Enhanced miR-210 expression promotes the pathogenesis of endometriosis through signal transducer and activator of transcription 3 activation

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Running head: Enhanced miR-210 expression in endometriosis**Abstract**

Study question: What are the roles of the microRNA miR-210 — a miRNA that is upregulated in endometriotic cyst stromal cells (ECSCs) — in the pathogenesis of endometriosis?

Summary answer: Upregulated miR-210 expression in ECSCs is involved in their proliferation, resistance to apoptosis, and angiogenesis through signal transducer and activator of transcription (STAT) 3.

What is known already: In the pathogenesis of endometriosis, a number of roles for microRNAs (miRNAs) are becoming apparent.

Study design, size, duration: ECSCs and normal endometrial stromal cells (NESCs) were isolated from ovarian endometriotic tissues (patients aged 24–40 yrs undergoing salpingo-oophorectomy or evisceration for the treatment of ovarian endometriotic cysts, n=10) and the eutopic endometrial tissues without endometriosis (premenopausal patients aged 35–45 yrs undergoing hysterectomies for subserousal leiomyoma, n=13), respectively.

Participants/materials, setting, methods: We used a global gene expression microarray technique to identify downstream targets of miR-210, and we assessed compulsory miRNA expression in NESCs to determine the functions of miR-210 in the pathogenesis of endometriosis.

Main results and the role of chance: Gene expression microarray analysis revealed that one of the key target molecules of miR-210 is STAT3. In the NESCs, the compulsory expression of miR-210 resulted in the induction of cell proliferation, the production of vascular endothelial cell growth factor (VEGF), and the inhibition of apoptosis through STAT3 activation. In the ECSCs, inhibitors of STAT3 inhibited the cell proliferation and VEGF production, and induced the apoptosis of these cells.

Limitations, reasons for caution: The roles of aberrant miR-210 expression were investigated only in the stromal component of ectopic and eutopic endometrium. Control endometrial tissues were obtained from premenopausal patients who had subserosal leiomyoma and NESC gene expression patterns may be altered in these women. Furthermore, the effects of STAT3 inhibitors were evaluated only in ECSCs and not in NESCs.

Wider implications of the findings: The present findings indicate that miR-210 induces NESCs to differentiate into the endometriotic phenotype and we speculate that upregulated miR-210 expression in ECSCs is involved in the creation of the endometriosis-specific cellular dysfunctions through epigenetic

mechanisms. The data indicate that STAT3 inhibitors may be promising candidates for the treatment of endometriosis.

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Key words: endometriosis, microRNA, signal transducer and activator of transcription 3, vascular endothelial growth factor

Introduction

Endometriosis is an estrogen-dependent disease exhibiting the benign ectopic growth of proliferative endometrial tissue. As a disease most frequently observed in women of reproductive age, endometriosis is most commonly based in the peritoneum, ovaries, and rectovaginal septum (Giudice and Kao, 2004). Dysmenorrhea, chronic pelvic pain, subfertility, and/or dyspareunia are the main symptoms, often greatly decreasing the quality of life of the affected women (Giudice and Kao, 2004).

Although endometriotic tissues share many histological characteristics with normal proliferative endometrial tissues (Giudice and Kao, 2004), they show several interesting molecular differences such as those concerning gene expression and protein production, synthesis and responsiveness to steroids and cytokines, immune components, adhesion molecules, and proteolytic enzymes and their inhibitors; endometriotic tissues also have a tissue structure and cell proliferation rates that differ from those of normal endometrial tissues (Nasu *et al.*, 2011). The occurrence and development of endometriosis may be based on these aberrations in molecular processes, which have been rather stable and consistent in endometriosis, the involvement of some sort of cellular memory is thus a possibility. To identify the responsible mechanism(s) of the pathogenesis of endometriosis, our research has been focused on the dysregulation of microRNA (miRNA) expression (Abe *et al.*, 2013), histone modification (Nasu *et al.*, 2014), and DNA methylation (Nasu *et al.*, 2012) in endometriotic cells from the viewpoint of epigenetics. We hypothesized that the acquisition of aberrant gene expression by epigenetic mechanisms may induce the endometrial cells to differentiate into an endometriotic phenotype (Nasu *et al.*, 2014).

miRNAs are short RNAs that comprise a class of regulatory genes characterized as endogenous, single-stranded, noncoding RNA (Bartel, 2004). They function by regulating the translation of specific targeted protein-coding genes (Bartel, 2004). It has been estimated that, as components of epigenetic mechanisms, miRNAs regulate the expression of 50–60% of the human genes without changing DNA sequences (Pillai, 2005; Engels and Hutvagner, 2006). Dozens of genes can be targeted by a single miRNA and, by the perfect or partial base-pairing with the 3'-untranslated region (UTR) of the target mRNAs, various cellular functions are induced or promoted (Pillai, 2005; Engels and Hutvagner, 2006). A broad range of physiological and pathological processes have been shown to involve one or more essential roles for miRNAs: the entire cell cycle (including embryogenesis, development, differentiation and proliferation), metabolism, cell-cell communication, cell survival and apoptosis, immune responses, and oncogenesis (Bartel, 2004; Engels and Hutvagner, 2006; Burney *et al.*, 2009; Ohlsson Teague *et al.*, 2009; Ohlsson Teague *et al.*, 2010).

A group of miRNAs that are differentially expressed among normal endometrium without endometriosis, eutopic endometrial tissues with endometriosis, and endometriotic lesions have been demonstrated in microarray studies (Burney *et al.*, 2009; Ohlsson Teague *et al.*, 2009; Ohlsson Teague *et al.*, 2010; Pan *et al.*, 2007; Toloubeydokhti *et al.*, 2008; Filigheddu *et al.*, 2010; Kuokkanen *et al.*, 2010; Hawkins *et al.*, 2011; Braza-Boïls *et al.*, 2014), indicating the importance of miRNAs in the pathogenesis of endometriosis. In our recent miRNA microarray analysis (Abe *et al.*, 2013), we identified a number of miRNAs that are aberrantly expressed in human endometriotic cyst stromal cells (ECSCs) compared to human normal endometrial stromal cells (NESCs) in primary culture. We found that miR-196b, one of the repressed miRNAs in the ECSCs, had anti-proliferative and pro-apoptotic functions in these cells.

We designed the present study to evaluate the role of miR-210, one of the upregulated miRNAs in ECSCs (Abe *et al.*, 2013), in the pathogenesis of endometriosis. Using the compulsory miRNA expression in NESCs, we observed the proliferative, anti-apoptotic, and angiogenic functions of miR-210 and the possible downstream targets of this miRNA. We also evaluated the efficacy of signal transducer and activator of transcription (STAT) 3 inhibitors as promising drugs for the treatment of endometriosis.

Materials and Methods

ECSC and NESC isolation procedure and cell culture conditions

Endometriotic tissues were obtained from patients with regular menstrual cycles who had undergone a salpingo-oophorectomy or evisceration for the treatment of ovarian endometriotic cysts (n=10, aged 24–40 yrs), as described (Nishida *et al.*, 2004). For ethical reasons it was difficult to obtain endometrial tissues from healthy women, therefore eutopic endometrial tissues were obtained from premenopausal patients who had undergone hysterectomies for subserousal leiomyoma and had no evidence of endometriosis (n=13, aged 35–45 yrs), as described (Nishida *et al.*, 2004). None of the patients had received any hormonal treatments for at least 2 years prior to the operation. All of the specimens were confirmed as being in the mid- to late-proliferative phases according to pathological observation and/or menstrual cycles. The patients in the present study were chosen without randomization. This study was approved by the institutional review board (IRB) of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients.

ECSCs and NESCs were isolated from ovarian endometriotic tissues and the eutopic endometrial tissues, respectively, by enzymatic digestion with collagenase as previously described (Nishida *et al.*, 2004). Isolated ECSCs and NESCs were cultured in Dulbecco's modified eagle medium supplemented with 100 IU/ml of penicillin, 50 mg/ml of streptomycin, and 10% heat-inactivated fetal bovine serum (all obtained from Gibco-BRL, Gaithersburg, MD, USA) at 37°C in 5% CO_2 in air. ECSCs and NESCs in the monolayer culture after the third passage were >99% pure as determined by immunocytochemical staining with antibodies to vimentin, CD10, cytokeratin, factor VIII, and leukocyte common antigen (Nishida *et al.*, 2004). Each experiment was performed in triplicate and repeated at least three times with the cells from at least three separate patients, except for gene expression microarray study.

Reverse transfection of miRNA precursors

NESCs were transfected with precursor hsa-miR-210 (Pre-miR miRNA precursor- hsa-miR-210, Ambion, Austin, TX, USA) or negative control precursor miRNA (Pre-miR miRNA precursor-negative control #1, Ambion) at a final concentration of 10 nM, using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) using the reverse transfection method, as previously described (Abe *et al.*, 2013).

Isolation of total RNA and the gene expression microarray analysis

Forty-eight hours after transfection, total RNA from cultured NESCs transfected with precursor hsa-miR-210 (n=3) and NESCs (n=3) transfected with negative control precursor miRNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA, USA). The quality of the extracted RNA was confirmed by measuring the absorbance at 230 nm, 260 nm, and 280 nm using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA) and by an Experion System (Bio-Rad Laboratories, Hercules, CA, USA). The samples were then subjected to a gene expression microarray analysis with a commercially available human mRNA microarray (G4845A, Human Gene Expression 4x44K v2, Agilent Technologies, Santa Clara, CA, USA), which consists of 44,000 probes for 27,958 human RNAs, based on RefSeq Build 36.3, Ensemble Release 52, Unigene Build 216, and GenBank (April 2009).

Briefly, the total RNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligomicroarray according to the manufacturer's instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw signal intensities and Flags for each probe were calculated from hybridization intensities (gProcessedSignal), and spot information (gIsSaturated), according to the procedures recommended by Agilent Technologies. The flag criteria on the GeneSpring Software were, Absent (A), "Feature is not positive and significant" and "Feature is not

above background"; Marginal (M), "Feature is not Uniform," "Feature is Saturated," and "Feature is a population outlier;" Present (P), others. The raw signal intensities of six samples were then $\log_2$-transformed and normalized by a quantile algorithm with the 'preprocessCore' library package on Bioconductor software (Gentleman *et al.*, 2004).

We selected probes that call the 'P' flag in both of two samples. To identify up or down-regulated genes, we calculated Z-scores and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparison between NESCs transfected with precursor hsa-miR-210 and NESCs transfected with negative control precursor miRNA (Quackenbush, 2002). We then established the criteria for regulated genes: Z-score ≥2.0 and ratio ≥1.5-fold for up-regulated genes, and Z-score ≤–2.0 and ratio ≤0.66 for down-regulated genes. All data are available at Gene Expression Omnibus via the National Center for Biotechnology Information under Accession No. GSE56854 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56854).

Ingenuity pathways analysis (IPA)

To investigate possible biological interactions of differentially expressed genes, we imported datasets representing genes with an altered expression profile derived from the microarray analyses into the IPA software (Ingenuity Systems, Redwood City, CA, USA) with the IPA knowledgebase (IPA Winter Release 2012).

Assessment of the cell viability of NESCs after miR-210 transfection and ECSCs after treatment with STAT3 inhibitors

WP1066 (573097; Merck Millipore, Darmstadt, Germany), S3I-201 (573102; Merck Millipore), and cryptotanshinone (79852; Sigma-Aldrich Co., St. Louis, MO, USA) were chosen as the representative STAT3 inhibitors in the present study. These STAT3 inhibitors were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical, Osaka, Japan) at the concentration of 100 mM as the stock solutions. The cell viability of NESCs after miRNA transfection and ECSCs after treatment with STAT3 inhibitors [WP1066 (1–8 μM), S3I-201 (20–160 μM), and cryptotanshinone (8–64 μM)] was determined by a modified methylthiazoletetrazolium (MTT) assay using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Briefly, 5×10^3 NESCs transfected with precursor hsa-miR-210 or negative control precursor miRNA by reverse transfection method were placed on 96-well flat-bottomed microplates (Corning, New York, NY, USA) and incubated for 72 h, as previously described (Abe *et al.*, 2013). ECSCs (5×10^3 cells) were placed in 96-well flat-bottomed microplates and incubated with STAT3 inhibitors and 0.1% DMSO for 72 h. Thereafter, 20 μl of CellTiter 96® AQ_{ueous} One Solution Reagent was added to each well and the cells were further incubated for 1 h. Cell viability was determined by measuring absorbance at 490 nm.

Assessment of the apoptosis of NESCs after miR-210 transfection and ECSCs after treatment with STAT3 inhibitors

We determined the apoptosis of NESCs after miRNA transfection and ECSCs after treatment with STAT3 inhibitors [WP1066 (1–8 μM), S3I-201 (20–160 μM), and cryptotanshinone (8–64 μM)] by the direct determination of nucleosomal DNA fragmentation using an enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection ELISA, Roche Diagnostics) (Abe *et al.*, 2013). Briefly, 5×10^3 NESCs transfected with precursor hsa-miR-210 or negative control precursor miRNA were placed on 96-well flat-bottomed microplates (Corning). ECSCs (5×10^3 cells) were placed on a 96-well flat-bottomed microplates and incubated with STAT3 inhibitors and 0.1% DMSO. After 72 h of culture, the cells were lysed according to the manufacturer's instructions, followed by centrifugation (200×g, 5

min). The mono- and oligonucleosomes contained in the supernatants were determined using an anti-histone-biotin antibody. The concentration of nucleosomes-antibody was evaluated by measuring the absorbance at 405 nm using 2,2'-azino-di(3-ethylbenzthiazoline-sulphonate) as a substrate.

Assessment of the activities of caspase-3 and caspase-7 in NESCs after miR-210 transfection and ECSCs after treatment with STAT3 inhibitors

The apoptosis of NESCs after miRNA transfection and ECSCs after treatment with STAT3 inhibitors [WP1066 (1–8 μM), S3I-201 (20–160 μM), and cryptotanshinone (8–64 μM)] was evaluated by the Caspase-Glo 3/7 Assay (Promega) as described (Abe *et al.*, 2013). The assay is a luminescent assay that measures the activities of caspase-3 and caspase-7. Briefly, 5×10^3 NESCs transfected with precursor hsa-miR-210 or negative control precursor miRNA were placed on 96-well flat-bottomed microplates (Promega). Whereas, 5×10^3 ECSCs placed on a 96-well flat-bottomed microplates were incubated with STAT3 inhibitors and 0.1% DMSO. After 72 h of culture, Caspase-Glo 3/7 reagent was added to each well. The plates were gently shaken for 60 min at room temperature, and then we measured the luminescence in a plate-reading luminometer.

Assessment of the effects of miR-210 on the STAT3 and VEGF-A mRNA expression in NESCs

The effects of miR-210 on the expressions of possible downstream target genes in NESCs were evaluated by quantitative RT-PCR. The STAT3 and vascular endothelial growth factor A (VEGF-A) were chosen as candidate molecules for evaluation. Briefly, NESCs transfected with precursor hsa-miR-210 or negative control precursor miRNA were cultured in 10-cm culture dishes (Corning). Forty-eight hours after incubation, total RNA was extracted from the cultured NESCs using a miRNeasy Mini kit (Qiagen), as described above. cDNA was then synthesized from 1 μg of total RNA using the Reverse Transcription System (Promega).

The quantitative RT-PCR was carried out with a LightCycler 480 (Roche Diagnostics GmbH, Penzberg, Germany) using TaqMan Universal PCR Master Mix II with specific primers for STAT3 (Assay ID: Hs00374280_m1, Applied Biosystems, Carlsbad, CA, USA), VEGF-A (Assay ID: Hs00900055_m1, Applied Biosystems), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: Hs02758991_g1, Applied Biosystems), as described (Abe *et al.*, 2013). The expression levels of STAT3 and VEGF-A mRNA relative to GAPDH mRNA were calculated from a standard curve.

Assessment of the effects of miR-210 on the phosphorylated and total STAT3 protein levels in NESCs

The effects of miR-210 on the phosphorylated and total STAT3 protein levels in NESCs were also evaluated, using the Cell-Based Human/Mouse/Rat STAT3 (Tyr705) Phosphorylation ELISA Kit (RayBiotech, Inc, Norcross, GA, USA). Briefly, 3×10^4 cells transfected with precursor hsa-miR-210 or negative control precursor miRNA were placed on a white-walled 96-well microplate (RayBiotech). After 48 h of culture, the plates were processed to measure the absorbance at 405 nm, according to the manufacturer's instructions.

Assessment of the VEGF-A protein levels in the supernatant of NESCs after miR-210 transfection and ECSCs after treatment with STAT3 inhibitors

The VEGF-A protein levels in the supernatant of NESCs after miRNA transfection and those of ECSCs after treatment with STAT3 inhibitors [WP1066 (1–8 μM), S3I-201 (20–160 μM), and cryptotanshinone (8–64 μM)] were determined by ELISA according to the manufacturer's instructions.

Briefly, subconfluent NESCs after miRNA transfection or ECSCs after treatment with STAT3 inhibitors were cultured in 24-well culture plates (Corning). After a 24-h culture, the supernatants were

then collected and stored at –70°C until assay. The concentration of VEGF-A was determined in each supernatant using commercially available ELISA kits (Human VEGF Immunoassay, R&D systems, Minneapolis, MN, USA). The sensitivity of the assay for VEGF and was 9.0 pg/ml.

Statistical analysis

Data were obtained from triplicate samples and are presented as percentages relative to the corresponding controls, as mean ± SD, and were appropriately analyzed by the Bonferroni test and the Student *t*-test with Sigmaplot 11.2 (Systat Software, San Jose, CA, USA). Values of $p < 0.05$ were considered significant.

Results

Identification of candidate genes regulated by compulsory miR-210 expression in NESCs

Among the 27,958 mRNAs examined by the gene expression microarray, 94 upregulated mRNAs and 229 downregulated mRNAs were identified statistically by using the criteria described above. Using the IPA software to find the known target and candidate downstream signaling networks of miR-210, we identified 35 downstream molecules, four molecular complexes, and eight molecular groups (Table 1).

Of the eight transcription factors detected (Table 1), we focused on STAT3 as a key molecule regarding the pathogenesis of endometriosis (Figure 1) and used it in the further experiments.

The mRNA expression of some molecules, such as EGR2, HOXA1, SREBF1, and PTPN1, seem to be unaffected by miR-210 transfection. It is speculated that miR-210 may affect the function of these downstream target molecules without changing their mRNA expression.

Enhanced cell viability of NESCs by compulsory miR-210 expression

The effects of miR-210 on the cell viability of NESCs were evaluated by a modified MTT assay. As shown in Figure 2A, the viable cell number was significantly increased by the transfection of miR-210 precursor.

Inhibition of apoptosis of NESCs by compulsory miR-210 expression

The anti-apoptotic effects of miR-210 on NESCs were determined by the Cell Death Detection ELISA assay. As shown in Figure 2B, the transfection of miR-210 precursor significantly inhibited the apoptosis of ECSCs.

Inhibition of caspase-3/7 activity in NESCs by compulsory miR-210 expression

The anti-apoptotic effects of miR-210 on ECSCs were also assessed by evaluating the activities of caspase-3 and caspase-7. As shown in Figure 2C, the transfection of miR-210 precursor significantly inhibited the activities of caspase-3 and caspase-7 in ECSCs.

Enhanced VEGF mRNA and protein expression in NESCs by compulsory miR-210 expression

The effects of compulsory miR-210 expression on the VEGF mRNA and protein expression in NESCs were assessed by RT-PCR and ELISA, respectively. The transfection of miR-210 precursor significantly enhanced the VEGF mRNA and protein expression in NESCs (Figure 2D, E).

Induction of STAT3 expression and activation in NESCs by compulsory miR-210 expression

The effects of compulsory miR-210 expression on the STAT3 mRNA and protein expression and STAT3 activation in NESCs were assessed. As shown in Figure 2F–H, the transfection of miR-210 precursor significantly enhanced the STAT3 mRNA and protein expression and STAT3 activity in NESCs.

Suppression of cell viability of ECSCs by STAT3 inhibitors

The effects of STAT3 inhibitors on the cell viability of ECSCs were evaluated by a modified MTT assay. As shown in Figure 3A, the viable cell number was significantly decreased by the addition of STAT3 inhibitors.

Induction of apoptosis of ECSCs by STAT3 inhibitors

The effects of STAT3 inhibitors on the apoptosis of ECSCs were determined by the Cell Death Detection ELISA assay. As shown in Figure 3B, the STAT3 inhibitors significantly induced the apoptosis of ECSCs.

The pro-apoptotic effects of STAT3 inhibitors on ECSCs were also assessed by evaluating the activities of caspase-3 and caspase-7. The STAT3 inhibitors significantly enhanced the activities of caspase-3 and caspase-7 in ECSCs (Figure 3C).

Suppression of VEGF production in ECSCs by STAT3 inhibitors

The effects of STAT3 inhibitors on the VEGF protein secretion of ECSCs were assessed by ELISA. As shown in Figure 3D, the STAT3 inhibitors significantly decreased the VEGF protein levels in the supernatant of ECSC culture.

Discussion

We conducted the present study to identify the role of miR-210, one of the upregulated miRNAs in ECSCs (Abe *et al.*, 2013), in the pathogenesis of endometriosis. By examining the compulsory miRNA expression in NESCs, the gene expression microarray technique, and an IPA, we found a variety of candidate molecules as the downstream targets of miR-210. We then focused on STAT3 and performed further functional experiments. We found that miR-210 promoted the proliferation, resistance to apoptosis, and VEGF production through STAT3 activation in NESCs, whereas STAT3 inhibitors inhibited the proliferation and VEGF production of ECSCs and induced the apoptosis of these cells. These findings suggest that miR-210 induces NESCs to differentiate into the endometriotic phenotype, which is characterized by proliferative, anti-apoptotic, and angiogenic features. We also suggest that upregulated miR-210 expression in ECSCs is involved in the creation of the endometriosis-specific cellular dysfunctions as part of epigenetic mechanisms. It is considered that activation of the miR-210-STAT3-VEGF axis is important in the pathogenesis of endometriosis. Based on our findings, we speculate that STAT3 inhibitors could be promising for the treatment of endometriosis.

As summarized in Table 2, the roles of several miRNAs in the pathogenesis of endometriosis have been demonstrated (Abe *et al.*, 2013; Lin *et al.*, 2012; Adammek *et al.*, 2013; Shen *et al.*, 2013; Hsu *et al.*, 2014; Lin *et al.*, 2014; Shi *et al.*, 2014). We showed that miR-196b, one of the repressed miRNAs in ECSCs, has anti-proliferative and pro-apoptotic functions in these cells by targeting c-myc and Bcl-2.Given the diverse roles that miRNAs play in numerous aspects of cellular functions, it is not surprising that they play key regulatory roles in the pathogenesis of endometriosis. All the miRNAs listed in Table 2 belong to a different cluster from miR-210. VEGFA was the only target molecule shared with miR-210 and miR-199a-5p. Evaluations of the functions of each aberrantly expressed miRNA are necessary in further research on endometriosis.

Several studies showed that in various cell types, hypoxia was followed by the induction of miR-210 expression through hypoxia-inducible factors (Crosby *et al.*, 2009; Zhang *et al.*, 2009; Huang *et al.*, 2010). A variety of tumors exhibit increased miR-210 expression (Gee *et al.*,, 2010; Stephen and Joseph, 2010; Buffa *et al.*, 2011; Yuk *et al.*, 2012). The identified functions of miR-210 include the modulation of cell proliferation, differentiation, cell cycle arrest, cell migration, DNA repair, chromatin remodeling, apoptosis, angiogenesis, and metabolism (Fasanaro *et al.*, 2008; Crosby *et al.*, 2009; Zhang *et al.*, 2009; Huang *et al.*, 2010). A large number of target molecules of miR-210 have been reported: AIFM3, caspase-8 associated protein-2 (CASP8AP2), DAPK1, E2F3, ephrin-A3, FGFRL1, HOXA1, HOXA3, iron-sulfur cluster scaffold proteins (ISCU), the myc antagonist MNT, PTPN1, RAD52, TP53I11, VEGF, and VEGFR2 (Fasanaro *et al.*, 2008; Chan *et al.*, 2009; Crosby *et al.*, 2009; Zhang *et al.*, 2009; Huang *et al.*, 2010; Noman *et al.*, 2012). Since miR-210 is well known as an angiogenic miRNA, we focused on the regulatory mechanism of VEGF expression by miR-210. In this setting, we found miR-210-STAT3-VEGF axis to be an important pathway (Figure 1). Interestingly, STAT3 has been also shown to regulate cell proliferation and apoptosis (Siveen *et al.*, 2014).

The treatment of endometriosis has been a challenge. Non-steroidal anti-inflammatory agents, agonists of GnRH, progestogens, androgens, and contraceptive steroids have all been tried for patients with endometriosis (Committee of the American Society for Reproductive Medicine, 2004), and several surgical and medical strategies have been conducted, with varying degrees of efficacy. The results of the present study suggest that STAT3 inhibitors have potential as a treatment for endometriosis. In fact, several STAT3 inhibitors are now in preclinical use as anticancer drugs (Furqan *et al.*, 2013). BBI608 (Langleben *et al.*, 2013) is now in phase III trial, whereas OPB-31121 (ClinicalTrials.gov Identifier: NCT00955812, Food and Drug Administration, USA) and BBI503 (Laurie *et al.*, 2014) are in phase I trial.

Siveen *et al.* (2014) demonstrated that the activation of STAT3 inhibits apoptosis and induces

cell proliferation, angiogenesis, metastasis and invasion. The STAT family of transcription factors, in their inactive form, is initially located in the cytoplasm of the cell. With the stimulation provided by extracellular signals, such as cytokines, Janus kinases (JAKs), growth factors and hormones are activated thereby inducing the phosphorylation of STAT3 on a tyrosine residue in its COOH terminus (Siveen *et al.*, 2014). Phosphorylated STAT3 proteins activate the transcription of their target genes after they dimerize, translocate into the nucleus, and bind with DNA. As shown in Figure 1, it is suggested that miR-210 induces STAT3 mRNA and protein expression/phosphorylation through PTPN1. Interestingly, as shown in Table 1, PTPN1 mRNA expression was not affected by compulsory miR-210 expression in NESCs. Further examinations are necessary to elucidate the precise mechanisms. In the present study, we chose three representative STAT3 inhibitors with diverse mechanisms of action. WP1066 inhibits STAT3 activity by inhibiting its upstream transcription factor JAK (Ferrajoli *et al.*, 2007), whereas S3I-201 inhibits STAT3 activity by inhibiting STAT3 dimerization (Fletcher *et al.*, 2009). Cryptotanshinone inhibits STAT3 activity by inhibiting its phosphorylation (Lu *et al.*, 2013). These STAT3 inhibitors show stronger effects in comparison with miR-210 transfection, suggesting that STAT3 can regulate cell viability by a mechanism independent of miR-210. Additional studies are necessary to fully understand the action of STAT3 inhibitors.

Another promising potential strategy for treating endometriosis is miRNA-targeting therapeutics in part because the use of small miR-inhibitor oligomers (e.g., DNA, DNA analogs, and RNA) can readily accomplish miRNA inhibition (Hemida *et al.*, 2010; Kota and Balasubramanian, 2010). In addition, the DNA analogs known as peptide nucleic acids (PNAs) — in which the sugarphosphate backbone is replaced by N-(2-aminoethyl)glycine units (Gambari *et al.*, 2011) — efficiently hybridize with their target miRNAs and inhibit their functioning. The in vitro and in vivo effects of PNAs targeting miR-210 have been described (Gambari *et al.*, 2011).

Angiogenesis is a critical step in the establishment of endometriosis, and endometriotic lesions are highly vascularized (Donnez *et al.*, 1998). Endothelial cell proliferation and migration for neovascularization are stimulated by VEGF, the main sources of which are endometriotic tissue and peritoneal macrophages, and mesothelial cells (Donnez *et al.*, 1998). As yet another endometriosis treatment strategy, anti-angiogenic therapy against VEGF holds great promise. miR-210-targeting molecules and STAT3 inhibitors can also act as anti-angiogenic argents by suppressing ECSCs' production of VEGF.

For ethical reasons it is difficult to obtain endometrial tissues from healthy women. Instead, control endometrial tissues were obtained from premenopausal patients who had undergone hysterectomies for subserosal leiomyoma and had no evidence of endometriosis. Gene expression patterns of NESCs may be altered by the presence of leiomyoma (Karmon *et al.*, 2014). Further limitations of the present study are that we have not performed a loss-of-function study using ECSCs transfected with miR-210-antagonist and the effects of STAT3 inhibitors were evaluated only in ECSCs and not in NESCs.

In conclusion, by using compulsory miRNA expression in NESCs,a gene expression microarray technique, and an IPA, we found that STAT3 is one of the key target molecules of miR-210. The compulsory expression of miR-210 directed the induction of cell proliferation and VEGF production and the inhibition of apoptosis in NESCs through STAT3 activation, whereas STAT3 inhibitors blocked the proliferation and VEGF production of ECSCs and induced apoptosis of these cells. These findings suggest that upregulated miR-210 expression in human ECSCs is involved in the creation of cellular dysfunctions that are disease-specific features of endometriosis, and we propose these may involve epigenetic mechanisms. In addition, STAT3 inhibitors are promising candidates for the treatment of endometriosis. Further studies on the repertoire of aberrantly expressed miRNAs, interacting miRNA-target mRNA associations, and the regulation and mechanisms of action of miRNA may provide

401 useful information about the pathogenesis of endometriosis.

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Authors' roles

K.N., M.M. and H.N. participated in the study design, analysis and manuscript drafting. M.O., W.A., Y.A., Y.K., and K.K. executed the study.

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Conflict of interest

None declared.

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Figure Legends

Figure 1. The downstream network of the miR-210-STAT3 signaling pathway detected by IPA. The genes that are shaded were determined to be significant from the statistical analysis. The genes in red are upregulated and those in green are downregulated. The intensity of the shading shows to what degree each gene was up- or downregulated. A solid line represents a direct interaction between the two gene products, and a dotted line means that there is an indirect interaction.

Figure 2. (A) Cell viability, (B) apoptotic cells, (C) caspase-3/7 activity, (D) VEGF mRNA levels, (E) VEGF protein levels, (F) STAT3 mRNA levels, (G) total STAT3 protein levels, and (H) phosphorylated STAT3 protein levels of NESCs after miR-210 transfection (n=3). The data are presented as percentages relative to the values of NESCs transfected with negative control precursor miRNA (n=3). *$p<0.05$, **$p<0.005$, ***$p<0.0005$ versus negative controls (Student *t*-test). Representative results are shown.

Figure 3. (A) Cell viability, (B) apoptotic cells, (C) caspase-3/7 activity, and (D) VEGF protein levels of ECSCs treated with STAT3 inhibitors [WP1066 (1–8 μM), S3I-201 (20–160 μM), and cryptotanshinone (8–64 μM)] (n=3). The data are presented as percentages relative to the values of untreated ECSCs (n=3). *$p<0.05$, **$p<0.005$, ***$p<0.0005$ versus negative controls (Bonferroni test). Representative results are shown.