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ENDOMETRIOSIS: ORIGINAL ARTICLE

hsa-miR-199a-3p Inhibits Motility, Invasiveness, and Contractility of Ovarian Endometriotic Stromal Cells

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Abstract

It is suggested that aberrantly expressed microRNAs are involved in the pathogenesis of endometriosis. Our previous study demonstrated that expression of the microRNA hsa-miR-199a-3p is attenuated in human endometriotic cyst stromal cells (ECSCs). The current study aimed to define the roles of hsa-miR-199a-3p in the development of endometriosis. ECSCs and normal endometrial stromal cells (NESCs) were isolated from ovarian endometrioma and normal endometrial tissues, respectively. We evaluated the effect of transfected hsa-miR-199a-3p on the migration, invasion, and contractility of ECSCs using Transwell migration assays, in vitro wound healing assays, Transwell invasion assays, and collagen gel contraction assays. We also examined the downstream target of hsa-miR-199a-3p with an online public database search and luciferase reporter assay. Expression of hsa-miR-199a-3p in ECSCs was significantly lower than that in NESCs, whereas the expression of p21-activated kinase 4 (PAK4) mRNA was significantly higher. Transfection of hsa-miR-199a-3p inhibited the migration, invasion, and contractility of ECSCs via inhibition of PAK4 mRNA expression. PAK4 was confirmed to be the direct target of hsa-miR-199a-3p. Transfection of PAK4 small interfering RNA and the PAK4 inhibitor PF-3758309 also inhibited ECSC migration, invasion, and contractility. These findings suggest that hsa-miR-199a-3p may act as a tumor suppressor in endometriosis development. Attenuation of hsa-miR-199a-3p expression was favorable for ECSCs to acquire the highly invasive, motile, and contractile characteristics of endometriotic cells and is involved in the development of endometriosis. Accordingly, PAK4 inhibitors may be promising for the treatment of endometriosis.

Keywords Endometriosis · hsa-miR-199a-3p · p21-activated kinase 4 · Invasion · Motility · Contractility

Introduction

Endometriosis is an estrogen-dependent benign disease and occurs in 6–10% of reproductive age women [1]. Although rare, this disease is also recognized as the origin of secondary malignant ovarian neoplasms, including low-grade serous, clear cell, and endometrioid carcinomas [2]. The etiology of endometriosis has not yet been elucidated; however, emerging evidence in recent years indicates that epigenetic mechanisms, including aberrant microRNA (miRNA) expression, may be involved in its pathogenesis [3–7].

In an effort to unveil the pathogenesis of endometriosis and establish novel therapeutic agents from the view point of epigenetics, we previously investigated miRNA expression in endometriosis [3–7]. Our previous microarray study identified a number of aberrantly expressed miRNAs in endometriosis [3]. We demonstrated that the upregulation of hsa-miR-100-5p [7] and hsa-miR-210 [4] and downregulation of hsa-miR-196b [3] and hsa-miR-503 [5] contribute to the pathogenesis of endometriosis. The invasion and motility of human normal endometrial stromal cells (NESCs) are enhanced by hsa-miR-100-5p [7], while hsa-miR-210 induces vascular endothelial cell growth factor (VEGF) production and cell proliferation of NESCs and inhibits apoptosis of these cells [4]. We also

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showed that hsa-miR-196b inhibits the proliferation of human endometriotic cyst stromal cells (ECSCs) and induces apoptosis of these cells [3]. G0/G1 cell-cycle arrest and apoptosis are induced by hsa-miR-503 and VEGF production, cell proliferation, and contractility of ECSCs are inhibited [5].

Recent studies have shown that hsa-miR-199a-3p functions as a tumor suppressor in a variety of malignant tumors, such as hepatocellular carcinoma [8, 9], thyroid carcinoma [10], cutaneous squamous cell carcinoma [11], endometrioid carcinoma [12], renal cell carcinoma [13], testicular germ cell tumor [14], osteosarcoma [15], ovarian carcinoma [16], and breast carcinoma [17]. Accordingly, hsa-miR-199a-3p is downregulated in endometrial carcinoma [12], ovarian carcinoma [16], renal cell carcinoma [18], bladder carcinoma [19], prostate cancer [20], malignant melanoma [21], hepatocellular carcinoma [22], thyroid carcinoma [10], osteosarcoma [15], breast carcinoma [23], and cutaneous squamous cell carcinoma [11].

In the current study, we examined the roles of hsa-miR-199a-3p in the pathogenesis of endometriosis, which is a miRNA that is downregulated in ECSCs [3]. We evaluated the effect of transfected hsa-miR-199a-3p on the motility, invasiveness, and contractility of ECSCs and the expression of p21-activated kinase 4 (PAK4), which is a downstream target of hsa-miR-199a-3p in ECSCs. We also accessed the therapeutic potential of PAK4 inhibitors as a novel medical treatment of endometriosis.

Materials and Methods

Human ECSC and NESC Isolation Procedures and Cell Culture Conditions

Ovarian endometrioma tissues were obtained during surgery from patients who had regular menstrual cycles (n = 24, age 24–49 years), as previously described [5, 6, 24]. Clinical characteristics of the patients with endometriosis are summarized in Table 1. Normal endometrial tissues were collected during hysterectomy from patients with subserous or intramural leiomyoma and that had no evidence of endometriosis (n = 23, age 40–50 years), as previously described [24]. Low-dose oral contraceptive is the first choice for the treatment of endometriosis in Japan; however, these drugs are not familiar to the patients yet compared with those in western countries. Most of the patients were newly diagnosed and none had received hormonal treatments for at least 2 years prior to their surgery. All specimens were confirmed to be in mid-to-late proliferative phases based on pathological examination and/or menstrual records. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Oita University (registration number: P-16-01). Written informed consent was obtained from all patients.

Table 1 Clinical characteristics of 24 patients with endometriosis

Clinical measures	Number (%)
Age (year)	37.8 ± 7.2
R-AFS stage	
III	9 (37.5%)
IV	15 (62.5%)
Main symptom	
Dysmenorrhea	7 (29.2%)
Pelvic pain	5 (20.8%)
Infertility	5 (20.8%)
Hypermenorrhea	1 (4.2%)
None	6 (25.0%)
Infertility	
Present	5 (20.8%)
Absent	19 (79.2%)

ECSCs were isolated from ovarian endometrioma using enzymatic digestion as previously described [5, 6, 24, 25]. Briefly, the tissues were minced in Hanks' balanced salt solution (GIBCO-BRL, Gaithersburg, MD, USA) and digested with 0.5% collagenase (GIBCO-BRL) in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) at 37 °C for 40 min. The dispersed cells were filtered through a 70-mm nylon mesh to remove undigested tissue pieces. The filtrated fraction was then further separated from epithelial cell clumps by differential sedimentation at unit gravity as follows. The cells were resuspended in 2 ml culture medium and slowly layered over 10 ml of medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37 °C for 30 min. After sedimentation, the top 8 ml of medium was collected. The medium containing the stromal cells was then filtered through a 40-mm nylon mesh. Final purification was achieved by allowing the stromal cells, which rapidly attach to culture plate surfaces, to adhere selectively to the culture dishes for 30 min at 37 °C followed by the removal of non-adhering epithelial cells. NESCs were also isolated by digesting endometrial tissue fragments with 0.5% collagenase as previously described [5, 6, 24, 25].

Isolated ECSCs and NESCs were cultured in DMEM supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 50 mg/ml streptomycin (all from GIBCO-BRL) at 37 °C in 5% CO_2. Monolayer culture ECSCs and NESCs after the third passage were determined to be >99% by immunocytochemical staining using antibodies specific for vimentin, CD10, cytokeratin, factor VIII, and leukocyte common antigen [25] and were used for the subsequent experiments. Each experiment was performed in triplicate and repeated at least three times using cells isolated from individual patients.

miRNA Target Prediction and Pathway Analysis

To identify the candidate downstream target genes of hsa-miR-199a-3p involved in the pathogenesis of endometriosis, we searched online public databases, TargetScanHuman (http://www.targetscan.org/, Release 7.0) and miRDB (http://mirdb.org/miRDB/). PAK4 was determined to be a putative target of hsa-miR-199a-3p.

Transfection of miRNA Precursors and Small Interfering RNAs

To evaluate hsa-miR-199a-3p and PAK4 functions, miR-199a-3p precursor (Pre-miR miRNA Precursor-hsa-miR-199a-3p, Ambion, Austin, TX, USA), Negative Control Precursor miRNA (Pre-miR miRNA Precursor-Negative Control #1; Ambion), PAK4 Silencer Pre-designed siRNA (Ambion), or Silencer® Select Negative Control #1 siRNA (Ambion) were transfected into ECSCs using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and the reverse transfection method, as previously described [3–5].

Quantitative Reverse Transcription-Polymerase Chain Reaction for hsa-miR-199a-3p and PAK4

For validation of the microarray data from our previous study [3], we performed quantitative reverse transcription-polymerase chain reaction (RT-PCR) with ECSCs (n = 10 samples) and NESCs (n = 10 samples) as described previously [3–5]. Reverse primers that were hsa-miR-199a-3p-specific (assay ID: 002304, Thermo Fisher Scientific, Waltham, MA, USA) or RNU44-specific (assay ID: 001094, Applied Biosystems) were used. The expression levels of hsa-miR-199a-3p were normalized to those of RNU44, quantitated using the ΔΔCT method, and are presented as relative expression in ECSCs compared to that in NESCs.

PAK4 mRNA expression levels in ECSCs (n = 10 samples) and NESCs (n = 10 samples) were also evaluated using quantitative RT-PCR with primers specific for *PAK4* (assay ID: Hs01100061_m1, Thermo Fisher Scientific) or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; assay ID: Hs02758991_g1, Applied Biosystems). Expression levels of PAK4 mRNA relative to those of *GAPDH* mRNA were calculated using a calibration curve and are presented as relative expression in ECSCs compared to that in NESCs.

The effects of miR-199a-3p precursor and PAK4 silencer transfection of ECSCs on hsa-miR-199a-3p and *PAK4* mRNA expression, respectively, were also evaluated using quantitative RT-PCR. The data were calculated from triplicate samples of ECSCs after transfection with miR-199a-3p precursor or PAK4 and are presented as percent values relative to ECSCs transfected with their respective negative controls.

Transwell Migration Assay

Cell motility of ECSCs after transfection with miR-199a-3p precursor or PAK4 silencer was analyzed using Transwell migration assays, as previously described [26]. Briefly, ECSCs after miRNA transfection (2×10^5 cells) were cultured in DMEM supplemented with 0.1% bovine serum albumin (BSA) on Transwell inserts with an 8-μm pore-size polycarbonate membrane (6.5 mm; Corning Inc., New York, NY, USA). After 3 h of incubation, the membranes were fixed with 100% methanol and Giemsa stained. The number of cells appeared on the undersurface of the polycarbonate membranes was visually scored using a light microscope at ×200 magnification.

Transwell Invasion Assay

Invasiveness of ECSCs after transfection with miR-199a-3p precursor or PAK4 silencer was evaluated using Transwell invasion assays, as described previously [26, 27]. NESCs after transfection of miR-199a-3p precursor or PAK4 silencer (2×10^5 cells) were cultured in DMEM supplemented with 10% charcoal-stripped heat-inactivated FBS on growth factor-reduced Matrigel-coated Transwell inserts with 8-μm pores (Corning Inc.). After 48 h of incubation, the membranes were fixed with 100% methanol and Giemsa stained. The number of cells appeared on the undersurface of the polycarbonate membranes was visually scored using a light microscope at ×200 magnification.

In Vitro Wound Healing Assay

Cell motility was also evaluated using an in vitro wound healing assays, as described previously [26, 27]. ECSCs were grown to confluence in 6-well plates (Corning Inc.), challenged overnight with serum-free medium, and then transfected with miR-199a-3p precursor or PAK4 silencer. The cell monolayers were wounded using a cell scraper and incubated in DMEM plus 0.1% BSA for 48 h. Thereafter, the cells were fixed with 3% paraformaldehyde, stained with Giemsa solution, and photographed. Wound healing was assessed by calculating size of the healed area in square micrometers comparing the lesion edges at 0 h and 48 h. The lesion analysis was performed using the public domain software Image J 1.44 developed at the US National Institutes of Health (Bethesda, MD, USA).

Collagen Gel Contraction Assay

Collagen gel contraction assays were performed as previously described [5, 28]. Forty-eight hours after transfection with miR-199a-3p precursor or PAK4 silencer, ECSCs were embedded in collagen gel (Cellmatrix type I-A; Nitta Gelatin,

Osaka, Japan) and cultured three-dimensionally for an additional 48 h. Thereafter, the collagen gels were photographed and the gel surface area measured using ChemiDoc™ XRS+ with the Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

Effect of PF-3758309 on ECSC Migration, Invasion, Motility, and Contractility

We chose PF-3758309 as a representative inhibitor of PAK4 for the current study. ECSCs were subjected to Transwell migration assays, Transwell invasion assays, in vitro wound healing assays, and collagen gel contraction assays in the presence of 5 nM PF-3758309 (Selleckchem, Houston, TX, USA). All assays were performed and analyzed as described above.

Luciferase Reporter Assay

The miTarget miRNA 3′ untranslated region (UTR) Target Clone PAK4 plasmid (Cat. No. HmiT117949a-MT06) and negative control plasmid were purchased from GeneCopoeia (Rockville, MD, USA). The reporter assay was performed using a Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia) using HEK293 cells (JCRB9068, Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), according to the manufacturer's protocol. At 24-h post transfection of HEK293 cells with 3 nM pre-miR-199a precursor or negative control, the reporter plasmid was cotransfected into the cells. Luciferase activity was measured using a luminometer. The firefly luciferase activity was normalized relative to the *Renilla* luciferase activity.

Statistical Analysis

All data were obtained from triplicate samples and are presented as mean percent values ± SD relative to their corresponding controls. Data were appropriately analyzed by the Student's *t*-test using the Statistical Package for Social Science software (IBM SPSS Statistics 24; IBM, Armonk, NY, USA). p-values < 0.05 were considered statistically significant.

Results

Expression of hsa-miR-199a-3p and PAK4 in ECSCs and NESCs

To validate our previous miRNA microarray data [3], the expression levels of hsa-miR-199a-3p in NESCs and ECSCs were evaluated using quantitative RT-PCR. As shown in Fig. 1A, the relative hsa-miR-199a-3p levels in ECSCs were significantly lower than those in NESCs (46.9 ± 40.5% vs. 100 ± 40.7%, respectively, $p < 0.005$), which was consistent with our previous miRNA microarray data [2].

The expression of PAK4 mRNA in NESCs and ECSCs was also evaluated using quantitative RT-PCR. As shown in

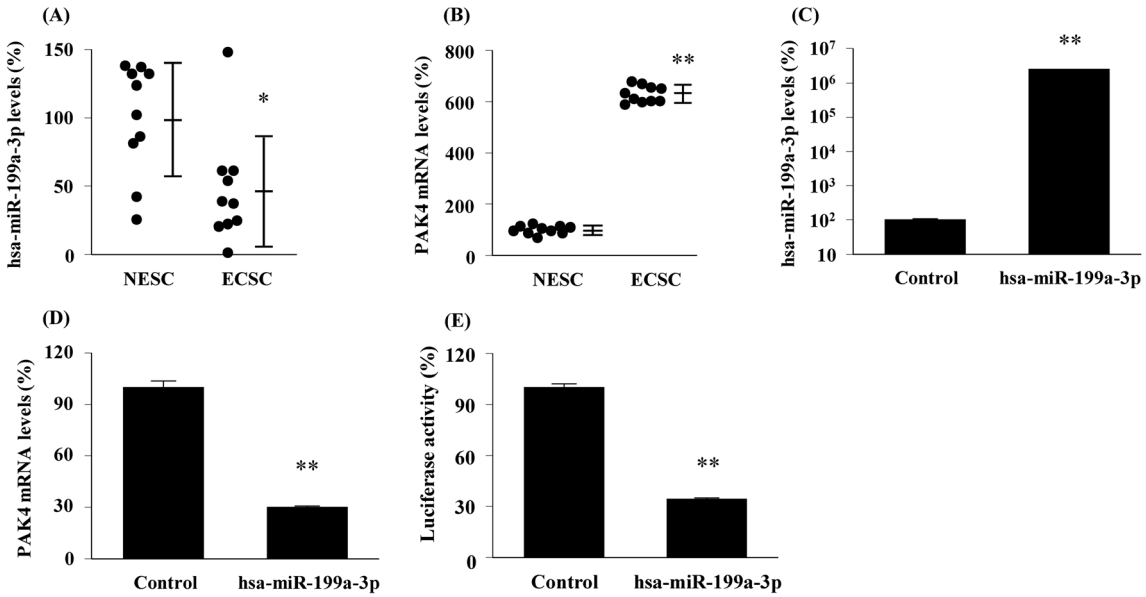

Fig. 1 hsa-miR-199a-3p and PAK4 expression in ECSCs and NESCs and the effects of miR-199a-3p precursor transfection. **A** Relative hsa-miR-199a-3p levels in ECSCs (n = 10 samples) and NESCs (n = 10 samples). **B** Relative PAK4 levels in ECSCs (n = 10 samples) and NESCs (n = 10 samples). **C** Relative hsa-miR-199a-3p levels after miR-199a-3p precursor transfection. The vertical axis is expressed as a logarithmic scale. **D** Relative PAK4 levels after miR-199a-3p precursor transfection. **E** Luciferase assay results. *$p < 0.05$, **$p < 0.005$ (Student's *t*-test). Data are shown as the mean ± SD. ECSCs, endometriotic cyst stromal cells; NESCs, normal endometrial stromal cells; PAK4, p21-activated kinase 4

Fig. 1B, the relative PAK4 mRNA levels in ECSCs were significantly higher than those in NESCs (629.3 ± 32.2% vs. 100 ± 16.1%, respectively, $p < 0.005$).

As shown in Fig. 1C, mature hsa-miR-199a-3p expression in ECSCs was significantly induced by transfection of miR-199a-3p precursor (24,500-fold increase, $p < 0.005$). Accordingly, we considered this experimental model appropriate for hsa-miR-199a-3p functional analyses. Finally, PAK4 mRNA expression in ECSCs was significantly inhibited by transfection of miR-199a-3p precursor (29.9 ± 0.7% vs. 100 ± 3.8%, respectively, $p < 0.005$, Fig. 1D).

Modulation of ECSC Cellular Functions by hsa-miR-199a-3p

We then investigated the effects of hsa-miR-199a-3p on the cellular functions of ECSCs. As shown in Fig. 2, hsa-miR-199a-3p transfection inhibited ECSC migration (106.7 ± 6.4 cells/membrane vs. 43.0 ± 9.8 cells/membrane, respectively, $p < 0.005$, Fig. 2A), invasion (1091.0 ± 85.7 cells/membrane vs. 684.0 ± 34.0 cells/membrane, respectively, $p < 0.005$, Fig. 2B), motility ($p < 0.05$, Fig. 2C), and contractility ($p < 0.005$, Fig. 2D).

Modulation of ECSC Cellular Functions by PAK4 siRNA

To confirm the hsa-miR-199a-3p functions were mediated by PAK4, we investigated the effects of PAK4 small interfering RNAs (siRNA) on the cellular functions of ECSCs. Transfection of ECSCs with PAK4 siRNA inhibited PAK4 mRNA expression ($p < 0.005$, Fig. 3A). Accordingly, we considered this experimental model appropriate for functional analyses of PAK4. As shown in Fig. 3, PAK4 siRNA transfection inhibited ECSC migration (483.0 ± 58.2 cells/membrane vs. 176.0 ± 57.9 cells/membrane, respectively, $p < 0.005$, Fig. 3B), invasion (437.0 ± 119.5 cells/membrane vs. 68.7 ± 45.6 cells/membrane, respectively, $p < 0.05$, Fig. 3C), motility ($p < 0.005$, Fig. 3D), and contractility ($p < 0.05$, Fig. 3E).

Binding of hsa-miR-199a-3p to the 3′ UTR of PAK4

The relative luciferase activity was significantly suppressed by transfection of HEK293 cells with miR-199a-3p precursor compared to cells transfected with the negative control miRNA ($p < 0.005$, Fig. 1E). This suggested hsa-miR-199a-3p targets the 3′ UTR of PAK4.

Modulation of ECSC Cellular Functions by PF-3758309

To develop a novel medical treatment of endometriosis, we accessed the effects of the PAK4 inhibitor PF-3758309 on the cellular functions of ECSCs. As shown in Fig. 4, PF-3758309 (5 nM) inhibited ECSC migration (367.0 ± 48.8 cells/membrane vs. 247.7 ± 27.8 cells/membrane, respectively, $p < 0.05$, Fig. 4A), invasion (961.0 ± 48.8 cells/membrane vs. 550.0 ± 46.9 cells/membrane, respectively, $p < 0.005$, Fig. 4B), motility ($p < 0.005$, Fig. 4C), and contractility ($p < 0.005$, Fig. 4D).

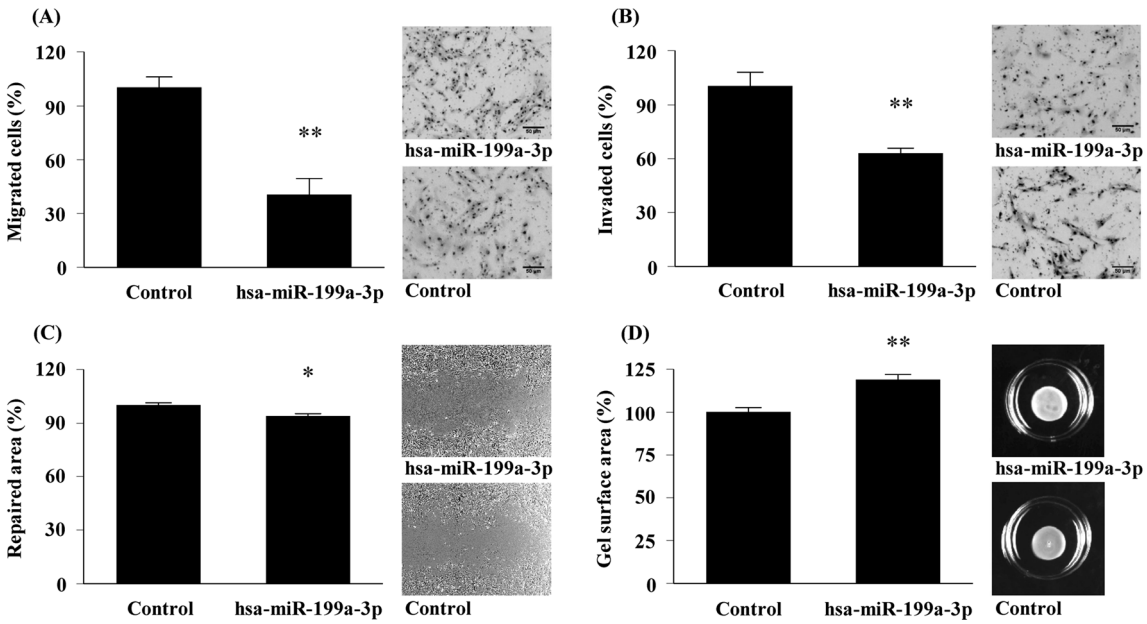

Fig. 2 hsa-miR-199a-3p expression in ECSCs and NESCs and the effects of miR-199a-3p precursor transfection. **A** Transwell migration assay results. **B** Transwell invasion assay results. **C** In vitro wound healing assay results. **D** Collagen gel contraction assay results. *$p < 0.05$, **$p < 0.005$ (Student's t-test). Data are shown as the mean ± SD. ECSCs, endometriotic cyst stromal cells; NESCs, normal endometrial stromal cells

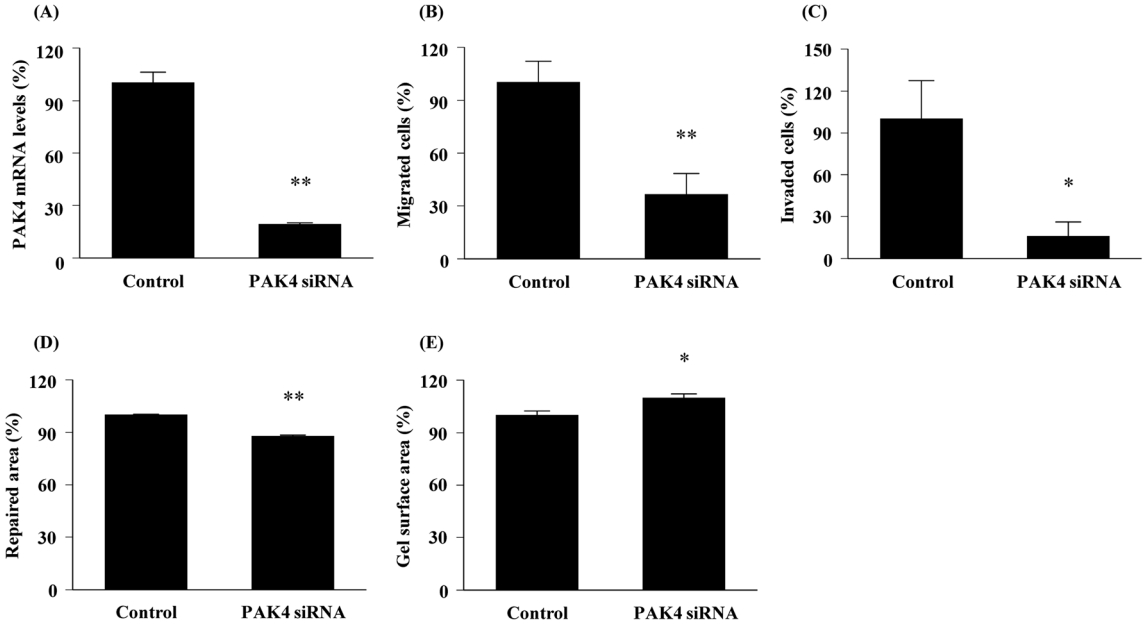

Fig. 3 Effects of PAK4 siRNA transfection on ECSC cellular functions. **A** Relative PAK4 levels after precursor PAK4 siRNA transfection. **B** Transwell migration assay results. **C** Transwell invasion assay results. **D** In vitro wound healing assay results. **E** Collagen gel contraction assay results. $*p < 0.05$, $**p < 0.005$ (Student's *t*-test). Data are shown as the mean ± SD. PAK4, p21-activated kinase 4. ECSC, endometriotic cyst stromal cell; PAK4, p21-activated kinase 4

Discussion

The results of the present study demonstrated the following: (1) Expression of hsa-miR-199a-3p in ECSCs was downregulated compared to that in NESCs; (2) expression of PAK4 mRNA in ECSCs was upregulated compared to that in NESCs; (3) hsa-miR-199a-3p transfection inhibited PAK4 mRNA expression in ECSCs; (4) hsa-miR-199a-3p transfection inhibited the migration, invasion, and contractility of ECSCs; and (5) PAK4 siRNA transfection and treatment with the PAK4 inhibitor PF-3758309 impeded these cellular functions. These findings suggest that hsa-miR-199a-3p may act

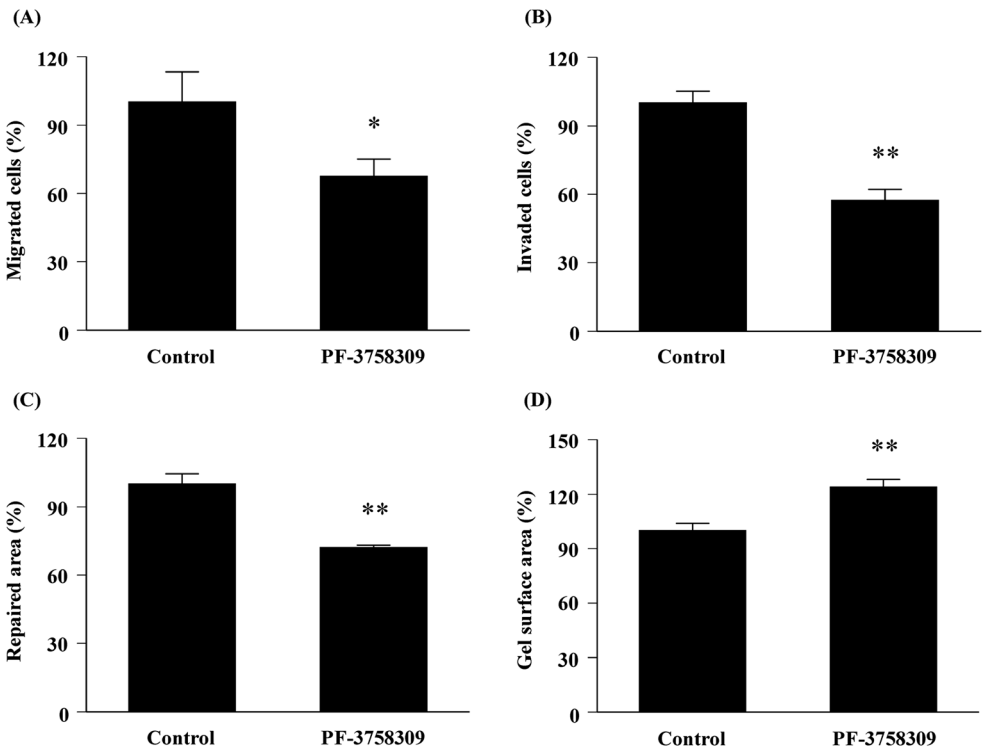

Fig. 4 Effects of PF-3758309 on ECSC cellular functions. **A** Transwell migration assay results. **B** Transwell invasion assay results. **C** In vitro wound healing assay results. **D** Collagen gel contraction assay results. $*p < 0.05$, $**p < 0.005$ (Student's *t*-test). Data are shown as the mean ± SD. ECSC, endometriotic cyst stromal cell

as a tumor suppressor in the development of endometriosis. Attenuation of hsa-miR-199a-3p expression was favorable for ECSCs to acquire the highly invasive, motile, and contractile characteristics associated with endometriosis and may be involved in the promotion of its development and progression.

In the current study, we confirmed that PAK4 is a direct target of miR-199a-3p. It has been demonstrated previously that miR-199a-3p negatively regulates PAK4 expression in breast cancer [17], esophageal cancer [29], gastric cancer [30], and hepatocellular carcinoma [9], which is consistent with our current findings. Furthermore, Dai et al. [31] found that the expression of miR-199a-3p in ovarian endometriotic cysts is significantly lower than that in eutopic endometrium with or without endometriosis, which is also consistent with our present findings. A limitation of their study was that they utilized eutopic endometrial stromal cells. As demonstrated in our current study, miR-199a-3p expression was higher in eutopic endometrial stromal cells compared to that in ovarian endometriotic stromal cells. Therefore, ECSCs are the appropriate target cells for miR-199a-3p transfection.

PAK4 belongs to the PAK family of serine/threonine protein kinases and regulates a variety of cellular functions, including cell motility, cell adhesion, cell survival, and cell proliferation [32, 33]. With respect to tumorigenesis, PAK4 acts as an oncogene by accelerating epithelial–mesenchymal transition, migration, invasion, metastasis, apoptosis, cell proliferation, and drug resistance [34]. Overexpression of PAK4 has been demonstrated in a variety of cancers, including ovarian cancer, uterine cervical cancer, breast cancer, prostate cancer, bladder cancer, gastric cancer, colorectal cancer, pancreatic cancer, and non-small cell lung cancer [35–45]. Furthermore, increased expression of PAK4 has also been reported in stromal cells of advanced-stage endometriosis [46].

PAK4 has been recognized as an attractive therapeutic target in a variety of cancers. Researchers have made advancements in the development of PAK4-specific inhibitors for use as anticancer drugs. A number of PAK4 inhibitors, such as KPT-9274 [44], KPR-7523 [44], KPT-7189 [44], PF-3758309, [44, 47], LCH-7749944 [48], CZh226 [49], fisetin [50], and LC-0882 [51], have been shown to have tumor suppressive properties. In the current study, we demonstrated that PF-3758309 was effective for the treatment of endometriosis. Further in vitro and in vivo studies are needed to confirm the therapeutic properties of PAK4 inhibitors.

In addition to the downregulation of hsa-miR-199a-3p, the expression of a number of miRNAs has been reported to be attenuated in endometriosis. These include hsa-miR-15a-5p [52], hsa-miR-16 [53], hsa-miR-23-a/b [54], hsa-miR-29c [55], hsa-miR-33b [56], hsa-miR-34c-5p [57], hsa-miR-93 [58], hsa-miR-126-5p [59], hsa-miR-141-3p [60], hsa-miR-142-3p [61], hsa-miR-145 [62], hsa-miR-183 [63], hsa-miR-196b [3], hsa-miR-199a-5p [64], hsa-miR-200-3p [65], hsa-miR-200c [66], hsa-miR-202 [67], hsa-miR-202-3p [68], hsa-miR-205-5p [69], has-miR-214 [70], hsa-miR-449b-3p [71], hsa-miR-503 [5], and hsa-miR-2861 [72]. Some of these miRNAs act as tumor suppressors and their expression is unfavorable, promoting the development and progression of endometriosis. Therefore, attenuation of miRNAs with tumor suppressor functions may help endometriotic cells transform to endometriosis-specific phenotypes with proliferative, anti-apoptotic, adhesive, motile, invasive, contractile, angiogenic, inflammatory, and estrogen-resistant properties.

A limitation of the present study is that we evaluated the roles of hsa-miR-199a-3p using only isolated stromal cells. As endometriotic tissues comprise a variety of cell types, further studies are necessary to fully define the pathogenesis of this disease. Furthermore, the stromal cells were only isolated from ovarian endometriomas. Since peritoneal endometriosis and deep infiltrative endometriosis are different entities, further studies are necessary using cells isolated from each of these lesions. Finally, the NESCs used in this study were isolated from patients with leiomyomas. The presence of leiomyoma may affect the cellular functions of endometrium; therefore, Pipelle endometrial sampling from healthy volunteers may be the most suitable to obtain truly normal endometrium. To confirm the findings of the current in vitro study, further in vivo studies are needed.

In summary, we confirmed that hsa-miR-199a-3p expression was attenuated in ECSCs. By transfecting hsa-miR-199a-3p into ECSCs, we determined that PAK4 was the direct downstream target of hsa-miR-199a-3p. Overexpression of hsa-miR-199a-3p, inhibition of PAK4 mRNA expression, or treatment with the PAK4 inhibitor PF-3758309 attenuated the migration, invasion, and contractility of ECSCs. These findings suggest hsa-miR-199a-3p is an additional miRNA whose expression is repressed in ECSCs and that is involved in ECSCs attaining endometriosis-specific features during the development and progression of endometriosis. Accordingly, PAK4 inhibitors may be useful for the treatment of endometriosis.

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Author Contribution R.Z. and K.N. participated in the study design, data analysis and interpretation, literature search, generation of figures, and writing and editing of the manuscript. N.H., M.Y., T.H., Y.A., and H.N. performed the data/case collection, experiments, data analysis, and interpretation. All authors read and approved the final manuscript.

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Declarations

Ethical Approval The study was approved by the Institutional Review Board of the Faculty of Medicine, Oita University (registration number: P-16-01).

Consent to Participate All subjects provided informed written consent.

Conflict of Interest The authors declare no competing interests.

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