In vitro & *in vivo* evaluation of anti-fibrotic effects of Rg3-enhanced extract and identification of the associated miRNAs in endometriosis

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Directed by Professor SiHyun Cho

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This certifies that the Doctoral Dissertation
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I dedicate this thesis to my family members. I am greatly thankful for my parents (In Ho Kim and Eun Joo Kim) for their constant love and support through prayers and advices. I also want to thank my sister (Min Ji Kim) for her great help with image editing. Last but not least, I praise God that this long journey is finally over!

M.K.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Objective: The potential therapeutic effects of ginsenoside Rg3 on endometriosis and its target miRNAs were identified.

Methods: An *in vitro* study using human endometrial stromal cells (HESCs) obtained from patients with endometriosis and an *in vivo* study using mouse endometriosis models were designed. HESCs were treated with Rg3-enhanced extract (Rg3E); real-time PCR, microarray profiling, transfection, and western blot were performed. Mouse endometriosis models were developed and supplemented with Rg3E for 8 weeks.

Results: RNA levels of Ki-67, Col-1, CTGF, fibronectin, TGF-β1, MMP2 and 9 were significantly decreased in Rg3E-treated HESCs. Microarray analysis revealed downregulation of miR-27b-3p, which is related to fibrosis modulation. Expression of miR-27b-3p was significantly higher in HESCs from patients with endometriosis than that of controls, and Rg3E treatment significantly decreased its expression. The transfection of miR-27b inhibitor revealed significant decreases in Col-1 and MMP9. The protein expression results from western blot were also comparable. The contraction and migration assay revealed significant reductions in both fibrosis and migration potential in Rg3E-treated HESCs from endometriosis patients. In Rg3E-treated mice, decreases in size and fibrotic character of endometrial lesions were observed.

Conclusion: Rg3E effectively alters fibrotic properties of HESCs from patients with endometriosis, which is presumed to be associated with miR-27b-3p modulation.

Key words : endometriosis, microRNA, red ginseng, ginsenoside, Rg3

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I. INTRODUCTION

Endometriosis is a common benign gynecological disorder, affecting up to 10% of all women of reproductive age and 20–50% of women with chronic pelvic pain and/or infertility.[1] It is defined as the proliferation of endometrial tissue outside of the uterine cavity.[2] The exact pathogenic mechanisms of endometriosis are not known and diagnosis is often delayed—making it more difficult to treat, both medically and surgically. However, several studies demonstrate that endometriosis has an invasive and fibrotic nature that is induced by inflammation,[3,4] which may represent a key pathophysiological target for treatment.

MicroRNAs (miRNAs) are a family of endogenous, small, noncoding, functional RNAs that control gene expression by translational repression or degradation of messenger RNA transcripts after targeting the 3'-untranslated region (3'-UTR).[5] Several studies have demonstrated that miRNAs are important regulators of development and cellular homeostasis through their control of diverse biological processes. Aberrant miRNA expression is known to be associated with various human diseases such as cancer, cardiovascular and inflammatory disorders, as well as gynecological diseases.[6-10] Previously, we showed that several circulating miRNAs such as let-7b, 7d, and 7f are

differentially expressed in the sera of endometriosis patients.[5] Several other studies also identified differential expression of miRNAs in eutopic and ectopic endometrial samples from endometriosis patients using microarray profiling.[11-13] It has been suggested that miRNAs act as potent regulators of gene expression in the pathogenesis of endometriosis and its associated reproductive disorders.[14]

Korean red ginseng (KRG) has been traditionally used as an herbal medicine to treat various diseases in Eastern Asia. Recent studies have shown that KRG has various biological activities such as immune enhancement, antioxidant, anti-inflammatory, neuroprotective, anti-metabolic syndrome, and anti-menopausal disorder effects.[15-19] Ginsenosides are biologically active components of KRG, and there are many different types of ginsenosides with different pharmacological activities.[20] Ginsenoside Rg3 is one of the major active components of KRG and has been shown to have various pharmacological benefits such as immunomodulatory, antioxidant, anti-inflammatory, anticancer, and anti-aging activities in several diseases and infections.[21,22] Previous studies demonstrated that Rg3-enhanced red ginseng extract (Rg3E) has anti-inflammatory effects in asthmatic lung tissue, brain, hepatic and renal injury.[23-25] Considering the anti-inflammatory activity of Rg3E, we hypothesized that Rg3E effectively inhibits the key pathophysiology of endometriosis.

The objective of this study was to investigate whether Rg3E affects any aspect of endometriosis pathogenesis *in vitro* and *in vivo*. Additionally, we analyzed the role of miRNAs in a pathogenic condition previously described[26] and evaluated the therapeutic effects of Rg3E by targeting specific miRNAs.

II. MATERIALS AND METHODS

1. Study population and sample collection

Thirty-six Korean female patients aged from 21-49 (premenopausal women) were enrolled in this study, and written informed consent was obtained before surgery in accordance with the study protocol, which was approved by the

Institutional Review Board of Gangnam Severance Hospital. Patients included in this study underwent laparoscopy for endometriosis or other benign ovarian cysts. Twenty-one patients were histologically confirmed to have endometriosis. Fifteen patients with histologically confirmed other benign ovarian cysts were included as controls. Postmenopausal women, previous hormone or gonadotropin-releasing hormone agonist users, and patients who had adenomyosis, endometrial cancer, endometrial hyperplasia or endometrial polyps, infectious diseases, chronic or acute inflammatory diseases, malignancy, autoimmune diseases, or cardiovascular diseases were excluded.

2. Culture of primary endometrial stromal cells and Ishikawa cell line

Eutopic endometrial tissue was obtained from 21 patients with endometriosis and 15 patients with benign ovarian cysts by endometrial biopsy before surgical procedures. Ectopic endometrial tissue was obtained from 21 patients with endometriomas who underwent cystectomy. The tissue samples were minced into smaller pieces and incubated in Hank's balanced salt solution including HEPES (25 mmol/mL), 1% penicillin/streptomycin, collagenase (1 mg/mL, 15 U/mg), and deoxyribonuclease (0.1 mg/mL, 1,500 U/mg) for 60 min at 37°C with agitation and pipetting. The cells were pelleted, washed, suspended in Ham F12:DMEM (1:1) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and then passed through a 40-μm cell strainer (Falcon™) and plated into 75 cm^2 Falcon tissue culture flasks (BD Biosciences, San Jose, CA, USA). Cultured human endometrial stromal cells (HESCs) at 3–5 passages were used for further analysis. Ishikawa cells were maintained in MEM (Invitrogen, Carlsbad, CA, USA) containing 2.0 mmol/L l-glutamine and Earl salts and supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin.[27]

3. Cell viability assay

Cytotoxicity was measured using the TACS® MTT cell proliferation assay kit (Trevigen, Gaithersburg, MD, USA). Cells were seeded onto 96-well plates and treated with indicated concentrations of Rg3E for 48 h. Post treatment, MTT (10 μL per well) was added, and the plates were incubated at 37°C. Dimethyl sulfoxide (DMSO, 100 μL) was added, and the dark blue formazan product was quantified using a microplate reader at 570 nm (with a 690 nm reference filter) (Molecular Device, Sunnyvale, CA, USA). Relative cell viability (%) is expressed as a percentage relative to non-treated control cells.[28]

4. Rg3E treatment

Rg3E powder was provided by Korea Ginseng Corporation. 1 g of Rg3E powder was diluted in PBS at the concentration of 1 g/mL by vortexing, keeping it in the water bath (37°C) for 30 min to 1 h, and then on a shaker for 20-30 min. The first filtration was done with 0.8 μm filters, and 0.2 μm filters were used for the second filtration of Rg3E.

HESCs from patients with endometriosis and Ishikawa cells were harvested from culture flasks using trypsin/EDTA (0.05%) and plated in 6-well plates (200 mL media) at 37°C with 95% air and 5% CO_2 in a humidified environment. HESCs were grown in Ham F12/DMEM (1:1) containing 10% FBS and 1% penicillin/streptomycin. Ishikawa cells were grown in MEM (Invitrogen) containing 2.0 mmol/L l-glutamine and Earl salts supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin. At 80% confluency, Rg3E was added at concentrations of 0 μL/mL and 400 μL/mL per the MTT assay results at 48 h.

5. MicroRNA microarray analysis

For quality control, RNA purity and integrity were evaluated using an OD 260 nm/280 nm ratio and analyzed with an Agilent 2100 Bioanalyzer (Agilent

Technologies, Palo Alto, USA). The Affymetrix GeneChip® miRNA 4.0 array process was conducted per the manufacturer's protocol. 1 μg RNA samples were labeled with the FlashTag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA). The labeled RNA was quantified, fractionated and hybridized to the miRNA microarray according to the standard procedures provided by the manufacturer. The labeled RNA was heated to 99°C for 5 min and then to 45°C for 5 min. RNA-array hybridization was performed with agitation at 60 rotations per min for 16–18 h at 48°C on an Affymetrix® 450 Fluidics Station. The chips were washed and stained using a GeneChip™ Fluidics Station 450 (Affymetrix, Santa Clara, California, United States). The chips were then scanned with an Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, California, United States). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip® Command Console® Software (AGCC). The CEL files import, miRNA level RMA+DABG-All analysis and result export using Affymetrix® Expression Console™ Software. Array data were filtered by probes annotated species. A comparative analysis between test and control samples was carried out using fold-change.[29-32]

6. RNA extraction and quantitative real-time polymerase chain reaction

To assess RNA levels, total RNA was extracted from cultured cells with Qiagen RNeasy isolation kit (Qiagen, Hilden, Germany). All samples were treated with RNase-free DNase (Ambion) to remove the possibility of genomic DNA contamination. RNA samples were analyzed with the use of a Nanodrop ND 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Using 1 μg of total RNA, cDNA was synthesized with oligo-dT in a Superscript III kit (Invitrogen) with the use of C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The resultant cDNA mixtures were stored at -20°C. Then, using a template of 2 μl

of synthesized cDNA, Quantitative real-time polymerase chain reaction (PCR) amplification was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with the use of the Power SYBR Green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Woolston Warrington, UK). Reaction mixture included cDNA template, forward and reverse primers, ribonuclease free water, and the SYBR Green PCR master mix, for a final reaction volume of 20 μL. The thermal cycling conditions were performed by procedures at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Threshold cycle (Ct) and melting curves were acquired with the use of 7300 software program of the Applied Biosystems. Each reaction was performed in triplicate. The mRNA levels of each sample were normalized to GAPDH expression.[28]

To assess miRNA expression levels, RNA was extracted from cultured cells using the miRVana RNA Isolation Kit (Ambion by Life Technologies, Carlsbad, CA, USA) per the manufacturer's specifications and eluted with 30 μL nuclease-free water. Isolated RNA (10 ng) and Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Baltics, UAB, Lithuania) were used. Quantitative real-time PCR for miRNAs was performed using a Taqman® Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA, USA) with sets for miR-27b-3p and U6 snRNA (Applied Biosystems, Foster City, CA, USA). All real-time PCR reactions were conducted in triplicate with a 7300 real time PCR system, and 40 cycles of amplification were performed. Relative expressions were calculated using the comparative threshold cycle method, and miRNA levels were normalized to U6 levels.[27] Relative miRNA levels were determined using the formula $2^{-\Delta Ct}$. Primers used in this experiment are described in Table 1.

Table 1. Primer sequences

	Forward	Reverse
miR27b-3p	TTCACAGTGGCTAAGTTCTGC	
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'
Ki-67	5'-GAAAGAGTGGCAACCTGCCTTC-3'	5'-GCACCAAGTTTTACTACATCTGCC-3'
Caspase 3	5'-GGAAGCGAATCAATGGACTCTGG-3'	5'-GCATCGACATCTGTACCAGACC-3'
CTGF	5'-CATTAAGAAGGGCAAAAAGTGC-3'	5'-CACACCCCACAGAACTTAGCC-3'
Col-1	5'-GAGAGCATGACCGATGGATT-3'	5'-CCTTCTTGAGGTTGCCAGTC-3'
Fibronectin	5'-CCATCGCAAACCGCTGCCAT-3'	5'-AACACTTCTCAGCTATGGGCTT-3'
TGF-β1	5'-TGGAAACCCACAACGAAATC-3'	5'-GGGTTCAGGTACCGCTTCTC-3'
MMP2	5'-ACCGCGACAAGAAGTATGGC-3'	5'-CCACTTGCGGTCATCATCGT-3'
MMP9	5'-CGATGACGAGTTGTGGTCCC-3'	5'-TCGTAGTTGGCCGTGGTACT-3'

7. Transfection of miRNA

Cells were cultured to 70%–80% confluence after being seeded onto 6-well plates and were transfected with hsa-mir-27b-3p inhibitor, a chemically synthesized double-stranded RNA that inhibits mature endogenous miRNA, or hsa-mir-negative as a control (Ambion by Life Technologies) with the use of Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions, at a final concentration of 50 nmol/L. The transfected cells were harvested 48 h after transfection.[27]

8. Western blot

The protein extracts were prepared using RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing freshly added protease and phosphatase inhibitor cocktail (Thermo Scientific). The concentrations of total cell lysates were measured using a BCA protein assay kit (Thermo Scientific). A total of 20 μg total protein was mixed with 5 x sample buffer and heated at 95°C for 5 min. The samples were loaded onto 8 - 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrotransferred to a polyvinylidene fluoride membrane

(Millipore Corporation, Billerica, MA, USA) using a Transblot apparatus (Bio-Rad, Hercules, CA, USA). The membranes (Millipore Corporation, USA) were blocked using 5% non-fat skim milk in Tris-buffered saline solution (10 mmol/L Tris-HCl (pH 7.4) and 0.5 mol/L NaCl) and adding Tween-20 (0.1% vol/vol).

The blots were probed using primary antibodies: Col-1 (1:1000, Santa Cruz biotechnology, Dallas, TX, USA), CTGF (1:1000, Santa Cruz biotechnology), Fibronectin (1:1000, Santa Cruz biotechnology), TGF-β1 (1:1000, Santa Cruz biotechnology), MMP2 (1:500, Santa Cruz biotechonology), MMP9 (1:500, Santa Cruz biotechnology) and β-actin (1:1000, Abcam, Cambridge, UK), followed by horseradish peroxidase conjugated secondary anti-mouse or anti-rabbit antibody (1:2000; Thermo Scientific). The proteins were detected using enhanced chemiluminescence (Santa Cruz Biotechnology). The experiment was repeated three times for analysis.[28]

9. Migration assay

The migration assay for transfected cultured cells was carried out using 8-mm pore size polycarbonate membranes (Millipore, Billerica, MA, USA) and 24-well plates. Freshly trypsinized and washed cells were suspended in serum-free medium, and cells (200 mL, 5×10^4 cells/well) were placed in the top chamber of each insert; medium (600 mL) containing 10% FBS was added into the lower chambers. After incubating for 24 h at 37°C in a 5% CO_2 humidified incubator, cells were fixed and stained with hematoxylin. Cells in the inner chamber were removed with the use of a cotton swab, and cells attached to the bottom side of the membrane were counted and imaged under an inverted microscope (Olympus Corp., Shinjuku, Tokyo, Japan) at × 200 magnification over ten random fields in each well.[27]

10. Collagen gel contraction assay

A sterile solution of bovine Type I Collagen (Cell Biolabs, Inc., San Diego, USA) was prepared per the manufacturer's instructions. HESCs were embedded in collagen gel and cultured three-dimensionally. Briefly, HESCs were suspended in the collagen solution (3.0×10^6 cells/mL). The collagen/cell mixture (2 mL/plate) was dispensed into 35-mm culture plates (Corning, New York, NY, USA); the mixture was polymerized at 37°C for 1 h. Immediately after polymerization, 1.0 mL of culture medium was added to each plate. After incubating for 72 h, the collagen gels were photographed and the gel surface area was measured.[33,34]

11. Mouse model for endometriosis

The mouse models for endometriosis were prepared using 6-week-old C57bl6 female mice as previously described.[35] This study was approved by the institutional committee on animal care and was conducted in accordance with its accepted standards. Rg3E was diluted in PBS as mentioned previously. Rg3E-treated mice were divided into two groups: a high-dose and a low-dose group. There were 10 mice in each group. The high-dose group was given 0.2 mg/g Rg3E and the low-dose group was given 0.1 mg/g Rg3E by daily oral gavage; the control group was given an equivalent amount of distilled water, also by oral gavage. The total feeding period was 8 weeks.

After 8 weeks of Rg3E treatment, the mice were sacrificed. Endometriotic implants were measured and collected, fixed in 10% formalin-acetic acid, and embedded in paraffin for histopathological examination. Paraffin-embedded tissue sections were stained with Masson's trichrome stain. Masson's trichrome staining detects collagen fibrils that are deposited in the matrix.[36] To quantify severity of fibrosis in stained tissue sections, staining scores were calculated by multiplying the percentage of positive cells and staining intensity as previously described.[36,37]

12. Statistical analysis

Data are presented as means ± SEM. Data from the *in vitro* experiments were assessed by Kolmogorov-Smirnov test or Shapiro-Wilk test to evaluate whether they were normally distributed. Continuous variables were compared using student's t-test or Mann-Whitney U test, when appropriate. SPSS v.23.0 and R statistical language v.2.15.0 were used for statistical analyses, and $P < 0.05$ was considered statistically significant.

III. RESULTS

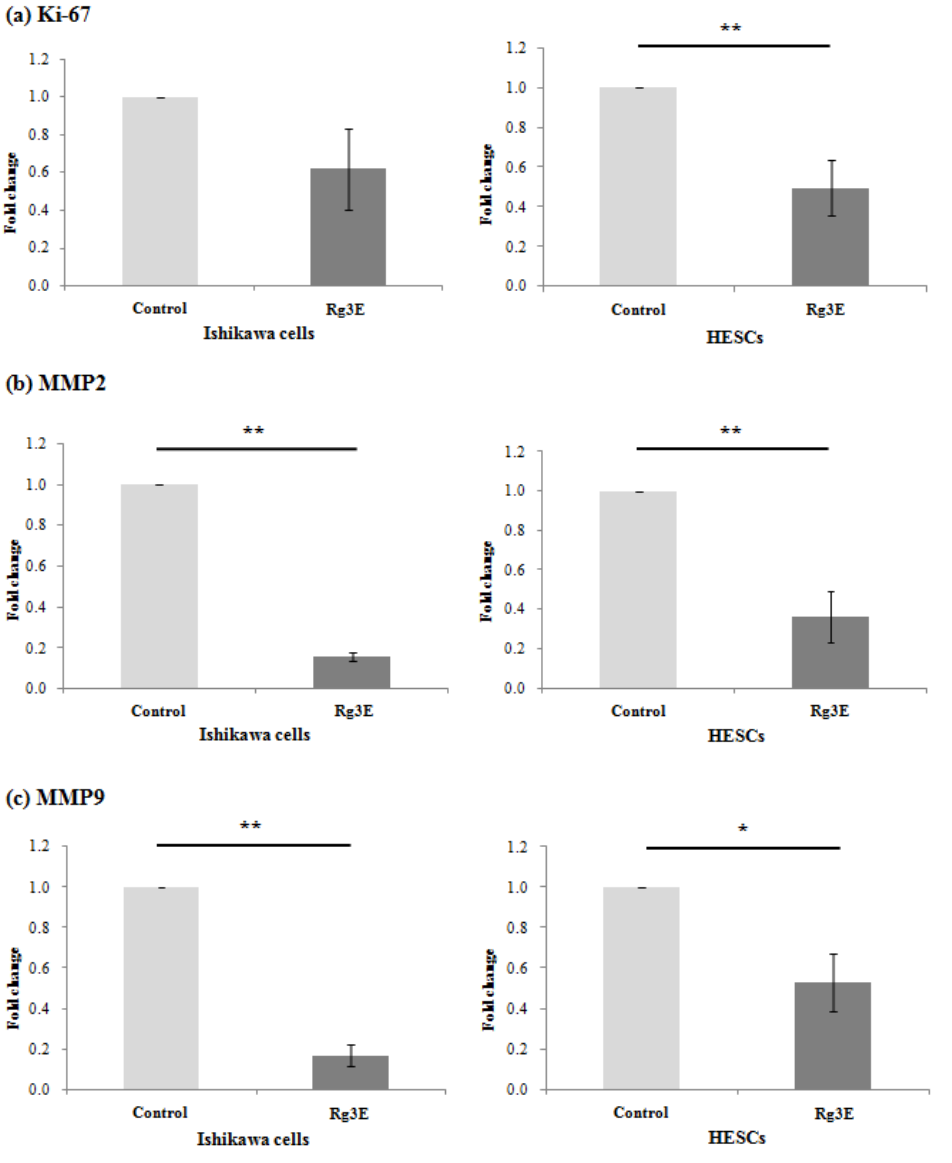
1. Patient Characteristics

There were no significant differences in age and body mass index (BMI) between the endometriosis group and controls. However, the endometriosis group had significantly lower gravidity (0.64 ± 0.34 vs. 2.18 ± 0.50, $P = 0.019$) and parity (0.45 ± 0.25 vs. 1.36 ± 0.28, $P = 0.024$) than those of the controls, and serum CA-125 levels were significantly higher than those of the controls (69.18 ± 17.56 vs. 15.14 ± 2.9, $P = 0.015$).

2. Effects of Rg3E on cell proliferation, invasion, apoptosis, and fibrosis markers

The MTT assay, used to test the toxicity of Rg3E at concentrations of 0, 400, 800, 1200, and 1600 μg/mL, showed significant decreases in cell viability at 800 μg/mL. Therefore, 400 μg/mL Rg3E was used for all subsequent experiments (data not shown). Ki-67, a cell proliferation marker, showed significantly decreased level in Rg3E-treated HESCs; however, no significant changes were observed in Ishikawa cells. Levels of MMP2 and MMP9, markers of invasion, significantly decreased in Rg3E-treated HESCs and Ishikawa cells. In contrast, caspase 3, a marker of apoptosis, was not significantly changed in either the Ishikawa cells or HESCs after Rg3E treatment. Markers of fibrosis, including Col-1, CTGF, fibronectin, and TGF-β1 showed significantly decreased levels after

treatment with Rg3E in both HESCs and Ishikawa cells (Figure 1).



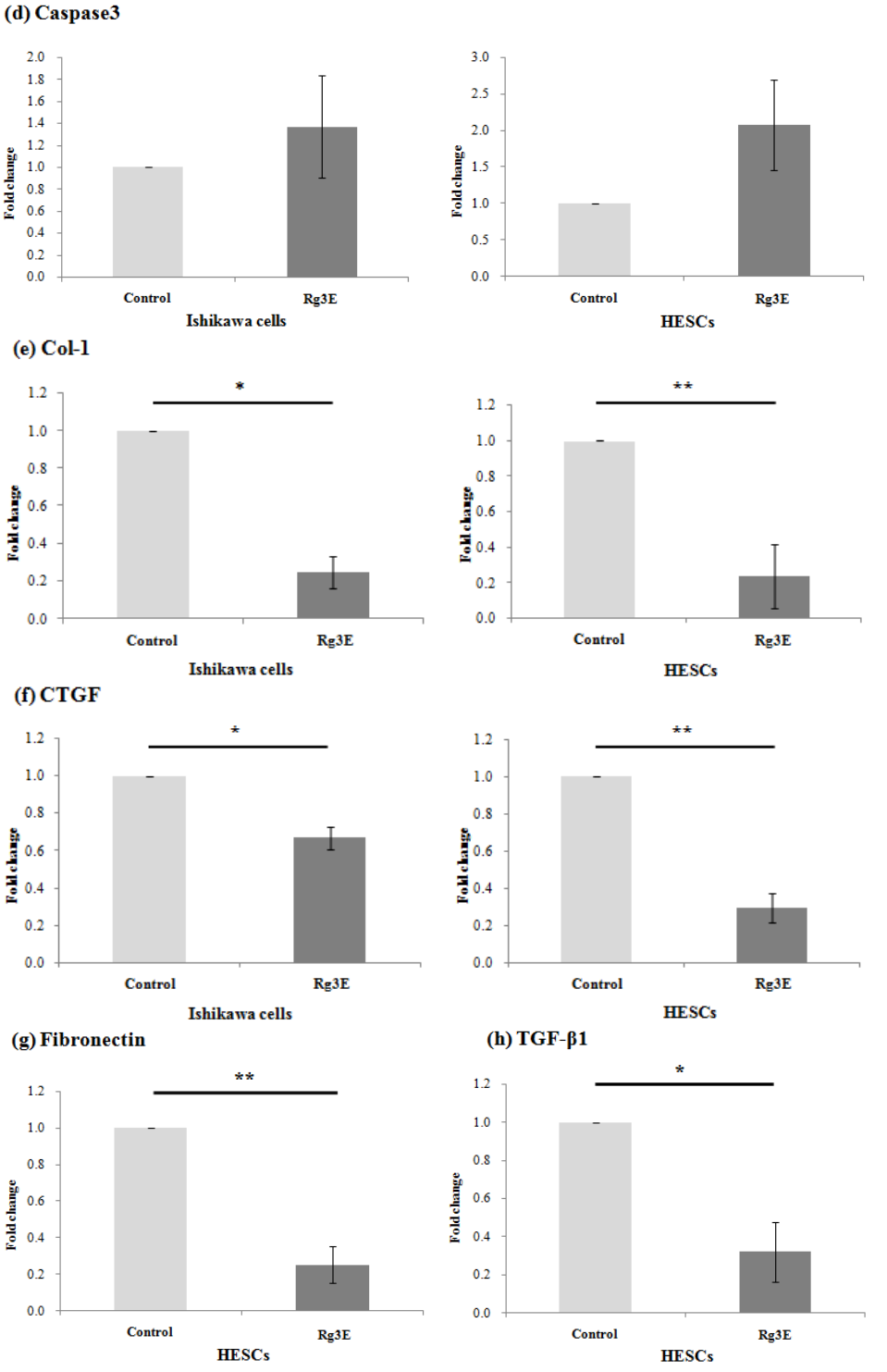


Figure 1. Expressions of Ki-67, MMP2, MMP9, caspase 3 and fibrosis markers after Rg3E treatment in Ishikawa cells and HESCs in patients with endometriosis.

(a) Ki-67, (b) matrix metalloproteinase (MMP)-2, (c) MMP9, (d) caspase 3, (e) type 1 collagen (Col-1), (f) connective tissue growth factor (CTGF), (g) fibronectin, (h) transforming growth factor (TGF)-β1.

(*, $P < 0.05$. **, $P < 0.01$. N = 8).

3. MicroRNA profiling after Rg3E treatment and validation of miR-27b-3p in endometriosis

To evaluate the effects of Rg3E on miRNAs associated with endometriosis, miRNA profiling was performed after Rg3E treatment of HESCs from patients with endometriosis. MicroRNA microarray analysis of samples from patients with endometriosis revealed several upregulated miRNAs. After Rg3E treatment, 20 miRNAs were significantly upregulated more than two-fold and six miRNAs were significantly downregulated more than two-fold; miR-27b-3p was one of the most downregulated miRNAs after Rg3E treatment in HESCs from patients with endometriosis. The miR-27b-3p is specifically related to fibrosis and is an important characteristic of endometriosis. To validate the miRNA microarray results, expression levels of miR-27b-3p were compared between eutopic endometria from patients with endometriosis and those without the disease. In the eutopic endometrium of patients with endometriosis, miR-27b-3p expression was approximately two-fold higher than that observed in patients without the disease (fold change 0.28 vs. 0.15, $P = 0.004$) (Figure 2(a)). In contrast, miR-27b-3p levels were lower in ovarian endometrioma cells than those observed in benign ovarian cyst cells (Figure 2(b)). After Rg3E treatment, miR-27b-3p expression significantly decreased in HESCs (0.51 vs. 1.00, $P = 0.029$) (Figure 3(b), Table 2); however, similar changes were not observed in Ishikawa cells.

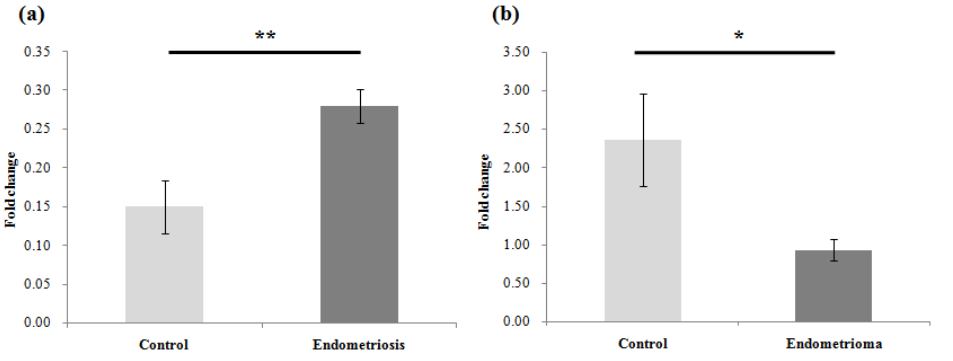


Figure 2. Expressions of miR-27b-3p in patients with and without endometriosis.

(a) mir-27b-3p expressions in eutopic endometrium of the patients with and without endometriosis.

(b) mir-27b-3p expressions in endometriomas and non-endometriotic ovarian cysts.

(*, $P < 0.05$. **, $P < 0.01$. N = 10).

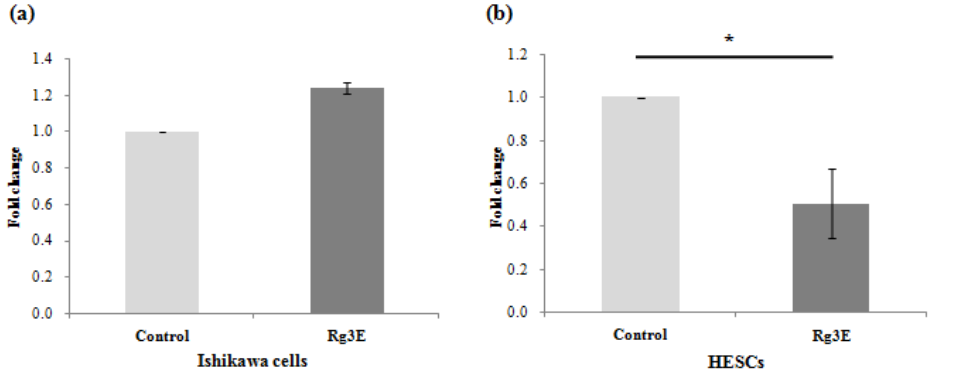


Figure 3. Expressions of miR-27b-3p in (a) Ishikawa cells and (b) HESCs from the patients with endometriosis after Rg3E treatment.

(*, $P < 0.05$. N = 6).

Table 2. Microarray analysis and significant fold changes of miRNAs before and after 48h-Rg3E treatment in HESCs of patients with endometriosis

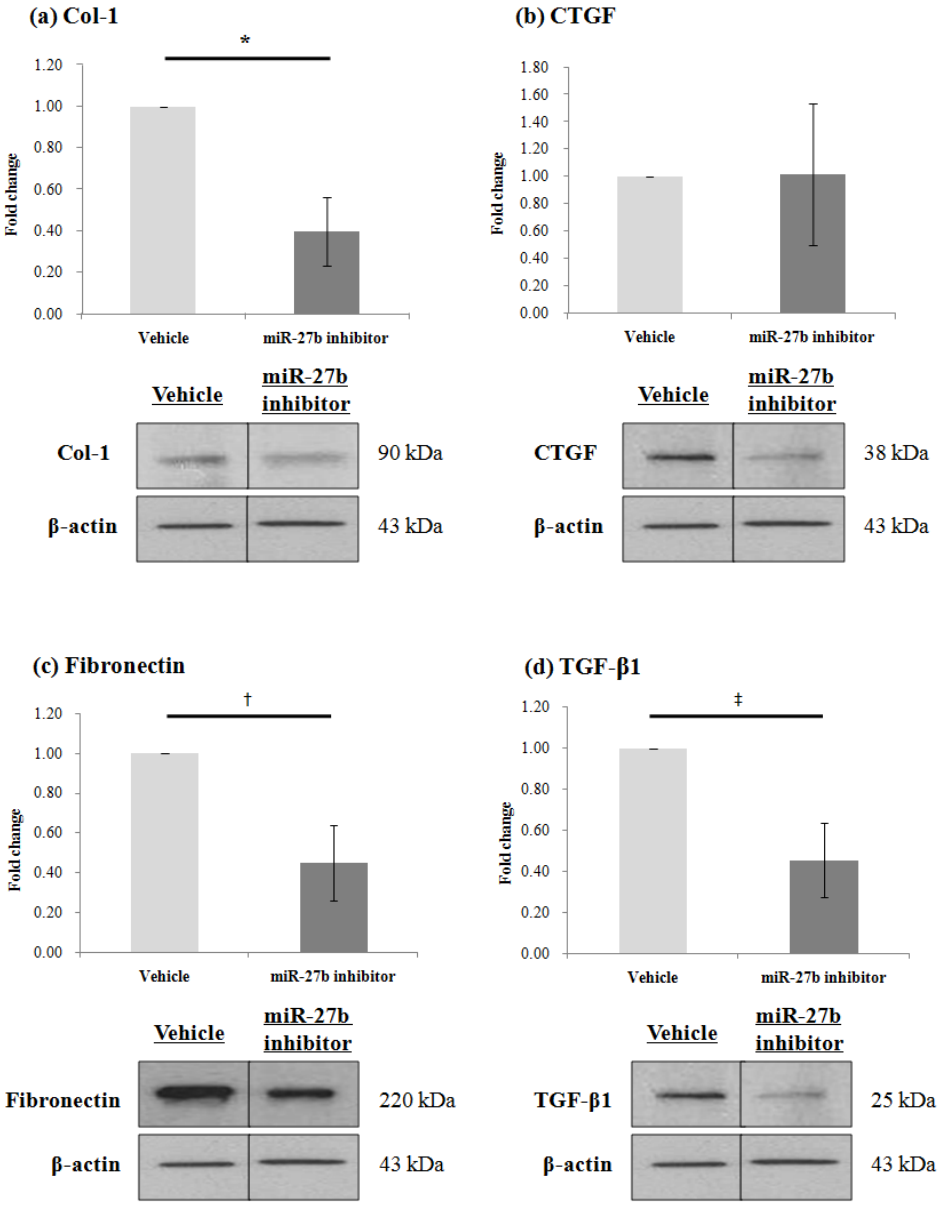
Up-regulated			**Down-regulated**		
miRNA	**FC***	***P*-value**	**miRNA**	**FC***	***P*-value**
hsa-miR-3188	3.121276	0.030	hsa-miR-22-5p	-7.511362	0.011
hsa-miR-4674	3.102003	0.049	hsa-miR-154-5p	-3.965245	0.009
hsa-miR-6805-5p	2.813756	0.026	hsa-miR-654-3p	-2.518972	0.041
hsa-miR-4516	2.734496	0.030	**hsa-miR-27b-3p**	**-2.454641**	**0.029**
hsa-miR-6765-5p	2.675679	0.041	hsa-miR-28-3p	-2.216515	0.049
hsa-miR-8089	2.599531	0.046	hsa-miR-140-5p	-2.207144	0.033
hsa-miR-6821-5p	2.59466	0.044			
hsa-miR-3621	2.545324	0.034			
hsa-miR-4649-5p	2.539717	0.0001			
hsa-miR-1908-5p	2.488741	0.048			
hsa-miR-4486	2.44996	0.033			
hsa-miR-8069	2.369964	0.034			
hsa-miR-663a	2.33128	0.035			
hsa-miR-6781-5p	2.241129	0.026			
hsa-miR-4690-5p	2.17942	0.035			
hsa-miR-6729-5p	2.146922	0.029			
hsa-miR-149-3p	2.140209	0.027			
hsa-miR-6125	2.111751	0.031			
hsa-miR-4745-5p	2.092432	0.047			
hsa-miR-937-5p	2.086316	0.038			

There were six significantly decreased miRNAs and miR-27b-3p (previously reported to be associated with modulating fibrosis) was included. Functions of

other decreased miRNAs were yet to be discovered or unrelated to the major pathogenic characteristics of endometriosis. Additionally, there were twenty miRNAs that were significantly increased after Rg3E treatment. However, these miRNAs' roles were not yet clarified. FC > 2 and $P < 0.05$ were considered significant. *FC: fold change.

4. miR-27b-3p inhibitor transfection and western blot

To evaluate transfection efficacy, miR-27b-3p expression was measured 48 h after transfection with an hsa-miR-negative control and hsa-miR-27b-3p inhibitor in HESCs. Expression of miR-27b-3p was 100- to 200-fold lower after treatment with the hsa-miR-27b-3p inhibitor than that observed after treatment with the hsa-miR-negative control (data not shown). Transfection of the miR-27b-3p inhibitor downregulated markers of fibrosis in HESCs (Figure 4). Col-1 RNA and protein expression significantly decreased after treatment with the miR-27b-3p inhibitor (0.40 vs. 1.00, $P = 0.035$), whereas that of CTGF (1.02 vs. 1.00, $P = 0.979$), fibronectin (0.45 vs. 1.00, $P = 0.063$), and TGF-β1 (0.45 vs. 1.00, $P = 0.057$) did not significantly change. However, fibronectin and TGF-β1 showed decreasing trend. MMP2 and MMP9, markers of invasion, were also affected by transfection of the miR-27b-3p inhibitor. Both RNA and protein expression of MMP9 were significantly downregulated (0.40 vs. 1.00, $P = 0.035$), and MMP2 also decreased; however, the results for MMP2 were not significant.



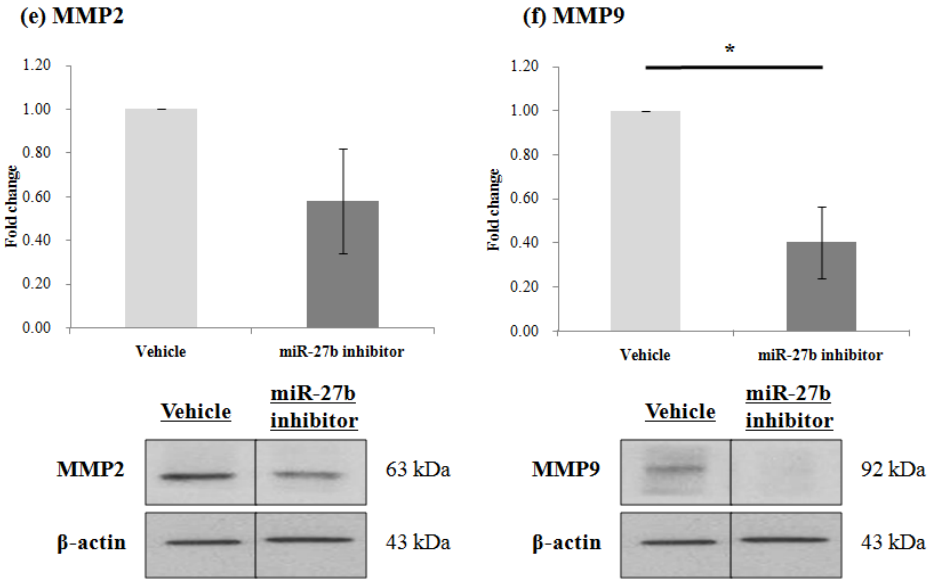


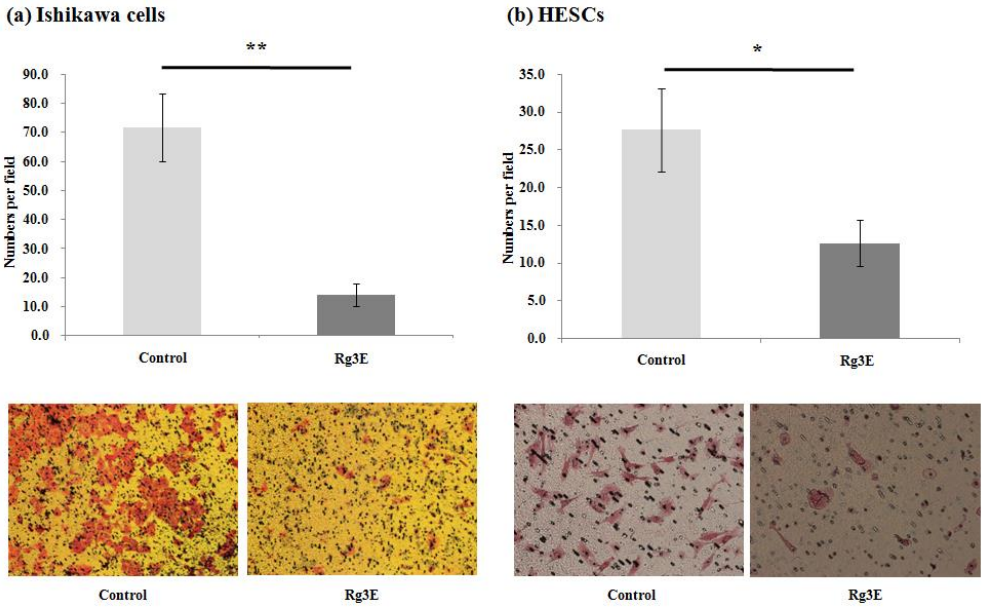
Figure 4. RNA and protein expressions of Col-1, CTGF, fibronectin, TGF-β1, MMP2 and MMP9 in HESCs from the patients with endometriosis after mir-27b-3p inhibitor transfection.

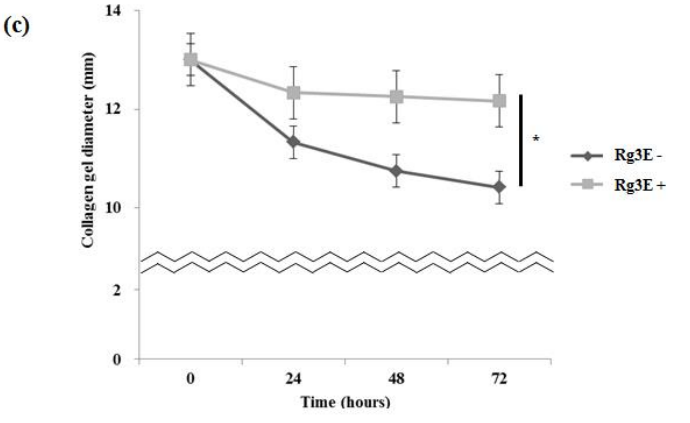
(a) Col-1, (b) CTGF, (c) fibronectin, (d) TGF-β1, (e) MMP2, (f) MMP9.

(*, $P < 0.05$. †, $P = 0.063$. ‡, $P = 0.057$. N = 4).

5. Effects of Rg3E on cell migration and collagen gel contraction

The migration assay showed a significant decrease in cell number after Rg3E treatment for both Ishikawa cells (cell count: 71.75 vs. 14.15, $P = 0.003$, N = 7) and HESCs (cell count: 27.61 vs. 12.62, $P = 0.026$, N = 12) (Figure 5(a)(b)). Collagen gel contractility of HESCs was evaluated using the collagen gel contraction assay. After treatment with Rg3E for 72 h, collagen gel contraction was significantly less than that of the control group that did not receive Rg3E treatment (contraction gel diameter: 10.42 vs. 12.17, $P = 0.012$, N = 6) (Figure 5(c)).





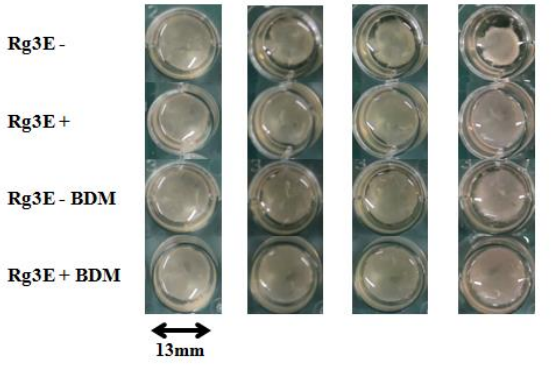


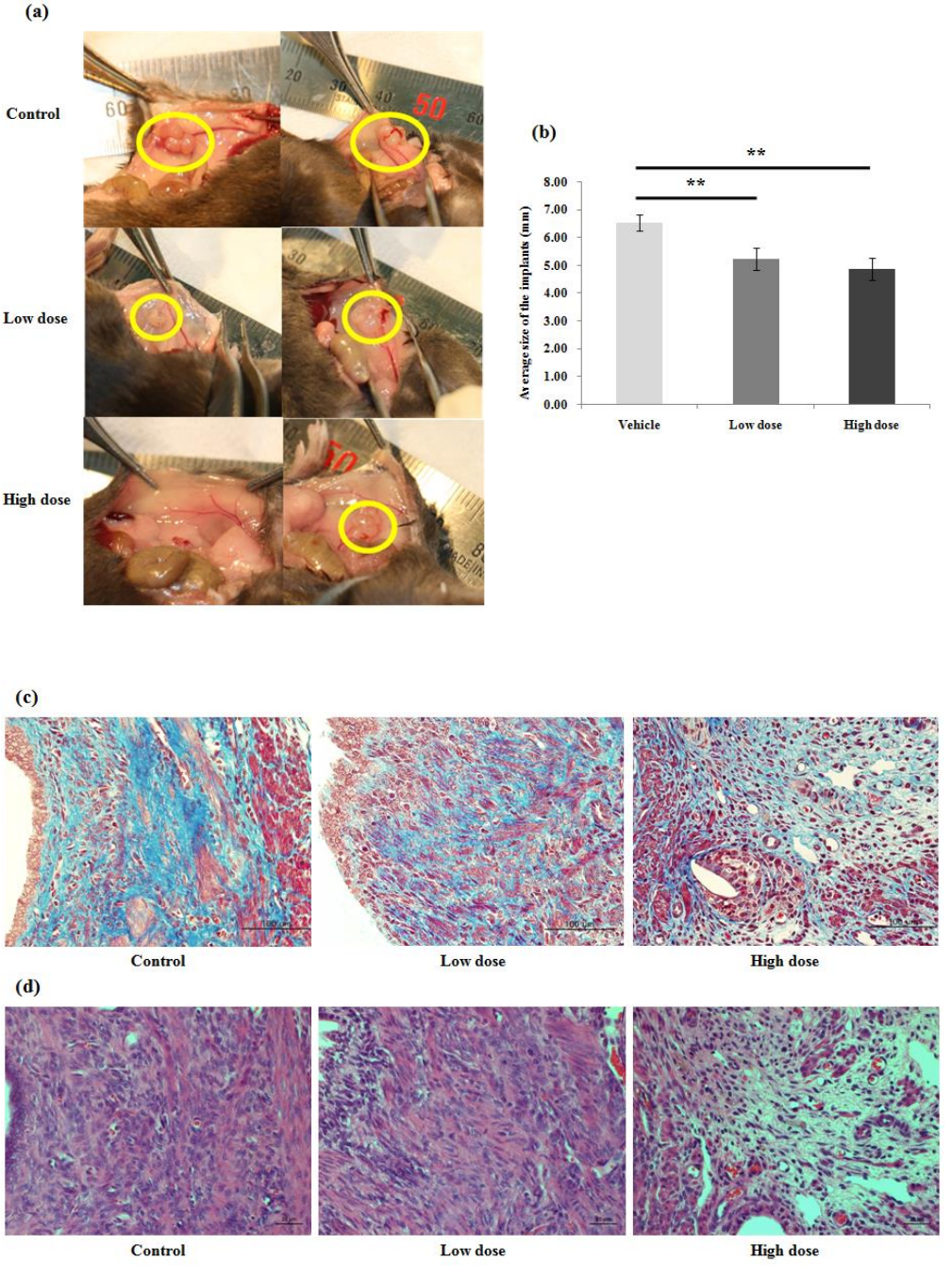
Figure 5. Migration assay of (a) Ishikawa cells and (b) HESCs of the patients with endometriosis after 48 h-Rg3E treatment and (c) contraction gel assays of HESCs of the patients with endometriosis before and after 72 h of Rg3E treatment. (*, $P < 0.05$. **, $P < 0.01$).

(a)(b) Migration assays in Ishikawa cells (cell count: 71.75 vs. 14.15, $P = 0.003$, N = 7) and HESCs (cell count: 27.61 vs. 12.62, $P = 0.026$, N = 12) of patients with endometriosis showed significantly decreased cell count. (c) Collagen gel contraction assay after 72 h of Rg3E treatment revealed significantly less collagen gel contraction compared to the control group. (Collagen gel diameter (mm): 12.17 vs. 10.42, $P = 0.012$, N = 6) (Rg3E -: without Rg3E treatment; Rg3E +: with Rg3E treatment; BDM: 3-Butandione manaxime treatment).

7. Mouse model of endometriosis

After 8 weeks of Rg3E treatment, the mice were sacrificed and their endometriotic lesions were obtained. All transplanted endometriotic lesions were found in the peritoneum of the sacrificed mice. The mean diameter of lesions in the vehicle, low-dose, and high-dose groups was 6.55 mm, 5.25 mm, and 4.88 mm, respectively. The average implant size in each group decreased as the dose of Rg3E was increased (Figure 6(a)(b)). Lesions from the treatment groups were significantly smaller than those from the vehicle group (vehicle vs. low dose, $P = 0.007$, vehicle vs. high dose, $P = 0.006$, N = 10 per group). However, no significant differences were noted between treatment groups.

Endometriotic lesions were stained with Masson's trichrome stain, and mean staining scores were calculated. Similar to the results observed for lesion size, mean staining scores for implants from each group decreased as the dose of Rg3E was increased. Mean staining scores of the vehicle, low-dose, and high-dose groups were 5.45, 4.36, and 4.18, respectively. Treatment group scores were significantly smaller than those of the vehicle group (vehicle vs. low dose, $P = 0.006$, vehicle vs. high dose, $P = 0.011$) (Figure 6(c)-(e)).



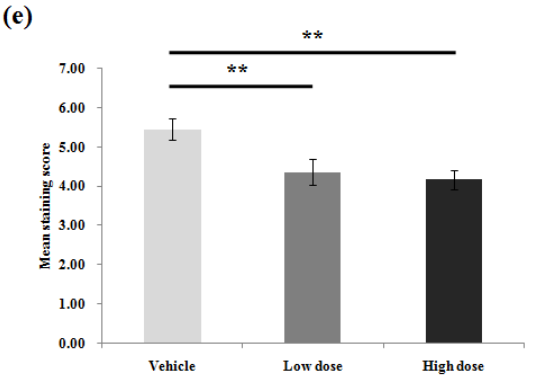


Figure 6. Mouse endometriosis model.

(a) Photographs showing endometriotic implants in mouse models fed with vehicle, low dose (0.1 mg/g), and high dose (0.2 mg/g) of Rg3E for 8 weeks. (b) Comparisons of the size of the endometriotic implants according to different treatment dose. (c) Masson's trichrome staining of endometriotic implants according to different treatment dose (magnification x 400). (d) Hematoxylin and eosin staining of the implants (magnification x 400). (e) Comparisons of Masson's trichrome staining scores according to different treatment doses.
(**, $P < 0.01$. N = 10 per group).

IV. DISCUSSION

This study demonstrates that Rg3E prepared from KRG significantly alters several major pathogenic characteristics of endometriosis, both *in vitro* and *in vivo*. To our knowledge, this report is the first study to evaluate the effects of Rg3E on endometriosis. The treatment mechanism involved changes in several miRNAs, including miR-27b-3p. In this study, miR-27b-3p expression is elevated in the eutopic endometrium of patients with endometriosis. In addition, Rg3E effectively reduces expression of this miRNA in HESCs from patients with endometriosis. Modulation of miR-27b-3p is associated with alteration of several cellular characteristics of endometriosis, including cell proliferation and invasion. Among these changes, the most profound effect was seen in fibrosis formation.

Rg3E and miR-27b-3p inhibition effectively reduce endometriosis fibrotic potential using a contraction gel assay and an *in vivo* mouse model.

Endometriosis is considered a benign disease; however, it often presents characteristics of malignancy such as proliferation and invasion,[38] and previous studies have shown that Rg3 inhibits such characteristics. A Chinese study demonstrated that Rg3 stimulates apoptosis and exhibits antitumor activity against lung cancer cells *in vitro*.[22] Rg3 is also known to suppress pro-angiogenic (TNF-α) and immunosuppressive cytokine (TGF-β) secretion, which may promote Rg3-induced immunogenic tumor cell death.[39] The invasive properties of endometriosis are related to an increase in proteolytic activity and matrix remodeling. MMPs are important for degrading the extracellular matrix, which takes part in endometriosis development.[40] This study shows that Rg3E significantly downregulates the expression of MMP2 and MMP9, blocking a significant pathogenic pathway of endometriosis.

The most interesting point of this study is the inhibition of fibrosis by Rg3E. Fibrosis is also an important pathogenic characteristic of endometriosis that aggravates infertility and pelvic pain. A previous study demonstrated that the Wnt/β-catenin signaling pathway is involved in regulating the cellular and molecular mechanisms of fibrosis in endometriosis, and the Tcf/β-catenin complex decreases fibrotic markers.[36] Another recent study claimed that endometriotic mesenchymal stem cells significantly promote fibrogenesis in ovarian endometrioma by paracrine production of TGF-β1 and Wnt1.[41] The present study shows Rg3E's anti-fibrotic effects in HESC-culture experiments and a collagen gel contraction assay, as well as fibrosis-related miRNA transfection. Previously, only one animal study reported the relationship between Rg3 and fibrosis, showing that Rg3 inhibits hepatic fibrosis in murine *Schistosomiasis japonica* models.[42] In this study, Rg3E greatly decreased expression of all fibrosis-related markers, including CTGF, fibronectin, Col-1, and TGF-β. Results differed among the control and Rg3E-treated groups, as shown by *in vitro* experiments and *in vivo*

through Masson's trichrome staining of mouse endometrial lesions. Although we discovered the miRNA related to this phenomenon, further studies will be needed to confirm the specific mechanism of Rg3's anti-fibrotic activity.

Recently, several studies have described the relationship between miRNAs and endometriosis pathogenesis. A recent study revealed that lower levels of miR-200b, miR-15a-5p, miR-19b-1-5p, miR146a-5p, and miR-200c, and higher levels of miR-16-5p, miR-106b-5p, and miR-145-5p are related to the modulation of vascular endothelial growth factor A (VEGFA), epidermal growth factor receptor 2 (EGFR2), phosphatase and tensin homolog (PTEN), and C-X-C chemokine receptor type 4 (CXCR4) expression, which are important in the pathogenesis of endometriosis.[43] Another study showed that miR-503, an miRNA that is repressed in endometriosis, induces apoptosis and inhibits cell proliferation, angiogenesis, and contractility of human ovarian endometriotic stromal cells.[44]

Among the miRNAs examined in this study, miR-27b was particularly highly expressed in the endometrium of patients with endometriosis. Several previous studies suggest that miR-27b is involved in fibrosis development. Overexpression of miR-27b promotes hypertrophic cardiomyocyte growth, while its suppression leads to inhibition of hypertrophic cell growth.[45] miR-27b expression significantly increased in both the sclerotic intima and serum samples of arteriosclerosis obliterans patients.[46] In pulmonary fibrosis, overexpression of miR-27b increased the expression of α-smooth muscle actin (α-SMA).[47] All these studies have shown that miR-27b expression is induced by TGF-β1, which is also related to fibrosis. Therefore, increased expression of miR-27b in endometriosis may be related to its fibrotic characteristics. More importantly, decreased miR-27b by Rg3E treatment demonstrates that Rg3 may be effective for reducing the fibrotic nature of the disease.

In contrast to endometrium, expression levels of miR-27b in endometrioma were significantly lower than in non-endometriotic cysts. This may reflect the notion that eutopic and ectopic endometriosis may not always share the

same molecular characteristics.[48] Furthermore, since miR-27b is involved in follicular maturation by regulation of activin A receptor type 1 (ACVR1) and DNA-binding protein inhibitor-2 (ID2) in mares, we hypothesize that it may reflect low ovarian reserves in endometriomas.[49] Another explanation can be derived from the fact that the control group was not normal ovarian tissue but mostly dermoid cysts. Although it is not clarified whether significant change of miR-27b is present in dermoid cysts, previous study show the differential profile of other 16 miRNAs in dermoid cysts compared to normal tissue.[50] The aberrant miRNA expressions determine the pathogenicity of dermoid cyst and miR-27b expression may also be completely different compared to both endometriomas and normal tissues.

V. CONCLUSION

Rg3E is shown to have beneficial effects for reducing the proliferative, invasive, and fibrotic nature of endometriosis. Among the endometriosis-affected miRNAs, miR-27b-3p was especially related to the development of fibrosis and inhibition of miR-27b significantly reduced fibrosis. In addition, Rg3E downregulated miR-27b-3p expression in HESCs. Therefore, Rg3 and modulation of associated miRNAs may provide a new therapeutic approach for endometriosis.

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ABSTRACT (IN KOREAN)

자궁내막증에서 Rg3 강화 추출물의 항섬유화 효과에 대한 생체내, 외 평가 및 이와 연관된 miRNA 규명

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김 민 경

연구목적: 홍삼에서 추출된 Rg3의 자궁내막증에 대한 치료적 효과와 이에 관련된 miRNA를 알아보고자 하였다.

연구방법: 생체외 실험으로는 사람의 자궁내막 기질 세포를 (HESCs) 자궁내막증 환자로부터 얻어 Rg3 강화 추출물 (Rg3E)로 처리하였으며 real-time PCR, microarray profiling, transfection, 그리고 western blot을 진행하였다. 생체내 실험으로는 자궁내막증 쥐 모델을 만들어 8주간 Rg3E 투약해 보았다.

결과: Rg3E 처리한 HESCs에서 Ki-67, MMP2, MMP9, Col-1, CTGF, fibronectin, 그리고 TGF-β1이 통계적으로 유의하게 감소하였다. 또한 microarray analysis상 miR-27b-3p가 감소함을 보였는데, 이는 섬유화 작용과 관련이 있다. 자궁내막증 환자의 HESCs에서 대조군과 비교하였을 때 miR-27b-3p가 유의하게 높게 발현하였으며, Rg3E 는 이를 유의하게 감소시켰다. miR-27b inhibitor로 transfection시킨 후 Col-1과 MMP9의 RNA 및 단백질 발현이 유의하게 감소하였다. Contraction과 migration assay에서 HESCs를 Rg3E 처리 후 세포 섬유화와 이동 가능성이 유의하게 감소하였다. 생체내 실험에서도 Rg3E를 투약한 군에서는 자궁내막증 병변의 크기와 섬유화 특성이

유의하게 줄어든 것을 확인하였다.

결론: Rg3는 자궁내막증 환자로부터 얻은 HESCs의 섬유화 특성을 효과적으로 변형시키며, 이는 miR-27b-3p 조절에 의한 것일 가능성이 있다.

핵심되는 말 : 자궁내막증, 마이크로RNA, 홍삼, 진세노사이드, Rg3