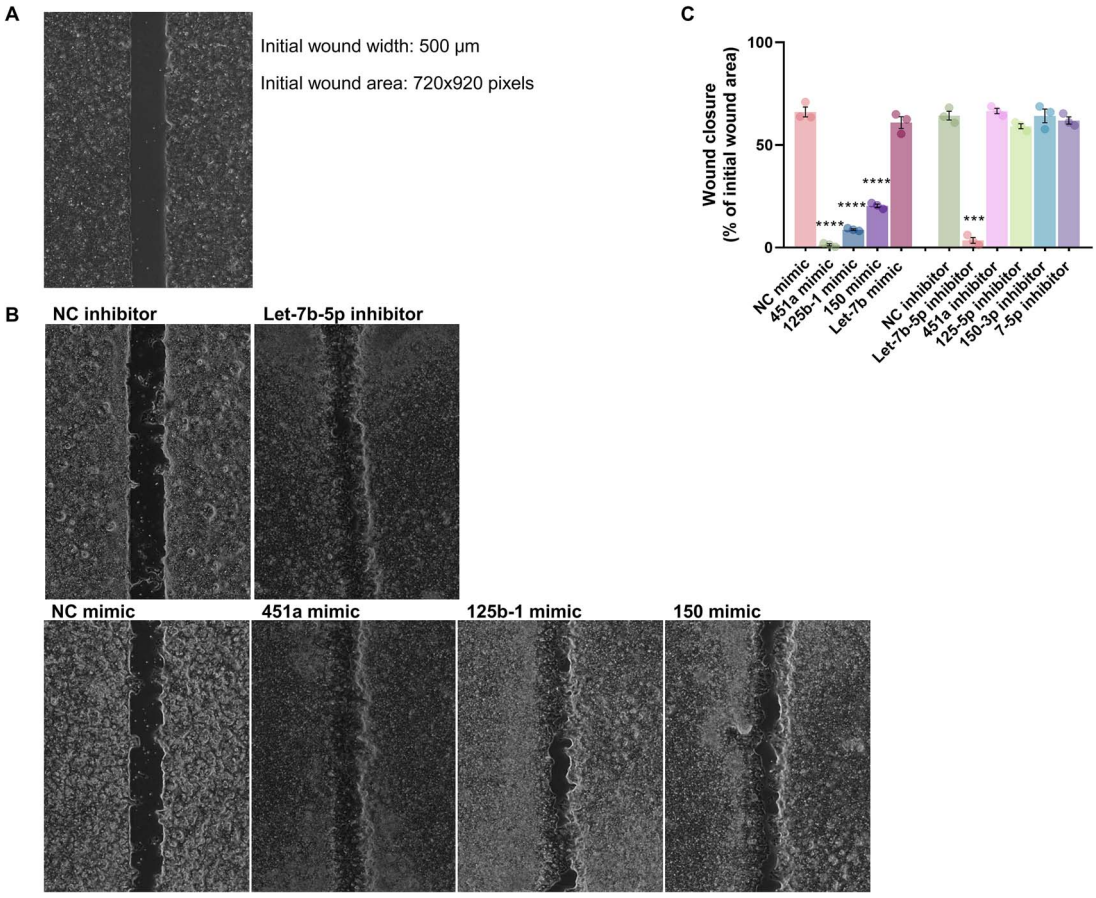


Figure 2. Wound healing assays. (A) Representative image showing the cell-free gap (initial wound area of 720 × 920 pixels) created upon insert removal. (B) Representative images of wound areas 24 and 48 h after scratching Ishikawa cell monolayers. Images were captured at 4× magnification. (C) Quantification of wound closure (%) over time, analyzed using Keyence microscope software (***$P < 0.001$; ****$P < 0.0001$; ns: non-significant). NC: negative control.

Wound migration assay

Culture inserts (Ibidi, Gräfelfing, Germany) split into 2 chambers were used to determine cell migration. A cell suspension (150,000 cells in 70 μl of 0.22 μm filtered MEM supplemented with 10% HI FBS and 1% anti-anti) was added to each chamber. After 24 h, when the cells had reached 80% confluency, the inserts were removed to create 500 μm cell-free gaps (initial wound width) corresponding to an initial wound area of 720 × 920 pixels (Figure 2A). To prevent cell proliferation, 70 μl of 0.22 μm-filtered MEM without HI FBS was added. Wound healing assays were carried out in triplicate with 2 images acquired per condition at 3 different timepoints (0, 24 and 48 h) using the Keyence fluorescence microscope (Keyence BZ-X 800, Osaka, Japan). For each condition, the mean wound area was calculated and compared to the initial wound area to determine the rate of wound closure. Data are presented as bar graphs.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from transfected Ishikawa cells using the RNeasy Plus Micro kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). One μl RNA per sample was loaded into the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for quantification. RNA (500 ng) was then reverse-transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) and the T100 thermal cycler machine (Bio-Rad, Hercules, CA, USA).

Gene expression levels of human Ki67, CASP-3, MMP-2, MMP-9, BAX, BCL2, CDH1, CDH2, SNAI1, SNAI2, AKT1 and PTEN (W.M. Keck Oligonucleotide Synthesis Facility, Yale University, New Haven, CT, USA) were evaluated using qRT-PCR SYBR Green (CFX Connect Real-Time system, Bio-Rad, Hercules, CA, USA). Gene expression was normalized to human glyceraldehyde 3-phosphate dehydrogenase (GADPH) and actin beta (ACTB) as internal controls (W.M. Keck Oligonucleotide Synthesis Facility, Yale University, New Haven, CT, USA). Each gene was tested in quadruplicate. Nuclease-free water served as an NC, replacing the cDNA template. Primer sequences are shown in Supplementary Table S1.

Quantitative RT-PCR was performed using 39 amplification cycles involving denaturation at 95°C for 5 s and annealing at 60°C for 30 s, following an initial activation step at 95°C for 2 min. Relative gene expression levels were determined using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$).

ELISA immunoassay

Human MMP-2 and MMP-9 ELISA kits (Invitrogen, Waltham, MA, USA, USA) were used to quantify protein

levels in supernatants of transfected Ishikawa cells. Analyses were conducted on cell culture supernatants, as MMP-2 and MMP-9 are secreted gelatinases that are released into the extracellular space, where they are activated and exert their function in ECM degradation. Assays were conducted according to the manufacturer's instructions. *Briefly*, standards and samples were added to pre-coated 96-well plates and incubated to allow binding of target proteins to captured antibodies. After washing, a biotinylated detection antibody was included, followed by horseradish peroxidase (HRP)-conjugated streptavidin. The signal was developed using a chromogenic substrate, namely 3,3′,5,5′ tetramethylbenzidine dihydrochloride (TMB), and the reaction was stopped with sulfuric acid. Absorbance was measured at 450 nm using the Azure Imaging Biosystems microplate reader (Dublin, CA, USA). Protein concentrations were calculated based on the 4-parameter logistic (4PL) standard curve from serial dilutions of recombinant human MMP-2 and MMP-9, respectively. To ensure that concentrations fell within the linear range of the standard curve, three dilutions of each sample (1×, 0.75× and 0.50×) were tested. Each ELISA was performed in triplicate and technically duplicated per condition to yield robust data for statistical analysis.

Statistical analysis

GraphPad Prism version 8 (San Diego, CA, USA) was used for statistical analysis. One-way ANOVA was applied to compare both mimic and inhibitor NCs among transfected samples. For normally distributed variables, data were represented as mean ± SEM. P-values <0.05 were considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

Results

Wound migration assays

Wound healing assay results demonstrated that Ishikawa cells transfected with the Let-7b-5p inhibitor, as well as miR-451a, miR-125b-1 and miR-150 mimics, exhibited enhanced wound closure over a 48-h period following a scratch in the cell monolayer, compared to cells transfected with the respective NC inhibitor or mimic (Figure 2B and C). By contrast, transfection with the Let-7b mimic or inhibitors of miR-451a, miR-125b-5p and miR-150-3p did not result in any significant difference in wound closure relative to their respective NCs. These findings indicate that EEC migration is promoted by inhibition of Let-7b-5p and overexpression of miR-451a, miR-125b-1 and miR-150.

Quantitative real-time polymerase chain reaction

Alterations in expression levels of Ki67, CASP-3, MMP-2, MMP-9, BAX, BCL2, CDH1, CDH2, SNAI1, SNAI2, AKT1 and PTEN *were evaluated in Ishikawa cells by qRT-PCR after liposomal transfection*. These genes were selected based on their proposed involvement in the pathogenesis of adenomyosis, particularly in cell migration, myometrial invasion and subsequent lesion establishment and development. They are key regulators of EMT, extracellular matrix remodeling, apoptosis resistance and proliferation, all of which contribute to the invasive phenotype of adenomyotic lesions.

Ishikawa cells transfected with the miR-451a mimic exhibited significantly higher Ki67 expression levels ($P = 0.022$) than those transfected with the NC mimic. Conversely, cells transfected with miR-451a inhibitor ($P < 0.0001$), miR-125b-5p inhibitor ($P < 0.0001$) and Let-7b mimic (*P* -value<0.0001) displayed significantly lower Ki67 expression values relative to their respective NCs (Figure 3A). M*iR-451a* ($P = 0.0345$; $P = 0.0312$), *miR-125b-1* ($P = 0.0292$; $P = 0.0234$) and *miR-150* ($P = 0.0339$; $P = 0.289$) *mimics* induced significantly stronger expression of *MMP-2* and *MMP-9* than did NC mimics. *Let-7b-5p inhibitor* ($P = 0.0005$) also boosted *MMP-9* expression, while *Let-7b mimic* ($P = 0.0358$) reduced *MMP-9* values, relative to their corresponding controls (Figure 3B and C). No statistically significant changes were observed in expression levels of CASP-3, BAX, BCL2, CDH1, CDH2, SNAI1, SNAI2, AKT1 or PTEN compared to their corresponding NCs (Supplementary Figure S2). These molecular findings are consistent with the wound healing assay results. Upregulation of MMP-2 and MMP-9 points to enhanced degradation of the ECM, which likely facilitates improved cell motility by creating space for migration. This is supported by the observed increase in wound closure in conditions with elevated MMP values, linking miRNA regulation to both gene expression and functional cell behavior.

ELISA immunoassay

To investigate the impact of miRNA transfection on MMP secretion, we measured MMP-2 and MMP-9 protein levels in the supernatant of Ishikawa cells by ELISA. We found significantly increased MMP-2 and MMP-9 values in cells transfected with miR-451a mimic (MMP-2, $P = 0.0002$; *MMP-9*, $P = 0.0004$), miR-125b-1 mimic (MMP-2, $P = 0.0004$), miR-150 mimic (MMP-2, $P = 0.0002$) and Let-7b-5p inhibitor (MMP-2, $P < 0.0001$; *MMP-9*, $P = 0.0002$) compared to the NC mimic and NC inhibitor (Figure 4A and B).

Discussion

Extensive efforts have been made to shed light on the roles of ESCs and immune components in adenomyosis development and progression [23, 25, 26, 27], but the functional contribution of EECs remains underexplored. Epithelial gland migration and invasion are key processes in myometrial infiltration, so understanding the molecular regulators of these behaviors is critical to elucidating disease pathogenesis. MiRNAs are emerging as important post-transcriptional regulators in various gynecological diseases, including endometriosis and adenomyosis [17, 28]. They have been shown to modulate a range of cellular processes like proliferation, apoptosis, inflammation and ECM remodeling [12]. While some studies have identified dysregulated miRNA profiles in eutopic endometrium from women with adenomyosis [13, 14, 18], the direct functional repercussions of these miRNAs on epithelial glands have not been ascertained.

In the present study, we investigated the effects of several miRNAs previously implicated in adenomyosis and endometriosis (Let-7b, miR-451a, miR-125b, miR-7 and miR-150) on EEC migration and MMP activity [15, 29, 30, 31], as these two diseases share biological and molecular features that may involve common miRNA-mediated pathogenic mechanisms. Using Ishikawa cells, a widely established human endometrial epithelium-like cell line, we conducted gain- and loss-of-function experiments through transfection with

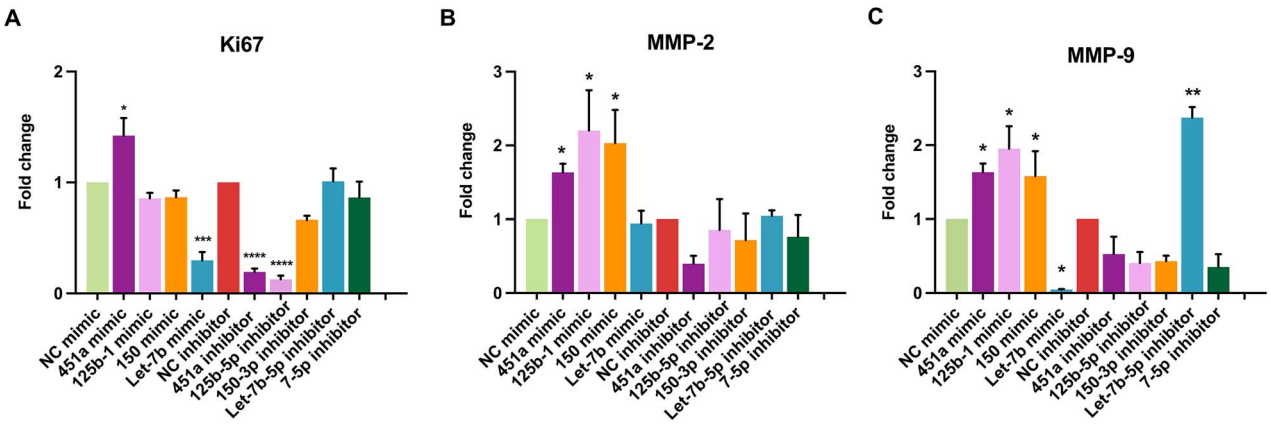


Figure 3. Gene expression analysis of Ki67, MMP-2 and MMP-9 in transfected Ishikawa cells. Effect of selected miRNAs on (A) Ki67, (B) MMP-2 and (C) MMP-9 gene expression in transfected Ishikawa cells. Relative fold changes in gene expression were established using the comparative CT method ($2^{-\Delta\Delta CT}$). Each gene was tested in quadruplicate. Data are presented as mean ± SEM. Statistically significant differences were determined using *P*-values <0.05 (* $P < 0.05$; *** $P < 0.005$). NC: negative control.

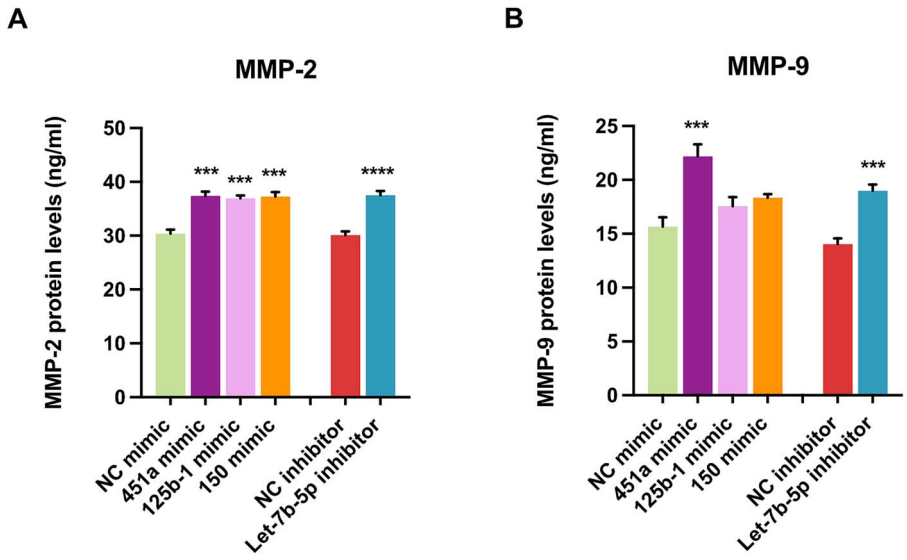


Figure 4. Quantification of MMP levels in supernatants of transfected Ishikawa cells. Bar plot showing (A) MMP-2 and (B) MMP-9 protein levels measured by ELISA in cell culture supernatants of Ishikawa cells transfected with miR-451a, miR-125b and miR-150 mimics, Let-7b-5p inhibitor and their respective NCs. Protein values were calculated based on the 4PL standard curve generated from serial dilutions of recombinant human MMP-2 and MMP-9, respectively. Each bar represents the mean value of technical replicates (n = 3). Statistically significant differences were determined using *P*-values <0.05 (*** $P < 0.005$; **** $P < 0.0001$). NC: negative control.

specific miRNA mimics and inhibitors. Wound healing assays revealed that transfection with Let-7b-5p inhibitor and miR-451a, miR-125b-1 and miR-150 mimics significantly boosted EEC migration. This was corroborated by molecular data showing statistically significant upregulation of MMP-2 and MMP-9 in terms of mRNA expression when Ishikawa cells were transfected with the same miRNAs. ELISA results further confirmed increased secretion of these proteins into the extracellular environment. These findings are consistent with the wound healing assay and qRT-PCR results, where the same miRNAs promoted faster wound closure and higher MMP expression levels. Elevated MMP-2 and MMP-9 secretions likely reflect enhanced ECM remodeling associated with heightened migratory activity. All in all, these data suggest that miR-451a, miR-125b-1 and miR-150 mimics as well as Let-7b-5p inhibitor could enhance EEC motility via MMP upregulation, thereby contributing to tissue remodeling processes involved in lesion development.

MMPs, zinc-dependent endopeptidases, play a crucial role in degradation and remodeling of the ECM. Among them, MMP-2 and MMP-9 are able to degrade most, if not all, components of the ECM at a neutral pH and activate or degrade several cytokines, chemokines and growth factors, thereby facilitating cell migration [32]. Studies have demonstrated increased expression of MMP-2 and MMP-9 in both eutopic and ectopic endometrial tissues from women with adenomyosis compared to normal endometrium. This overexpression correlates with enhanced angiogenesis, suggesting that MMPs may facilitate invasion of the myometrium by endometrial tissue and support formation of new blood vessels needed for lesion viability and persistence [33]. Our findings also align with previous studies showing stronger MMP expression in murine uterine tissue, despite most prior research focusing on stromal cells rather than epithelial glands [25]. Indeed, our results support the existence of a miRNA–MMP axis in EECs that may underlie a key pathological mechanism in

adenomyosis, contributing to the broader understanding of how EEC behavior might be modulated by miRNAs, promoting lesion development, persistence and growth.

This study has several strengths. First, we employed a multilevel approach, integrating phenotypic assays, gene expression analysis and protein quantification. Second, use of a human-derived cell line ensures reproducibility, laying the ground for mechanistic insights. However, there are limitations that must be acknowledged. While Ishikawa cells are widely used as a well-established model to study the endometrium, they may not fully reflect the behavior of primary EECs. In fact, the hormonal environment, cellular heterogeneity and immune context are known to play a role in the pathogenesis of adenomyosis [8, 34].

In summary, this study provides new evidence that adenomyosis- and endometriosis-associated miRNAs can enhance EEC migration through upregulation of MMP-2 and MMP-9. These findings highlight a potential mechanism by which MMPs participate in lesion formation in adenomyosis. Identification of a miRNA–MMP regulatory axis opens new avenues for research into disease progression and therapeutic targeting. Future studies should seek to validate these results in primary epithelial glands isolated from fresh endometrial biopsies, and explore the potential for miRNA-based therapies to mitigate lesion generation and improve clinical outcomes for patients with adenomyosis. MiRNA-based diagnostics or therapeutics could provide minimally invasive tools to identify or treat early-stage adenomyosis as demonstrated by their success in targeting endometriotic lesions in mouse models [35, 36]. Targeting MMP activity through tissue inhibitors of MMPs, otherwise known as TIMPs, and preventing invasion of the myometrium by endometrial tissue also hold promise for future therapeutic strategies aimed at mitigating development of this condition.

Supplementary data

Supplementary data are available at *BIOLRE* online.

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Author contributions

M.Z.: Conceptualization and design, methodology and experiment execution, formal analysis and interpretation of data, writing—original draft. A.C.: Review and editing. R.M.: Interpretation of data, supervision, review and editing. H.S.T.: Interpretation of data, supervision, review and editing. M-M.D.: Interpretation of data, supervision, review and editing.

Conflict of interest: The authors have nothing to disclose.

Data availability

All data needed to evaluate this article are provided in the main body or in the Supplementary Materials.

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