Effects of exosomes derived from eutopic endometrial cells on cell proliferation and apoptotic pathway in endometriosis and the discovery of novel exosome related biomarkers for endometriosis

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Effects of exosomes derived from eutopic endometrial cells on cell proliferation and apoptotic pathway in endometriosis and the discovery of novel exosome related biomarkers for endometriosis

Directed by Professor SiHyun Cho

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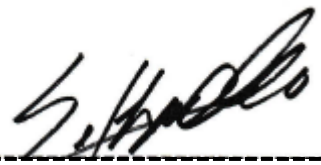
This certifies that the Master's thesis of
SeHee Kim is approved.



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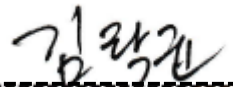

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김세희 올림

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ABSTRACT

Effects of exosomes derived from eutopic endometrial cells on cell proliferation and apoptotic pathway in endometriosis and the discovery of novel exosome related biomarkers for endometriosis

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(Directed by Professor SiHyun Cho)

Endometriosis (EMS) is an estrogen dependent disease, where tissue similar to the lining of the endometrium grows in other areas such as the ovaries, fallopian tubes, and peritoneal cavities. It commonly occurs in women of childbearing age and can cause complications such as infertility, but the pathogenesis has not been clearly identified, yet. Additionally, there is a high risk of recurrence, emphasizing the need for research on non-invasive biomarkers to enable urgent diagnosis. Exosomes, small extracellular vesicles with a diameter of 30-100 nm, are secreted from most cell types to reflect the internal state of cells and are known to play a crucial role in disease progression in various pathological processes. In addition, it encapsulates microRNA and is highly applicable as a diagnostic marker. Studies on the pathophysiology of endometriosis have partially reported the role of exosomes in mechanisms related to angiogenesis and immunity, but the mechanisms associated with cell proliferation and apoptosis remain unclear. In this study, we

hypothesized that exosomes play a role by the autocrine effect, and related biomarkers were identified to confirm their potential as diagnostic markers. We collected eutopic endometrial tissues from patients with endometriosis (EMS-EM) and without endometriosis (CTL-EM) and cultured to primary cells. Exosomes were extracted from EMS-EM and CTL-EM, respectively, and then treated *in vitro* endometriosis model for 24 hours. Results from CCK-8 and FACS assays showed that exosome extracted from EMS-EM (EMS-Exo) treatment induced cell proliferation and reduced apoptosis. In particular, JC-1 red/green dye was significantly increased, and the mitochondrial apoptosis signal pathway was regulated by EMS-Exo treatment. The examination of protein expression identified that proliferation was induced through ERK and AKT signaling, and mitochondrial apoptosis signaling pathway was regulated through PI3K/AKT mechanism. To identify related microRNAs, we performed miRNA array analysis on exosomes collected from EMS-EM and CTL-EM cell. The results showed differences in 16 miRNAs, with hsa-miR-200a-3p upregulated and 29a-3p down regulated. These 2 miRNAs were found to be related to the PI3K/AKT mechanism. Furthermore, qRT-PCR was performed on serum exosomes for in vivo diagnosis, and significant differences were observed in 5 miRNAs. Finally, ROC curve analysis was conducted to identify the potential of these miRNAs as diagnostic biomarker, and 4 miRNAs showed diagnostic performance. However, in order to be commercially viable, a combination with increased sensitivity and accuracy needs to be identified. In conclusion, these findings suggest that the exosome-related mechanism influences the development of endometriosis and highlights the potential of exosomes as novel diagnostic biomarkers for endometriosis.

Key words: endometriosis, exosome, autocrine effect, proliferation, anti-apoptosis, mitochondria, biomarker

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I. INTRODUCTION

Endometriosis (EMS) is a condition where tissue similar to the lining of the womb starts to grow in other places, such as the ovaries, fallopian tubes, and peritoneal cavities.[1] EMS is a chronic, inflammatory condition of high incidence and serious complications such as subfertility and pain. It occurs in about 10% of women in childbearing age, characterized by endometrial growth and endometrial infiltration, and 50% of these patients suffer from chronic pelvic pain (CPP).[2-3]

Numerous hypotheses have been proposed to explain development of EMS. Some studies suggest that the development of stable changes in cell shape is due to continuous stimulation, development from remnants of Müllerian ducts, and endometrial growth caused by retrograde

menstrual reflux. Recently, the tissue damage and recovery theory has been reported, indicating that the local production of estrogen by ectopic endometrial tissue infiltrates into the basal endometrial myometrium and abdominal cavity. This infiltration leads to nerve division and neuropathy, resulting in varying degrees of pain .[4-5]

Numerous hypotheses exist regarding EMS, yet the precise pathophysiology underlying its development remains unclear. EMS is reported as a multifaceted disease with evidence of dysregulation of signal transmission pathways, including angiogenesis, attachment, adherence, invasion, migration, proliferation, apoptosis, and inflammation.[6-8] A number of biochemical differences in blood, peritoneal fluid, urine and tissues in women with or without endometriosis have been reported.[9-11] It is usually diagnosed and treated through laparoscopic surgery, and long-term medical management is recommended after surgery due to the high recurrence rate. However accurate prediction of recurrence is challenging due to the lack of reliable biomarkers.[12-15] Therefore, continuous research aimed at identifying specific non-invasive diagnostic markers and developing effective treatments for patients with endometriosis is needed.

Exosomes are small extracellular vesicles, ranging from 30 to 100 nm in diameter, composed of a lipid bilayer produced from the cell membrane and various transmembrane proteins, which encapsulate miRNA, long non-coding RNAs, micromolecular proteins, and cytokines.[16-17] Exosomes are secreted by diverse cell types and are also present in biological liquids such as blood, cerebrospinal fluid, and urine.[18] Their main function is to facilitate cell-to-cell communication by delivering specific proteins, nucleic acids, and lipid cargoes

to recipient cells.[19] Consequently, exosomes have been implicated in disease progression of various disease conditions.[16] Recent studies have demonstrated that exosomes are released during cellular events, such as cell activation, autophagy, apoptosis, and cancer.[16,20-21] Moreover, exosome-derived microRNAs have been identified as biomarkers in the development of early diagnostic technologies for diseases such as cancer and brain disorders.[22,23]

Autocrine signaling refers to a type of cell signaling wherein hormones, growth factors, or genetic signals are released by a cell, binding to autocrine receptors on the same cell and inducing cellular changes.[24] Exosomes affect cellular functions in autocrine signaling through the encapsulation of microRNA/DNA and membrane-bound proteins.[25] For instance, studies have reported that prostate tumor cell exosomes increase autocrine proliferation by altering the gene expression of stromal cells and BMI1-containing exosomes promote carcinoma proliferation and metastasis through autocrine signaling.[26,27] Recent research has implicated exosomes in the pathogenesis of endometriosis, particularly in processes such as angiogenesis, inflammation, and invasion.[28-30] However, there have been no reports that exosomes are involved in cell proliferation, migration and apoptosis via autocrine effects. Therefore, we investigated this specific mechanism in EMS cells and mitochondrial-mediated apoptosis. Additionally, specific microRNA changes were identified to determine their potential as diagnostic markers in blood samples. Our findings suggest that exosomes can play a crucial role in the development of endometriosis through their autocrine effect.

II. MATERIALS AND METHODS

1. Study population and sample collection

Twenty women (age range, 15–52 years) participated in this study. All participants underwent laparoscopic surgery at Gangnam Severance Hospital (Seoul, South Korea) for endometriosis, or other benign gynecological diseases. During the surgery, all potential endometriotic lesions were excised and sent for pathologic evaluation. Based on the pathology results, each patient was assigned to a study group. Among the 20 women, 10 patients were assigned to the endometriosis group and 10 patients were assigned to the control group. In the control group, each patient had a different benign condition instead of endometriosis, such as dermoid cysts or simple serous cysts. Eutopic endometrial tissue samples were obtained from the 10 patients with endometriosis and the 10 patients with other benign ovarian cysts. All samples were rinsed in cold phosphate-buffered saline (PBS; Welgene, Gyeongsan, Korea) three times, sectioned into 4 mm^3 pieces, and then stored at -80 °C in vials containing RNAlater (Invitrogen, Carlsbad, CA, USA) for nucleic acid preservation.

A total of 50 serum samples (25 patients with endometriosis and 25 patients without the disease) were obtained from Gangnam Severance Hospital (Seoul, South Korea). The serum samples were collected in SST tubes and allowed to clot at room temperature for 30 min. Subsequently they were centrifuged at 3,000g for 10 min. All samples were extracted

within 1 hr after receiving whole blood and then immediately stored at -80 °C until further use.

The study was conducted with the approval from the Institutional Review Board of Gangnam Severance Hospital, Yonsei University College of Medicine (IRB approval no. 3-2018-0231). Each patient provided informed written consent and the hospital's ethics committees approved enrollment in the study. Written informed consent was obtained from all participants.

2. Endometrial primary cell isolation and culture

Endometrial primary cells were isolated from fresh human eutopic endometrial tissues with endometriosis (EMS-EM) and without endometriosis (CTL-EM). Tissue samples were minced and incubated in PBS, containing collagenase type I 2.0 mg/mL (Gibco, Waltham, MA, USA), for 2 hr at 37 °C and a 5% CO2 incubator. Cells were obtained after filtering through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA). The supernatant was removed and cells were resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Cytiva, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 2% penicillin-streptomycin (P/S; Cytiva, Marlborough, MA, USA) in a 5% CO2 atmosphere at 37 °C incubator. For passaging, when the cells reached 80~90% confluency, 2 ml 0.25% trypsin EDTA (Gibco, Waltham, MA, USA) was used to digest the cells for 5min in the incubator, then 1 ml

culture medium was added to stop the digestion. The primary cultured cells of from passages 3 to 5 were used for the experiments (Figure 1A).

3. Endometrial primary cell immunostaining

Microscope cover glasses (Marienfeld, Lauda-Königshofen, Germany) were inserted into a 12-well plate using forceps, after being sterilized. Poly-L-lysine solution (Merck, Darmstadt, Germany) was added to each well at a volume of 700 μl and left at least 3 hr. The plate was then washed with PBS three times. Cells were seeded and allowed to reach approximately 50% confluency. The cells were fixed with 4% paraformaldehyde (Forbio, Seoul, Korea) on a shaker (Daihan Scientific, Wonju, Korea) at 40 rpm for 15 min at room temperature. After being washed three times with PBS, a solution of 300 μl per well containing 4 % normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.2% Triton X-100 (Merck, Darmstadt, Germany) in PBS was added, and the plate was blocked on a shaker for 1 hr. The primary antibody CD44 (Abcam, Cambridge, UK) was diluted 1:100 in 4 % normal goat serum solution and added at a volume of 300 μl per well, and the plate was shaken overnight at 4 °C. After removing the primary antibody, the sample was washed three times with PBS for 5 min each time. The sample was then incubated with the secondary Alexa488-conjugated rabbit antibody (Abcam, Cambridge, UK) diluted 1:400 in PBS for 2 hr at room temperature. After washing with PBS, DAPI (Merck, Darmstadt, Germany) was added at a volume of 300 μl per well, and the plate was stained for 5 min.

After washing with PBS, a drop of mounting medium (Dako, Glostrup, Denmark) was added onto the slide glasses (Citotest Scientific, Jiangsu Province, China), covered with a coverslip, and allowed to dry completely. Nail polish was used to seal the coverslip. Finally, the sample was imaged using a confocal microscopy (LSM 980, Carl Zeiss, Oberkochen, Germany) (Figure 1B).

4. Extraction and treatment of Endometrial cell exosome

For the preparation of endometrial cell exosome, EMS-EM or CTL-EM cells were seeded 3×10^5 cell/well in a 6-well dish and cultured when reached 80~90% confluency. Before exosome isolation, the culture medium was exchanged to DMEM/F12 for 24 hr at 37 °C in a 5% CO_2 incubator. Exosome isolation using Exo-quick-TC (SBI, Palo Alto, CA, USA) was performed according to the manufacturer's specifications. Briefly, the culture medium was centrifuged at 1200g for 20 min to remove cells and debris, and the 5 ml media supernatant was mixed thoroughly with 1ml Exo-quick exosome precipitation solution and incubated for 24 hr at 4 °C. Media supernatant complex was centrifuged at 1,500g for 30 min, and then the supernatant was removed and centrifuged at 1,500g for 5 min again. The remaining exosome pellet was resuspended in DMEM/F12. Exosome concentration was measured using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Cells were treated with 20 μg/well of EMS-EM cells exosomes (EMS-Exo) or CTL-EM cells exosomes (CTL-Exo) for 24 hr (Figure 2A).

5. Cell Proliferation assay

EMS-EM cells were seeded at a density of 5×10^3 cells/well in 96-well plates. After 24 hr, the culture medium was exchanged to DMEM/F12 with 0.67 μg/well of EMS-Exo and CTL-Exo. The next day, cell proliferation assays were performed by dropping 100 μl of Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) into each well, and incubating at 37 °C for 2 hr. The supernatants measured at an optical density (OD) of 450 nm using a VersaMax microplate reader (Molecular Devices, San Jose, CA USA).

6. Cell Migration assay

A. Wound healing assay

EMS-EM cells were seeded at a density of 3×10^5 cells/well in 6-well plates. when cells reached 80~90% confluency, the culture medium was exchanged to DMEM/F12 with 20 μg/well of EMS-Exo and CTL-Exo. A sterile pipette tip was used to create a clear line. Initial area was measured using Image J. After 24 hr, Final area was also measured using Image J software (NIH, Bethesda, Maryland, USA). The percent of Wound healing area was calculated [(initial area – final area)/initial area] x 100.

B. Crystal violet migration assay

The 8 μm cell culture insert (Merck, Darmstadt, Germany) was placed in 24-well plates, and 700 μl of DMEM/F12 media containing 10% FBS and 2% P/S was added to the plates.

Exosome-treated cells were seeded at a density of 5×10^4 cells/insert with Serum free media. After 24 hr, the media in the insert was removed, and the insert was gently wiped with a cotton swab. To fix the insert, it was submerged in 75% EtOH (Merck, Darmstadt, Germany) and left for 10 min. Following this, the EtOH was removed, and the insert was left to dry. Next, 0.2% Crystal violet (Thermo Scientific, Waltham, MA, USA) was added to the insert and left for at least 15 min. After that, the insert was wiped with cotton swab and washed in water. Finally, after the insert was allowed to dry, a picture was taken with a microscope, and the migrating cell was counted using Image J software (NIH, Bethesda, Maryland, USA).

7. Annexin-v/propidium iodide labeling and flow cytometry assay for apoptosis

EMS-EM cells were seeded 3×10^5 cells/well and cultured in a 6-well plate. when reached 80~90% confluency, the culture medium was exchanged to DMEM/F12 followed by treatment with 20 μg/well of EMS-Exo and CTL-Exo for 24 hr. For Annexin V and Propidium Iodide (PI) staining in cell death assays, the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used. Briefly, after treatment cells were trypsinized, washed in PBS, and resuspended 1×10^5 cells/tube in Annexin V binding buffer. Standard samples for each staining dye were mixed, including a negative sample (only cells), an Annexin V staining standard sample, and a PI staining standard sample. Annexin V and PI were added to the test samples and incubated for 15min in the dark at

room temperature. Standard samples were used to determine accurate settings and then each 10,000events test sample were reading using FACS Canto II (BD biosciences, San Jose, CA, USA). The fluorescence was analyzed by with Flowing Software 2.5.1 (Flowing Software, Turku, Finland). The experiment was performed five times.

8. Assessment of mitochondrial membrane potential

The mitochondrial membrane potential was determined by the Mito Probe JC-1 assay kit (Invitrogen, Carlsbad, CA, USA). EMS-EM cells were cultured in a confocal flux bottom dish (SPL, Pochen, Korea). When the cells reached approximately 50% confluency, the culture medium was exchanged to DMEM/F12. EMS-EM cells were then treated with EMS-Exo and CTL-Exo for 24 hr. Subsequently, the cells were loaded with a final concentration of 2 μM of JC-1 at 37 °C for 15 minutes and 50 μM of DAPI (Sigma, St. Louis, MO, USA) at RT for 5 min. The cells were washed with PBS and imaged using confocal microscopy (LSM 980, Carl Zeiss, Oberkochen, Germany). JC-1 monomer and aggregate were quantified using Image J software (NIH, Bethesda, Maryland, USA).

9. Western blotting

Proteins were extracted using radioimmunoprecipitation assay buffer (RIPA buffer; Thermo Scientific, Waltham, MA, USA) with a protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). The protein concentrations were measured using the

bicinchoninic acid assay kit (Thermo Scientific, Waltham, MA, USA). Equal protein (20 μg) was mixed with 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Biosesang, Seongnam, Korea) and heated at 95 °C for 5 min. The samples were filtered through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and then moved to polyvinylidene fluoride membranes (PVDF; Merck, Darmstadt, Germany). Membranes were blocked with 5% non-fat skim milk in Tris-buffered saline solution (10mM Tris-HCl [Ph 7.4] and 0.5M NaCl) with Tween-20 (0.1 % v/v) at room temperature for 1 hr. The membranes were incubated with primary antibodies specific for CD63 (1:1000, Abcam, Cambridge, UK), Phospho-p44/42 MAPK (p-ERK 1/2) (1:500, Cell signaling technology, Danvers, MA, USA), p44/42 MAPK (ERK 1/2) (1:500, Cell signaling technology, Danvers, MA, USA), Phospho-AKT (p-AKT) (1:500, Cell signaling technology, Danvers, MA, USA), AKT (1:500, Cell signaling technology, Danvers, MA, USA), Bcl-2 (1:250, Santa Cruz, Dallas, TX, USA), Bax (1:500, Santa Cruz, Dallas, TX, USA), caspase-3 (1:200, Santa Cruz, Dallas, TX, USA) and GAPDH (1:2000, Santa Cruz, Dallas, TX, USA) overnight at 4 °C. The membranes were then incubated with Goat anti-Mouse IgG (H+L) or anti-Rabbit IgG (H+L) secondary antibody (1:3000, Thermo Scientific, Waltham, MA, USA) for 1 hr at room temperature. Detection was facilitated by Super Signal West Pico Plus Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) solution and imaged in a chemiluminescence imaging system (Image Quant LAS 4000; General Electric, Chicago, IL, USA). The bands were quantified by densitometry using Image J software (NIH, Bethesda, Maryland, USA).

10. miRNA analysis

A. Nano String nCounter Platform

miRNA presence and content of the samples were analyzed with the nCounter Human miRNA expression Assay kit (Nano String, Seattle, WA, USA) according to the manufacturer's instructions. 100 ng of exosomal cell RNA sample was mixed with 8μl of Master Mix. 2 μl of the capture probe Set was added to the solution, which was mixed thoroughly and then spun down. It was placed in a 65 °C thermocycler (Bio-Rad Laboratories Inc, Hercules, CA, USA) for 16 hr. The samples were transferred to the preparation station (Nano String Technologies, Seattle, WA, USA) with prepared nCounter Master Kit and a cartridge. The samples are binding to the cartridge. The preparation station 12 lanes per run in approximately 2.5 to 3 hr. The cartridges were transferred to the Digital Analyzer (Nano String Technologies, Seattle, WA, USA) for analysis. Cartridges were then scanned on the Digital Analyzer at 555 fields of view.

B. Normalization and Differential expression analysis

To quantify the miRNA expression values, first, background correction was skipped as default. Then global normalization was performed by TOP 100 methods which normalizes to total counts of the 100 most highly expressed miRNA targets across all samples. Second, differentially expressed miRNAs (DEmiRNAs) between the two selected biological conditions were analyzed by t-test on log2-transformed count data. All the expression

analyses described above were performed using nSolver Analysis software Ver 4.0 (Nano String Technologies, Seattle, WA, USA).

C. Functional analysis

To compare the expression profiles among the samples, normalized expression values of the differentially expressed miRNAs were plotted as a heatmap through unsupervised hierarchical clustering technique, using in-house R scripts. To get some insights on function of the DEmiRNAs between the compared biological conditions, gene-set enrichment tests with the target mRNAs(genes) of DEmiRNAs were performed using g: Profiler, for various functional gene-sets including KEGG pathways, etc. Target gene prediction was performed by miRDB databases (http://mirdb.org).

11. Precipitation of Serum exosome and RNA extraction

The precipitation of serum extracellular vesicles was performed using Exo-quick-TC (SBI, Palo Alto, CA, USA) according to the manufacturer's instructions. 500 μL of each EMS and CTL patients' serum was mixed with 4.5 ml PBS, and then 1 ml of Exo-quick was added according to the method. The total exosomal RNA was isolated from serum exosome pellet by the Trizol method. For this, 1 ml of Trizol reagent was added to the exosome pellet according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and

dissolved in 20 μL of RNase-free water. RNA was stored at -80 °C until further use (Figure 2B).

12. microRNA real-time quantitative reverse transcription PCR

Quantitative real-time PCR (qRT-PCR) was performed with specific primers for miRNAs to quantify the expression of hsa-miR-216a-5p, 147a, 29a-3p, 200a-3p, 191-5p, 10b-5p (Table 1). TaqMan advanced miRNA assay (Thermo Scientific, Waltham, MA, USA) was used for miRNA detection and quantification. The RT reaction was performed using a TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). miRNAs were confirmed by Taqman fast advanced master mix (Thermo Scientific, Waltham, MA, USA) with a Step One Plus Real-time PCR System (Applied Biosystems, Waltham, MA, USA). cel-miRNA-39-3p, a non-human miRNA, was spiked into RNA samples as a control for extraction and amplification steps. Threshold cycle (Ct) values were used to calculate the relative miRNA expression using the ΔΔCt method. Each reaction was run in Triplicate. A comparison between groups was performed using the student's *t*-test.

13. Statistical analysis

Image J (NIH, USA) was used to quantify the protein blot and image intensity. Data were analyzed using one-way ANOVA. Data were expressed as mean ± S.E.M and differences were deemed significant when $P < 0.05$. All experiments are triplicated. All statistical analysis and ROC curve data are analyzed using the Graph pad Prism program (GraphPad Software Inc, San Diago, CA, USA).

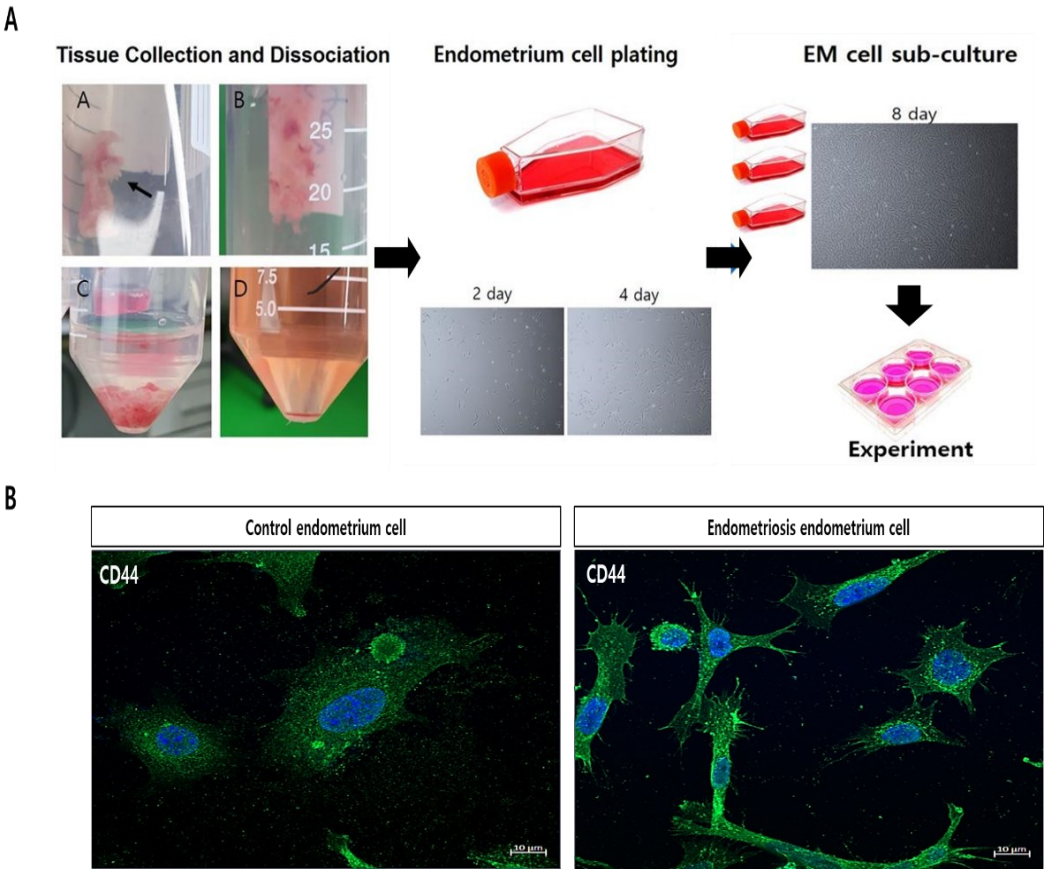

Figure 1. Schematic diagram of primary cell culture and characterization. (A) Schematic diagram of tissue collection and primary cell culture, (B) Immunostaining of endometrium cell marker CD44 (Green) and DAPI (Blue). (Scale bar = 10 μm)

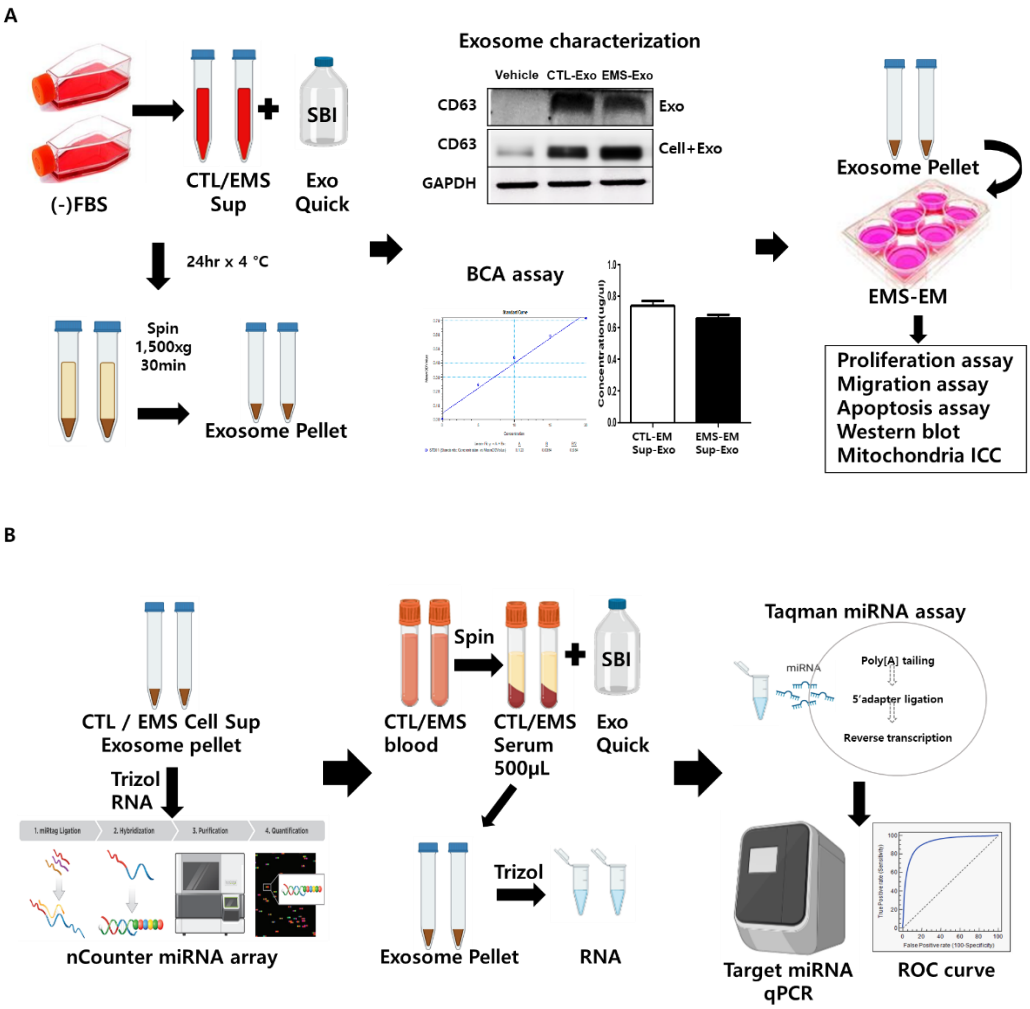

Figure 2. Schematic diagram of exosome pathology study and biomarker study. (A) Schematic diagram of exosome pathology study. Exosome characterization through CD63 transmembrane protein exosome marker. (B) Schematic diagram of exosome biomarker study

Table 1. A list of TaqMan advanced microRNA assays for qRT-PCR

miRNA	Assay ID	Mature miRNA sequence
Hsa-miRNA-216a-5p	478490_mir	UAACACUGUCUGGUAACGAUGU
Hsa-miRNA-147a	478514_mir	GUGUGUGGAAAUGCUUCUGC
Hsa-miRNA-29a-3p	478587_mir	UAGCACCAUCUGAAAUCGGUUA
Hsa-miRNA-200a-3p	478490_mir	UAACACUGUCUGGUAACGAUGU
Hsa-miRNA-191-5p	477952_mir	CAACGGAAUCCCAAAAGCAGCUG
Hsa-miRNA-10b-5p	478494_mir	UACCCUGUAGAACCGAAUUUGUG
Cel-miRNA-39-3p	478293_mir	UCACCGGGUGUAAAUCAGCUUG

III. RESULTS

1. EMS-Exo treatment induces cell proliferation.

To investigate the effect of EMS-Exo on cell proliferation, an *in vitro* endometriosis model was treated with EMS-Exo and CTL-Exo for 24 hr, respectively. The cell morphology appeared denser (Figure 3A) and the results from CCK-8 showed that EMS-Exo treatment significantly increased cell survival compared to the vehicle and CTL-Exo. The relative cell viability percentages were 100%, 103%, and 118% (Figure 3B). Therefore, these results suggest that EMS-Exo treatment induces cell proliferation in endometrial cells.

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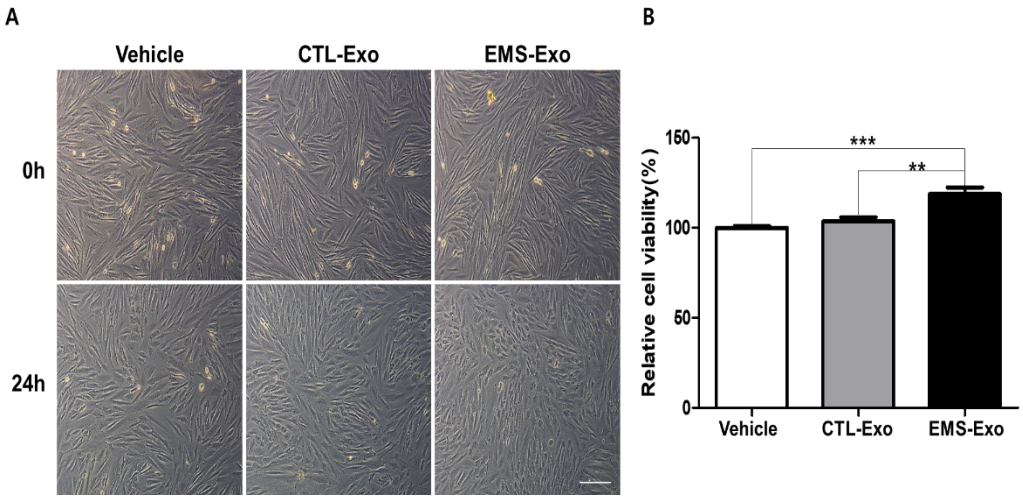

Figure 3. Cell proliferation assay. (A) Effects of exosome on cell viability. EMS-EM cells were treated with 20 μg of each exosome for 24 hr. EMS-EM cell morphology after treating vehicle, CTL-Exo, EMS-Exo (scale bar = 500 μm). CCK-8 assay was performed to measure cell viability. (B) Cell viability was significantly increased in the EMS-Exo treatment group compared to Vehicle and CTL-Exo. The results are shown as the mean ± S.E.M of three independent experiments. (n=3; **p<0.01, ***p<0.001).

2. EMS-Exo treatment does not affect cell migration.

The results of the wound healing assay showed that the percentage of wound closure for the vehicle, CTL-Exo, and EMS-Exo treated samples were 33%, 28%, and 30%, respectively, with no significant difference (Figure 4A, B). Similarly, the number of cells per field in the transwell migration assay was 17.5, 34.2, and 36.3ea for vehicle, CTL-exo, and EMS-exo treated samples, respectively, with no significant difference between CTL-exo and EMS-exo (Figure 4C,D). Therefore, the results suggest that EMS-exo treatment did not have any significant effect on cell migration.

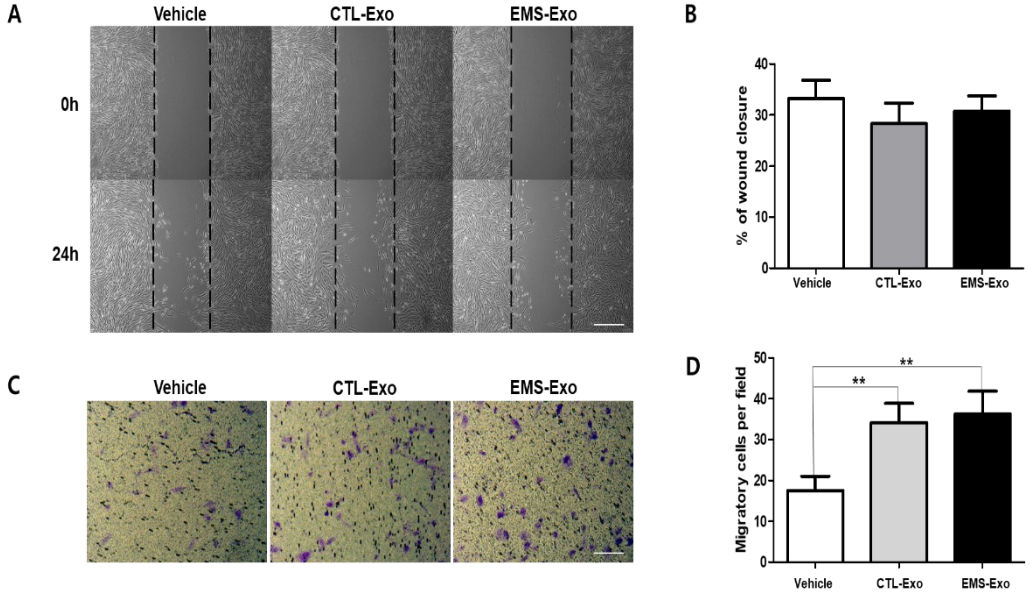

Figure 4. Cell Migration assay. (A) EMS-EM cells were treated with 20 μg of each exosome for 24 hr. Morphology of wound healing assay after treating vehicle, CTL-Exo, EMS-Exo (scale bar = 500 μm). (B) Percent of wound closure. (n=3, $p>0.05$) (C) Transwell migration assay after treatment each exosome (scale bar = 50 μm). (D) Migratory cells per field. The results are shown as the mean ± S.E.M of three independent experiments. (n=4, $^{**}p<0.01$)

3. EMS-Exo treatment reduces cell apoptosis through mitochondrial-mediated pathway

Flow cytometry analysis showed that both the proportion of early apoptosis and the late apoptosis were significantly decreased in the EMS-Exo treatment as compared to the vehicle and CTL-Exo (Figure 5A). The result of FACS demonstrated that the percentage of apoptotic cells in the vehicle, CTL-exo, and EMS-exo treated samples were 100%, 85.9%, and 61.3%, respectively (Figure 5B). Since we observed that EMS-Exo treatment reduces apoptosis, we hypothesized that it could regulate the *in vitro* endometriosis model through the mitochondrial apoptosis pathway. To test this hypothesis, mitochondrial membrane potential was determined with JC-1 staining. Upon treating cells with EMS-Exo, the JC-1 aggregation (red) was increased and the JC-1 monomer (green) was decreased compared to the vehicle and CTL-Exo (Figure. 5C). The Red/Green ratio increased up to 10-fold and up to 12.3-fold in EMS-Exo treatment compared to the vehicle and CTL-Exo (Figure. 5D). Therefore, these results suggest that EMS-Exo treatment reduces apoptosis in the *in vitro* endometriosis model through the mitochondrial-mediated apoptosis.

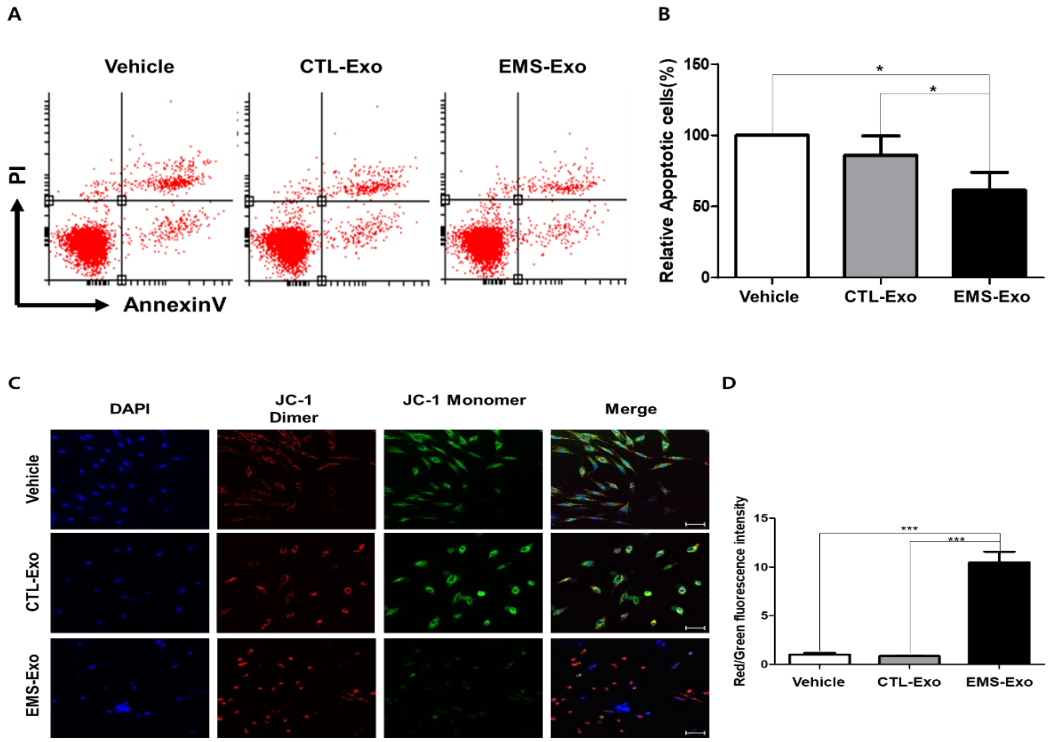

Figure 5. Cell Apoptosis assay. (A) Analysis of apoptosis by flow cytometry. Cells were incubated with each exosome for 24 hr, after which they were harvested, the DNA was stained with propidium iodide, and the cells were analyzed using FACS. Exosome decreased the proportion of apoptotic cells in the EMS-Exo treatment group compared to Vehicle and CTL-Exo. The results are shown as the mean ± S.E.M of three independent experiments (n=5; *p<0.05). (C) JC-1 expression *in vitro* endometriosis model after culturing with each exosome for 24 hr. The mitochondrial potential was observed via JC-1 staining. The red fluorescence of the JC-1 probe indicates the normal mitochondrial potential (anti-apoptosis), whereas the green fluorescence of the JC-1 probe means a defective mitochondrial potential (apoptosis) (Scale bar=50 μm). (D) The JC-1 fluorescence ratio was calculated by the average optical fluorescence density ratio of red/green. Data were shown as mean ± S.E.M of three independent experiments (n=3, ***p<0.0001).

4. EMS-Exo treatment regulates cell proliferation and mitochondria-mediated apoptosis via AKT and ERK pathway in *in vitro* endometriosis model

The AKT signaling pathway is one of the most critical pathways in regulating cell survival[31] while the ERK pathway regulates cell proliferation and cell differentiation[32]. We hypothesized that EMS-Exo might regulate cell proliferation through these pathways. Western blot results show that EMS-Exo treatment activated the phosphorylation of AKT and ERK (Fig. 6A). The p-AKT/AKT protein level increased to 3.09-fold and to 2.45-fold in EMS-Exo treatment compared to vehicle and CTL-Exo. The p-ERK/ERK protein level also increased to 1.65-fold and to 1.30-fold in EMS-Exo treatment compared to both (Fig. 6B). As mitochondrial-mediated apoptosis is characterized by the mitochondrial potential dissipation, we observed mitochondrial-related proteins, including Bcl-2, Bax, Cleaved Caspase 3 and the upstream AKT pathway (Fig. 6C). The Bax ratio decreased by 0.8-fold in EMS-Exo treatment compared to both. The Bcl-2 ratio exhibited an increasing tendency in EMS-Exo treatment. The Cleaved Caspase 3 ratio decreased by 0.5-fold in EMS-Exo treatment compared to vehicle and decreased tendency was noted compared to CTL-Exo (Fig. 6D). In conclusion, these data suggest that EMS-Exo regulates proliferation through AKT and ERK signaling and mitochondrial anti-apoptosis through affecting PI3K/AKT pathway.

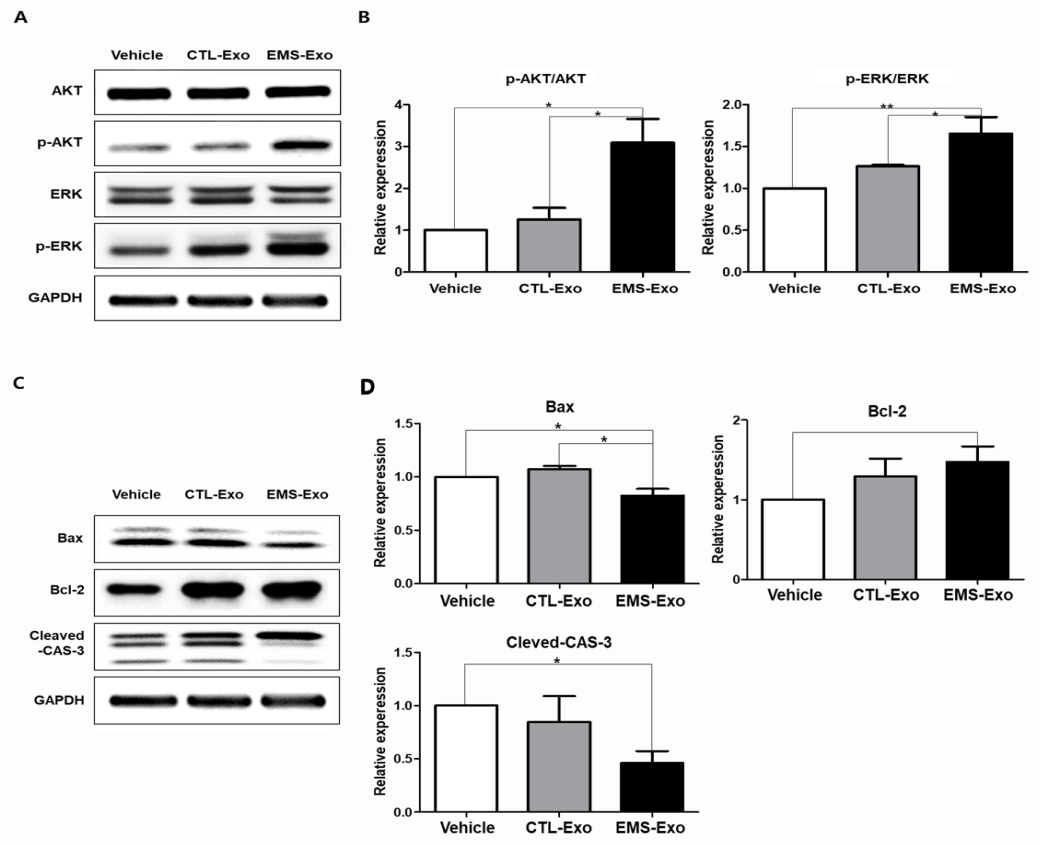

Figure 6. Cell proliferation-related protein AKT, ERK expression and Mitochondrial apoptosis related protein PI3K/AKT expression. (A) Proliferation related proteins AKT, P-AKT, ERK, and P-ERK were detected by western blotting after cells were treated with each exosome for 24 hr. (B) Calculated P-AKT/AKT, and P-ERK/ERK ratio. Data were shown as mean ±S.E.M of three independent experiments (*P<0.05, **p<0.01). (C) Apoptosis related proteins Bax, Bcl-2, and Caspase-3 were detected by western blotting after cells were treated with each exosome for 24 hr. (D) Calculated Bax/GAPDH, Bcl-2/GAPDH (vehicle vs EMS-Exo: p=0.07), Cleaved Caspase-3/GAPDH ratio. Each experiment was performed three times and all data were expressed as mean ±S.E.M. (*P<0.05).

5. Exosomal miRNA profiling reveals association between Hsa-miR-29a-3p and Hsa-miR-200a-3p with PI3K-AKT signaling Pathway.

To find related miRNA, we performed miRNA array analysis in EMS-EM and CTL-EM cell exosomes and confirmed 12 miRNAs were upregulated and 4 were downregulated in EMS-Exo compared to CTL-Exo (Figure 7A, Table 2). In TOP 10 singular enrichment analysis mirTarBase, Hsa-miR-29a-3p and Hsa-miR-200a-3p dominated the top rankings in the analysis (Figure 7B). In KEGG pathway, these microRNAs are also showed an association with the PI3K/AKT pathway (Figure 7C). In Target gene prediction, hsa-miR-29a-3p and hsa-miR-200a-3p regulation were related with caspase3 and Bcl2 (Figure 7D).

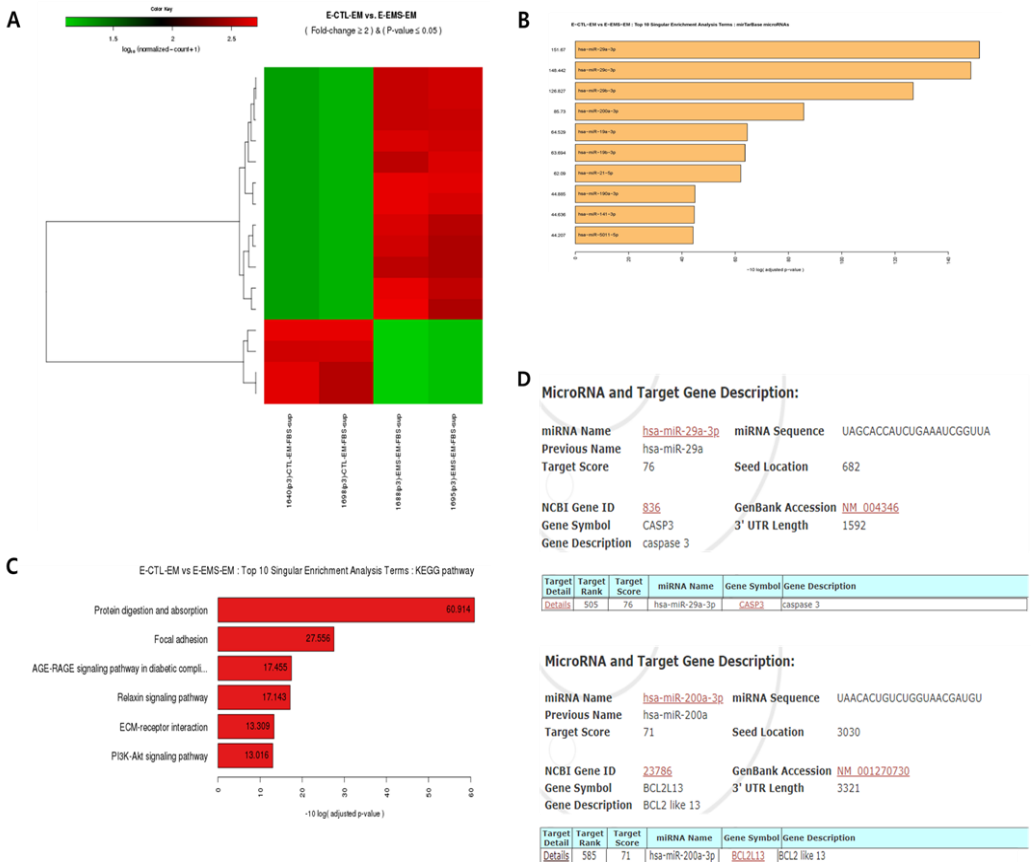

Figure 7. nCounter miRNA assay and Functional Study. (A) Heatmap of miRNA profiling between EMS-EM Exo vs CTL-EM Exo. Hierarchical clustering shows 14 significantly regulated miRNAs in EMS-EM and CTL-EM. A total of 12 miRNAs were upregulated and 4 was downregulated in the EMS group compared to those in the CTL group. The color code in the heat maps is linear, with green and red indicating the lowest and highest expression, respectively (fold-change ≥2, p-value < 0.05). (B) Top 10 singular enrichment analysis: mirTarBase. (C) Top 10 singular enrichment analysis: KEGG pathway. (D) microRNA and Target gene prediction: miRDB databases.

Table 2. Differentially expressed miRNAs in the endometriosis cell exosome.

miRNA	P-value	Log2FC
miR-10b-5p	0.009	4.66
miR-1180-3p	0.017	4.51
miR-1307-5p	0.033	4.61
miR-147a	0.001	4.79
miR-191-5p	0.010	4.86
miR-200a-3p	0.006	4.55
miR-216a-5p	0.006	4.55
miR-4707-5p	0.005	4.53
miR-514a-3p	0.005	4.66
miR-515-5p	0.003	4.32
miR-563	0.004	4.57
miR-587	0.005	4.43
miR-1299	0.011	-5.32
miR-29a-3p	0.018	-4.99
miR-5196-5p	0.010	-5.04
miR-639	0.018	-4.99

miRNA: microRNA, FC: Fold change

6. As a result of miRNA validation, Hsa-miR-200a-3p was upregulated and Hsa-miR-29a-3p was downregulated.

For *in vivo* endometriosis study, we confirmed the presence of these miRNAs in serum-derived exosomes using qRT-PCR. Our results showed that Hsa-miR-216-5p, 147a, 191-5p and 200a-3p were upregulated, while Hsa-miR-29a-3p was downregulated. These findings were consistent with the heatmap analysis. However, we did not observe a significant difference in Hsa-miR-10b-5p (Figure 8). In many studies, it was reported that the upregulation of Hsa-miR-200a-3p and downregulation of Hsa-miR-29a-3p in different diseases, following the Bax-Bcl2 apoptosis mechanism[33,34], which was also observed in our results.

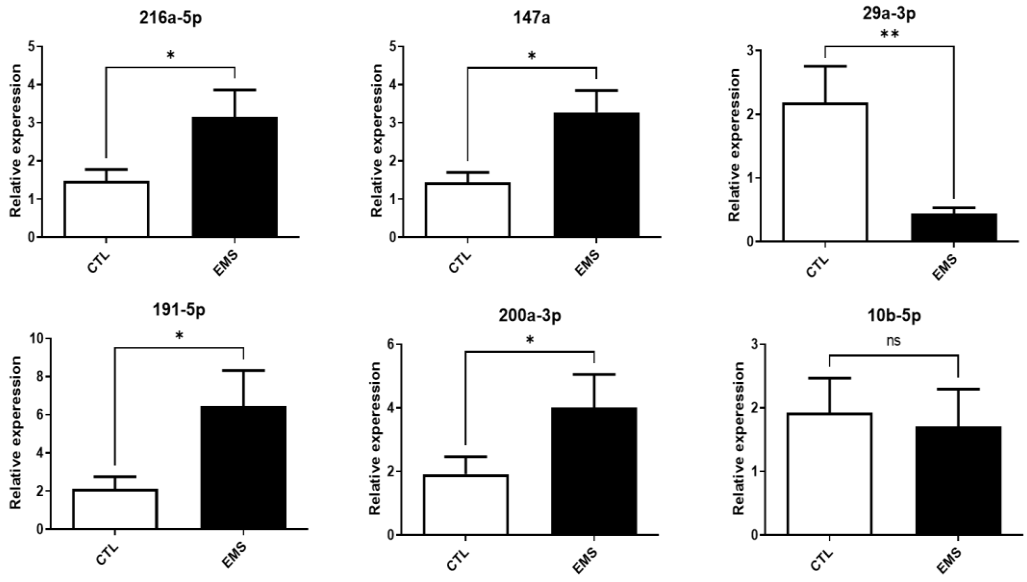

Figure 8. Expression level of serum derived exosome microRNA confirmed by qRT-PCR. Quantitative real-time-PCR analysis of hsa-miRNA-216a-5p, 147a, 29a-3p, 191a-5p, 200a-3p, 10b-5p. The expression level of Hsa-miRNA in EMS-Serum-Exo and CTL-Serum-Exo were determined. Each experiment was performed three times and all data were expressed as mean ± S.E.M. (n=18; *P<0.05, **P<0.01).

7. Diagnostic potentials of miR-216a-5p, miR-147a, miR-29a-3p, and miR191-5p for endometriosis

The receiver operating characteristic (ROC) curve analysis was conducted to predict the serological biomarkers. According to the ROC curve analysis, the area under the curve (AUC) for serum exosomal Hsa-miR-216a-5p, miR-147a, miR-29a-3p, and miR-191-5p were 0.7022 (95% CI: 0.5375-0.8669, P-value: 0.0331), 0.7377 (95% CI: 0.5766-0.8988, P-value: 0.0148), 0.7647 (95% CI: 0.5989-0.9305, P-value: 0.0084) and 0.7361 (95% CI: 0.6440-0.9407, P-value: 0.0036), respectively, indicating the potential to distinguish Endometriosis from CTL cases. However, AUC for Hsa-miR-200a-3p and 10b-5p were 0.6814 (P-value: 0.0558) and 0.5502 (P-value: 0.6175) (Figure 9, Table 3).

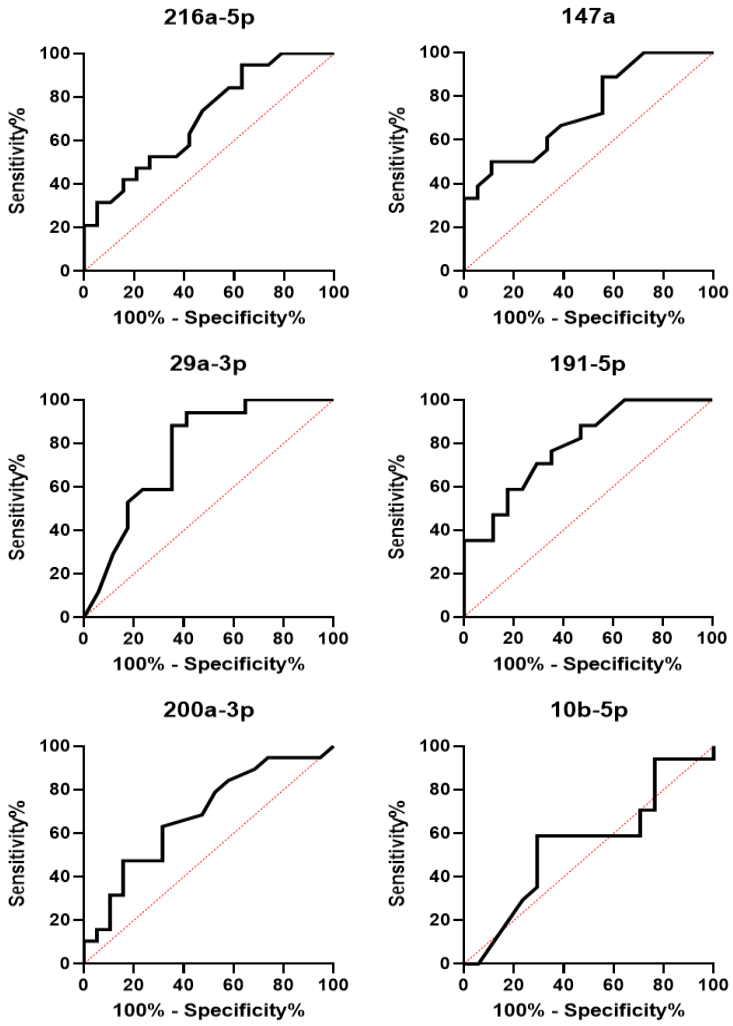

Figure 9. The receiver operating characteristic (ROC) curve analysis of serum exosomal miRNAs for distinguishing endometriosis. According to the ROC curve analysis, the area under the curve (AUC) for serum exosome were determined. (n=18)

Table 3. AUC and performance values for each biomarker

miRNA	AUC	Cutoff value	Sensitivity %	Specificity %	95% CI	P-value
216a-5p	0.7022	>2.6	47.37	78.95	0.5375-0.8669	0.0331
147a	0.7377	>2.95	50	88.89	0.5766-0.8988	0.0148
29a-3p	0.7647	<0.85	88.24	64.71	0.5989-0.9305	0.0084
191-5p	0.7924	>1.2	76.47	64.70	0.6440-0.9407	0.0036
200a-3p	0.6814	-	-	-	-	0.0558
10b-5p	0.5502	-	-	-	-	0.6175

IV. DISCUSSION

There are many hypotheses regarding the mechanism of endometriosis including the ectopic implantation of refluxed menstrual tissues.[4] It is crucial to effectively address endometriosis to minimize the associated infertility. Nevertheless, the challenge lies in the limited understanding of its pathogenesis.[12-14] In this study, we aim to provide sights into the growth and progression of the endometrium in endometriosis, as well as potential biomarkers for easier diagnosis.

Exosomes are extracellular vesicles that play critical roles in various diseases and cell-to-cell communication by autocrine or paracrine signaling.[16,25] Due to these characteristics, exosomes are studied for pathology and biomarkers. In this study, we hypothesized exosomes worsen endometriosis by autocrine signaling. Therefore, we treated an *in vitro* endometriosis model with EMS-Exo. As the result, we revealed, for the first time, that an exosomal mitochondrial anti-apoptotic regulatory network participated in endometriosis development. Prior studies have reported that ERK and AKT expression are increased and PI3K/AKT signaling was activated, inducing proliferation and inhibition of apoptosis in endometriosis .[35,36] Our results are in agreement with these findings, as EMS-Exo treatment induced an increase in pERK and pAKT expression. Changes in mitochondrial membrane potential are one of the main pathways regulating apoptosis through the Bcl family and caspase.[37] Bcl-2 and Bax play a key role in regulating mitochondrial membrane permeability and function.[38] Our study reports that internal exosomes can affect the

membrane potential of mitochondria by autocrine and the mechanism of anti-apoptosis through Bcl-2-Bax regulation.

This study presents novel findings regarding the involvement of exosomes, extracellular vesicles, in the pathological mechanisms of endometriosis. Exosomes have gained increasing attention due to their role in intercellular communication and disease processes. Notably, previous studies have highlighted the significance of miRNAs in the pathogenesis of endometriosis. Within the context of endometriosis, exosomes have emerged as crucial carriers of miRNAs, mediating their communication and functional transfer between cells. These miRNAs encapsulated within exosomes can be transferred to target cells, modulating gene expression and influencing cellular processes relevant to endometriosis development and progression. By shuttling miRNAs, exosomes have the potential to exert both autocrine and paracrine effects, affecting the microenvironment and cellular behavior associated with endometriosis.[39] In this study, we sought to elucidate the specific miRNAs carried by exosomes that may contribute to the pathogenesis of endometriosis. To achieve this, we performed miRNA array analysis on exosomes derived from EMS-EM and CTL-EM cells, representing endometriosis and control samples, respectively. The analysis revealed distinct miRNA profiles, with 12 miRNAs found to be upregulated and 4 miRNAs downregulated in EMS-Exo compared to CTL-Exo. Notably, among the top candidates, hsa-miR-29a-3p and hsa-miR-200a-3p exhibited significant enrichment according to singular enrichment analysis. The dysregulation of hsa-miR-29a-3p and hsa-miR-200a-3p in endometriosis-derived exosomes is intriguing, as these miRNAs have been implicated

in diverse cellular processes. Previous studies have shown that hsa-miR-29a-3p can induce apoptosis, while upregulation of hsa-miR-200a-3p has been associated with apoptosis inhibition through the Bax-Bcl2 pathway in various cellular contexts, including dermal fibroblasts, gastric cancer, and Alzheimer's disease.[33,40,41] In line with these findings, our results demonstrate the downregulation of hsa-miR-29a-3p and the upregulation of hsa-miR-200a-3p in EMS-Exo. Additionally, our KEGG analysis and miRDB database exploration revealed the association of these miRNAs with the PI3K/AKT pathway, further supporting their relevance in the context of endometriosis pathogenesis. We also validated these findings in serum-derived exosomes. These results suggest the possibility of inducing anti-apoptosis through the Bax-Bcl2 pathway in endometriosis cell-derived exosomes via autocrine effects. However, further studies are required to confirm the precise mechanism underlying the involvement of exosomal miRNAs in this process.

Furthermore, we evaluated exosomal microRNAs as potential biomarkers for diagnosing endometriosis. Although CA-125 is commonly utilized for diagnosis, its lack of specificity limits its effectiveness. Elevated levels of CA-125 can also be observed in other conditions such as ovarian cancer and uterine fibroids, and its levels can fluctuate due to factors like the menstrual cycle and pregnancy.[42] MiRNAs are short, single-stranded RNAs that regulate gene expression post-transcriptionally in a wide range of diseases. They exhibit high stability in biological fluids and possess the potential for tissue and disease specificity. Circulating miRNAs demonstrate important diagnostic potential.[43] Previous studies have reported that miR-22-3p, miR-320a as being related to endometriosis in serum exosome.[44]

However, our candidate group of miRNAs represents a novel set of exosomal miRNA biomarker candidates that have not been previously reported. To assess their diagnostic performance, we employed ROC curve analysis, with the area under the curve (AUC) commonly used to evaluate the discriminatory ability. According to the AUC criteria, 0.5 indicates no diagnostic ability, values ranging from 0.5 to 0.7 indicates poor discrimination ability, values from 0.7 to 0.8 indicates acceptable discrimination ability, and values from 0.9 to 1.0 indicates outstanding discrimination ability. Our results showed that Hsa-miRNAs 216a, 147a, 29a-3p, and 191a-5p exhibited AUC values of 0.7 or higher, indicating acceptable diagnostic performance. Notably, miRNA 29a-3p has high potential for utilization as it is also associated with anti-apoptotic mechanisms. However, further research involving various combinations is necessary for better accuracy and sensitivity. Additionally, using other clinically available samples, such as menstrual blood, is needed to discover more accuracy endometriosis study.

While these findings suggest a potential role for exosomal miRNAs in mediating anti-apoptotic effects in endometriosis, it is important to acknowledge the need for further investigations to fully elucidate the underlying mechanisms and confirm the specific contributions of exosomal miRNAs in endometriosis progression. Nonetheless, this study provides valuable insights into the association between exosomes and miRNAs, shedding light on their potential involvement in the complex molecular landscape of endometriosis.

V. CONCLUSION

In the present study, we investigated the effects of EMS-Exo on cellular proliferation and apoptosis in an in vitro endometriosis model. Our results demonstrated that EMS-Exo exerted proliferative effects on the cells by increasing the protein levels of AKT and ERK. Additionally, EMS-Exo promoted anti-apoptotic processes by enhancing mitochondrial membrane potential through the activation of the PI3K/AKT cell signaling pathway. Furthermore, we conducted an analysis of miRNAs associated with these effects and found that 12 miRNAs were upregulated while 4 miRNAs were downregulated. To explore the potential of utilizing these miRNAs for *in vivo* diagnosis, we validated the expression of 6 selected miRNAs in serum exosomes. Remarkably, 5 of these miRNAs exhibited consistency with the miRNA analysis of cell-derived exosomes, further strengthening their potential diagnostic relevance. Specifically, hsa-miR-29a-3p and hsa-miR-200a-3p were identified as miRNAs associated with the anti-apoptosis pathway. Moreover, we conducted ROC curve analysis to identify potential diagnostic biomarkers for endometriosis and identified 4 miRNAs that displayed good diagnostic performance.

In conclusion, our observations contribute to the understanding of the main mechanisms underlying exosome-related pathology in endometriosis. Moreover, we have identified novel miRNA candidates that show promise as diagnostic biomarkers for this condition. However, for these findings to be effectively translated into clinical applications, further studies and validations are required. The commercialization potential of these diagnostic

biomarkers should also be explored in future research endeavors.

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ABSTRACT (IN KOREAN)

자궁내막증 세포 유래 엑소좀의 세포증식 및 사멸 기전 효과와 연관 바이오 마커 발굴

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김세희

자궁내막증은 자궁내막과 유사한 조직이 난소, 나팔관, 복막강 등 다른 위치에서 자라기 시작하는 에스트로겐 의존성 부인과 질병이다. 이 질병은 가임기 여성에서 흔하게 발생하고 불임과 같은 합병증을 유발하지만 발병기전이 명확히 밝혀지지 않았다. 또한 재발위험이 높아 신속한 진단을 위한 비 침습적인 진단 마커에 대한 연구가 필요한 상황이다. 엑소좀은 직경이 30-100nm인 작은 세포 외 소포체이며 대부분 세포유형에서 분비되어 세포의 내부 상태를 반영하고 다양한 병리학 과정에서 질병진행에 중요한 역할을 한다고 알려져 있다. 또한 microRNA를 캡슐화 하여 진단 마커로의 활용도가 높다. 자궁내막증의 병태생리학 규명에 관한 연구에서 엑소좀의 역할에 대해 혈관 신생 및 면역에 관련한 기전에 대해선 일부 보고되어 있지만 세포 증식 및 사멸 관련 기전에서는 명확히 보고된 바가 없다. 본 연구에서는 엑소좀의 자가분비 효과에 의해 질병악화를 시킨다는 가설을 세우고 연관된 바이오 마커를 발굴하여

진단표지자로의 가능성을 확인하였다. 구체적으로 자궁내막증 환자와 대조군으로부터 자궁내막 조직을 수집하고 일차세포로 배양하였다. 세포에서 각각의 엑소좀을 추출한 뒤 자궁내막증 모델에 24시간 처리하여 분자생물학적인 기법을 통해 결과를 관찰하였다. CCK8과 FACS 분석 결과에서 자궁내막증 유래 엑소좀이 세포증식을 유도하고 세포사멸을 감소시키는 것으로 나타났다. 특히, JC1 red/green 비율이 유의미하게 증가하였고 미토콘드리아 연관 세포사멸이 경로가 조절되는 것으로 나타났다. 단백질 발현을 조사한 결과, ERK, AKT 신호전달을 통해 증식이 유도되었고, PI3K/AKT 기전을 통해 미토콘드리아 세포사멸 신호경로가 조절된 것이 확인되었다. 관련 miRNA를 파악하기 위해 miRNA array 분석을 수행하였다. 그 결과 16개의 miRNA 가 차이를 보였고, hsa-miR-200a-3p 가 상향 조절되고 29a-3p 가 하향 조절되었다. 특히, 이 두 miRNA는 PI3K/AKT 기전과 연관성이 있었다. 또한, 생체내 진단 활용을 위해 혈청 엑소좀의 qRT-PCR를 수행하였고, 5개의 miRNA 가 유의차가 있었다. 마지막으로 진단 바이오 마커로의 가능성 파악을 위해 ROC 커브 분석이 시행되었고, 4가지 miRNA 가 진단 성능을 보였다. 하지만 상용화를 위해선 민감도와 정확도를 높인 조합을 찾을 필요성이 있다. 결론적으로, 본 연구는 분자생물학적 기법을 통해 엑소좀 자가분비 관련 주요 기전을 밝히고 이와 연관된 자궁내막증 진단 바이오 마커의 새로운 후보군을 제시하였다.

핵심 되는 말: 자궁내막증, 엑소좀, 자가분비 효과, 세포증식, 항세포사멸, 미토콘드리아, 바이오 마커