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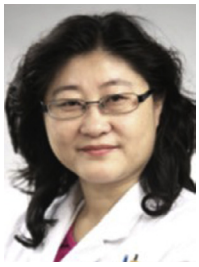
ARTICLE

Fibrinogen alpha chain is up-regulated and affects the pathogenesis of endometriosis



BIOGRAPHY

Xiaohong Chang received her PhD in obstetrics and gynaecology from Peking University, China, in 2003. Since then she has worked at the Peking University People's Hospital. Her research has focused on molecular mechanisms in endometriosis and ovarian cancer.

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KEY MESSAGE

High expression of fibrinogen alpha chain (FGA) in endometriosis is closely related to disease severity. The expression level of FGA in eutopic endometrial stromal cells may affect a cell's stromal phenotype, causing cytoskeletal remodelling and reducing the expression of MMP-2, which might play an important role in the pathogenesis of endometriosis.

ABSTRACT

Research question: In the group's previous study, fibrinogen alpha chain (FGA) was identified as an up-regulated differential protein that was highly expressed in women with endometriosis. The current study investigated the expression and effects of FGA in endometriosis. It also evaluated the effects of FGA on human endometrial stromal cells and studied the possible mechanism.

Design: This was a cross-sectional analysis of FGA expression in plasma and endometrial tissue of matched eutopic and ectopic samples from women with endometriosis undergoing laparoscopic surgery and samples from women without endometriosis. Forty-four patients with endometriosis and 32 healthy control subjects who donated plasma for FGA analysis, including 26 matched cases of eutopic and ectopic endometria from endometriosis patients and 22 endometria from healthy control subjects, were analysed. The effects of FGA were studied in a human endometrial stromal cell line after transfection with FGA short interfering RNA (siRNA).

Results: FGA concentrations in serum and expression in eutopic and ectopic endometrial tissue were significantly higher in women with endometriosis than controls ($P < 0.05$ and $P < 0.01$ respectively), whereas FGA expression was not significantly different in eutopic compared with ectopic endometrial tissues from the same patients. High FGA concentrations in serum were related to disease stage and ovarian involvement, but were not affected by age and menstrual cycle. The knockdown of FGA expression by FGA siRNA inhibited hEM15A cellular adhesion, migration and invasion, and attenuated matrix metalloproteinase-2 (MMP-2) expression.

Conclusions: High FGA expression in endometriosis was closely related to disease severity and affected cell adhesion, migration and invasion, which might play an important role in the pathogenesis of endometriosis.

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KEY WORDS
Endometriosis
Fibrinogen alpha chain
Invasion
Migration

INTRODUCTION

Endometriosis affects more than 10% of women of reproductive age. This disease often causes pelvic pain and infertility, with consequences that are not only physical, but also psychological and social in nature. Although the exact origin and pathophysiology of endometriosis remains unclear, it is widely accepted that endometriosis tissue originates from retrograde menstruation. Although retrograde menstruation, which is a common physiological process, occurs in more than 90% of menstruating women with patent fallopian tubes, endometriosis only affects approximately 10% of women of reproductive age (*Halme et al., 1984*; Krina et al., 2018), indicating that other factors may contribute to the development of endometriosis (*Li et al., 2013*; *Reis et al., 2013*). Recent studies have postulated that eutopic endometria appear to be genetically and biochemically different from normal endometria, which facilitates complete adhesion, invasion and angiogenesis, and ultimately the development of endometriosis (*Baranov et al., 2018*; *Liu and Lang, 2011*; *Matson et al., 2018*; *Vallvé-Juanico et al., 2017*). Even though endometriosis is defined as a benign disease, it exhibits many features similar to those of malignancy, such as distant foci and invasion of other tissue, with the subsequent damage that produces endometriosis (Ayala et al., 2007).

The current group previously identified a cluster of five peptides (4210, 5264, 2660, 5635 and 5904 Da), using three of these (4210, 5904 and 2660 Da) to discriminate women with endometriosis from healthy volunteers. One of these peptides, fibrinogen alpha chain (FGA), was shown to differ most between patients with endometriosis and controls (*Zhao et al., 2015*). FGA is one of three types of non-identical polypeptide chain consisting of fibrinogen as a cell adhesion molecule, which contains two arginyl-glycyl-aspartic acid (RGD) cell adhesion motifs. The RGD motif is the binding site for integrin $\alpha v\beta 3$ to mediate adhesion and migration between cells or extracellular matrix (ECM), and for signal transduction (*Nieberler et al., 2017*). Integrins are cell surface adhesion receptors that promote cell–cell, cell–matrix and cell–pathogen interactions, which play key roles in the attachment of cells to the ECM and are involved in cell–cell adhesion (*Xin et al., 2015*; *Wu et al., 2005*). Integrins may promote attachment of ectopic endometrial cells to the peritoneum. Integrins $\beta 1$ and $\beta 5$ were present in endometriosis lesions in a nude mouse model and shown to be of peritoneal origin (Osorio et al., 2014). However, the relationship between FGA and integrins in the pathogenesis of endometriosis remains unknown.

Nothnick and colleagues, using a mouse model, reported that a loss of microRNA miR-451 expression resulted in a lower number of ectopic lesions being established. Analysis of differential protein expression showed that the concentration of fibrinogen alpha polypeptide isoform 2 precursor was lower in miR-451-deficient than wild-type endometrial fragments. Fibrinogen alpha polypeptide isoform 2 precursor was associated with altered expression of the parent FGA mRNA and protein. This study revealed that FGA expression may play a role in the process of endometriosis (*Nothnick et al., 2014*). Above all, it suggested that FGA may play a role in the initial development of retrogradely shed endometrial fragments into endometriosis. More recently, several studies have reported that FGA expression increases during tumour progression as an ongoing inflammatory response to the tumour (*Bai et al., 2014*; *Shi et al., 2018*; *Tao et al., 2012*; *Yang et al., 2016*). Dalenc and colleagues reported that, compared with breast cancer patients who benefited from combined tipifarnib plus tamoxifen therapy after 8 weeks, FGA concentrations were significantly increased in patients exhibiting tumour progression (*Dalenc et al., 2010*).

Despite the above evidence, studies about the relationship between FGA and endometriosis are very limited. Until now, the role of FGA in endometriosis has been reported in only a single study (*Nothnick et al., 2014*). The effects of FGA in mouse endometrium have only been studied with regard to the formation of ectopic foci, and its other roles are poorly understood. The authors postulate that aberrant expression of FGA may be associated with the clinical parameters of endometriosis and affect cellular biological functions in the development of the condition. The study therefore aimed to investigate FGA expression A in both serum and endometrium from women with endometriosis, the relationship between FGA and the clinical characteristics and symptoms of endometriosis patients, and the effects of FGA on migration, invasion, adhesion and proliferation of endometrial stromal cells (ESC), as well as to further study its mechanism.

MATERIALS AND METHODS

Sample collection

Thirty-two asymptomatic women aged between 18 and 50 years with regular menstruation and normal transvaginal ultrasound results were recruited as healthy controls. The blood samples from the healthy controls were collected in all phases of the menstrual cycle. Forty-four patients aged between 18 and 50 years old with endometriosis that had been both surgically and histologically confirmed at Peking University People's Hospital donated blood samples, collected at the proliferative or secretory phase. Details of age, infertility, dysmenorrhoea, location of the ovarian lesions, peritoneal lesions, the revised American Fertility Society (rAFS) stage (American Fertility Society, *1985*) and recurrence followed up for 3 years were also collected.

In addition, tissue specimens were collected from another group of patients. The age of patients and controls ranged from 18 to 50 years. Matched eutopic and ectopic endometrial tissues were collected from 26 patients with surgically and histologically confirmed disease. Fifteen of the 26 endometrial samples for areas of endometriosis were taken in the proliferative phase, and the others during the secretory phase. Endometrial biopsy specimens were collected from 22 women with tubal infertility and confirmed by laparoscopy as having no areas of endometriosis; nine of the samples were from the proliferative phase and 13 from the secretory phase. The tissue samples were collected during the proliferative or secretory phase of the menstrual cycle. No participants were pregnant, were undergoing menopause or had a history of cancer, and none had used corticosteroid hormones within 3 months prior to study enrollment.

This study was approved by the Ethics Committee of the Peking University People's Hospital (2016PHB165-01). Informed consent was obtained from all enrolled individuals.

Enzyme-linked immunosorbent assay

Serum FGA concentrations were quantified in triplicate using the FGA enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life, China) following the manufacturer's protocol. The minimum detectable concentration of FGA using this kit is 6.5 ng/ml. The intra-assay coefficient of variation was less than 10%, and the inter-assay coefficient of variation less than 12%. Prior to each immunoassay, the serum samples were diluted 1:25 using the sample buffer provided.

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissue samples were sliced into 5 μm thick sections. The tissue slides were deparaffinized by xylene and hydrated with graded ethanol. The tissue slides were incubated with fresh prewarmed antigen retrieval buffer sodium citrate, and the buffer was heated to boiling. The tissue slides were then equilibrated to room temperature for 30 min. After being blocked by 10% goat serum for 30 min at 37°C, the tissue slides were incubated overnight at 4°C with a 1:100 dilution of primary rabbit polyclonal anti-FGA antibody (Santa Cruz, USA) in phosphate-buffered saline (PBS).

Next, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min at 37°C and visualized using diaminobenzamidine (DAB). The nuclei were counterstained with haematoxylin. For the negative controls, the primary antibody was replaced by non-immune rabbit serum (1:200 dilution; ZSGB-Bio, China). Cells exhibiting positive staining were counted in at least 10 representative fields (× 400 magnification), and the mean percentage of positive cells was calculated. The expression of FGA was evaluated based on the staining intensity and positive expression area. Five fields (× 400 magnification) were observed continuously under the microscope, and the cell count applied was 100.

The scoring method was as follows. No staining scored 0 points, light brown 1 point, brown 2 points, and dark brown 3 points. Fewer than 5% coloured cells scored 0 point, 6–25% 1 point, 26–50% 2 points, and 51% or more 3 points. Each tissue slice was evaluated for the degree of staining, and the score for average percentage of coloured cells was multiplied by the final score; less than 1 point was classified as negative (–), 1–2 points as positive (+), 3–5 points as ++, and 6 or more as +++. Immunostaining was assessed by two independent pathologists blinded to the clinical characteristics.

Cell culture and transfection

hEM15A is an immortalized stromal cell line derived from the eutopic endometrial tissue of endometriosis patients that is preserved at the China Center for Type Culture Collection and Cell Resource Center of Peking Union Medical (*Chen et al., 2016*). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics at 37°C in a humidified atmosphere with 5% carbon dioxide. hEM15A cells were seeded in culture media 24 h before transfection into six-well plates (2 × 105 cells per well) for western blotting and quantitative real-time reverse transcription (RT)-PCR. Short interfering RNA (siRNA) transfection was carried out in serum-free DMEM/F-12 by using Lipofectamine 3000 (Invitrogen, USA) and 20 nmol/l siRNA. Three different siRNA targeted to FGA and negative control siRNA were synthesized in GenePharma (Suzhou, China). The sequences are listed in Supplementary Table 1. siRNA were transfected into hEM15A cells using Lipofectamine 3000 (Invitrogen). Control siRNA or FGA siRNA were mixed into the culture media and incubated for 24 h for quantitative real-time RT-PCR or for 48 h for western blotting.

Western blot analysis

After transfection for 48 h, cells were washed with PBS and lysed using RIPA buffer (Cell Signaling Technology, USA) supplemented with phenylmethylsulphonyl fluoride (PMSF; Cell Signaling Technology) on ice in line with the manufacturer's instruction. Protein concentrations were determined using Coomassie Brilliant Blue (Bio-Rad, USA). Equal amounts of protein (25 μg) from hEM15A transfected with siRNA were subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (0.45 mm, Millipore, USA). After blocking for 1 h at room temperature with blocking buffer (0.1% Tris-buffered saline with Tween [TBST] with 5% fat-free milk powder), the blots were incubated with primary antibodies against FGA (diluted 1:1000, rabbit; Santa Cruz), E-cadherin (diluted 1:1000, rabbit; Cell Signaling Technology), N-cadherin (diluted 1:1000, rabbit; Abcam, USA), integrin αv (diluted 1;1000, rabbit; Cell Signaling Technology), integrin β3 (diluted 1:1000, rabbit; Cell Signaling Technology), matrix metalloproteinase-2 (MMP-2; diluted 1:1000, rabbit; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; diluted 1:1000, rabbit; Abcam) at 4°C overnight. The membranes were washed three times with TBST, for 10 min each time. The target proteins were visualized by the chemiluminescent gel imaging system (Bio-Rad, USA) after incubation for 60 min at room temperature with a secondary antibody (diluted 1:1000, goat; ZSGB-Bio). Protein expression was quantitatively analysed by

TABLE 1 SERUM FGA CONCENTRATION OF HEALTHY CONTROLS

	Number	Median (ng/ml)	Range (ng/ml)
Age (years)			
20–29	11	300.385	164.155–533.698
30–39	11	277.927	173.291–597.066
40–50	10	307.699	161.828–545.135
Cycle phase			
Menstrual	32	261.375	161.828–488.508
Proliferative	32	319.459	197.790–597.066
Secretory	32	288.804	179.809–542.787

No significant differences were found among any of the subgroups.

FGA, fibrinogen alpha chain.

densitometry and values normalized to those of GAPDH.

Real-time PCR analysis

Total RNA from hEM15A cells transfected with siRNA was isolated using TRIzol reagent (Invitrogen). The cDNA were synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with random primers. Real-time quantitative PCR was carried out using 2 × Taq PCR Master Mix (Tiangen Bio, China) in a 7900 Real-Time PCR System (Applied Biosystems, USA). The sequences of FGA and GAPDH primers used in this study are shown in Supplementary Table 2.

Cell proliferation and viability assay

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) according to the manufacturer's instruction. After being transfected with siRNAs, hEM15A cells were plated in 96-well pates at a density of 3000 cells in 100 µl of culture medium per well day 1. After a culture time of 0, 24 and 48 h, 10 µl of CCK-8 was added for an additional 2 h of incubation. Absorbance at 450 nm was measured to calculate the number of viable cells in each well. Absorbance relative to the blank control (hEM15A cells plus culture medium) at time 0 h was used to calculate the relative cell proliferation rate. Each experiment was repeated five times.

Apoptosis assay

hEM15A cells transfected with siRNAs 48 h were stained with an Annexin V/FITC kit (BD Biosciences, USA) and propidium iodide (BD Biosciences). Fluorescence-activated cell sorting was performed according to the manufacturer's instructions. Cells labelled with annexin V were considered apoptotic, and propidium iodide was used to detect dead cells. The apoptotic rate was the sum of annexin V-positive/propidium iodide-negative and annexin V-positive/propidium iodide-positive cells in two quadrants as a proportion of the total number of cells. Fluorescence-activated cells were quantified using a flow cytometer. Each experiment was performed in triplicate.

TABLE 2 CLINICAL RELEVANCE OF SERUM FGA CONCENTRATION IN ENDOMETRIOSIS PATIENTS

Characteristic	Number	FGA (ng/ml)	*P*-value
Age			
<35	19	382.61 (241.21–902.95)	NS
≥35	25	401.33 (242.90–573.45)	
Menstrual cycle			
Proliferative Proliferative phase	23	456.92 (241.21–902.95)	NS
Secretory phase	21	370.55 (242.90–522.73)	
Dysmenorrhoea			
Yes	25	372.77 (242.90–522.73)	NS
No	19	437.95 (241.21–902.95)	
Infertility			
Yes	8	347.22 (275.58–902.95)	NS
No	27	393.10 (241.21–594.64)	
Ovarian lesion			
Unilateral	28	348.98 (156.83–672.16)	0.010
Bilateral	16	464.52 (332.06–902.95)	
Peritoneal lesion			
Yes	24	431.0 (275.58–902.95)	NS
No	20	382.94 (241.21–522.73)	
rAFS stage			
I–II	13	305.19 (242.90–485.62)	0.003
III–IV	31	463.46 (241.21–902.95)	
Recurrence			
Yes	10	491.95 (275.58–594.64)	NS
No	26	377.69 (241.21–902.95)	

Results are given as median (range). rAFS stage: revised American Fertility Society stage.
NS, not statistically significant.

Human umbilical vein endothelial cell adhesion assay

hEM15A cells were pre-treated with CellTracker (Invitrogen) diluted with PBS at 3:1000 for 30 min. After washing three times with PBS, the labelled hEM15A cells were seeded in six-well plates and transfected with siRNA for 48 h. Human umbilical vein endothelial cells (HUVEC) were cultured in six-well plates and allowed to grow to confluence. A total of 2×10^4 CellTracker-treated hEM15A cells were added to the wells pre-coated with HUVEC cells for 30 min at 37°C. The wells were then gently washed with PBS to remove non-adherent cells. Five images were collected, and the number of hEM15A cells adhering to the HUVEC was then quantified using ImageJ software (NIH, USA).

Wound healing assay

Wound healing assays were performed to evaluate the cells' ability to migrate. After transfection with siRNA for 6 h, a total of 1×10^6 hEM15A cells were seeded into a six-well plate in culture medium. Cells grew to confluence 24 h later. Negative control siRNA or siRNA were added to the of hEM15A cell medium after creating an artificial wound in each well using a P10 pipette tip. The areas of the wounds were measured at 0 and 24 h with a microscope (Leica, Germany). The wound area was calculated using Image J software and the migration rate calculated (wound area after/before × 100). The assay was repeated three times.

Transwell assays

hEM15A cells transfected with siRNA or negative control siRNA were harvested, washed and suspended in DMEM/F-12 with 2×10^4 cells seeded to the upper chambers of Transwell inserts (8 µm pore; Corning, USA) in the migration assay. The upper chambers were coated with Matrigel (BD Biosciences) prior to inoculation with 2×10^4 hEM15A cells in the invasion assay. In both experiments, the lower compartments were filled with DMEM/F-12 with 5% FBS. Cells in the upper chamber were removed with a swab after incubation for 12 h. The cells

that migrated to or invaded the lower layer and attached to the membrane were stained with crystal violet and counted in five fields per well under a microscope (Leica DM IL LED; Wetzlar, Germany). The assays were repeated three times.

Cytoskeleton staining

The cytoskeleton and nuclei of hEM15A cells were stained with 50 μg/ml phalloidin (AAT Bioquest, USA) for 60 min to stain F-actin, and 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, respectively. Images were taken using a confocal microscope (FV1000; Olympus, Japan).

Transmission electron microscopy

hEM15A cells were digested with 0.25% trypsin and suspended at a concentration of 1.0×10^6/ml in 1.5% glutaraldehyde, and fixation was carried out at 4°C for 6 h with 1.5% glutaraldehyde. Later, ultrathin (100 nm) sections were prepared, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (H-600; Hitachi, Japan).

Statistical analysis

Statistical processing was performed using SPSS V21 software (IBM, USA). Serum FGA concentrations exhibited a skewed distribution so data were expressed as medians and ranges. The differences in FGA concentrations between groups were analysed using the Mann–Whitney rank sum test. The chi-squared test was used to analyse the results of immunohistochemical staining. A *P*-value <0.05 was considered to represent a statistically significant difference.

RESULTS

Expression of FGA in serum and tissue from healthy controls and endometriosis patients

To evaluate the expression profile of FGA in women with endometriosis and healthy controls, ELISA and immunohistochemistry were performed on serum and endometrial tissues, respectively. In healthy controls, the relationship between serum FGA concentrations and age and phase of the menstrual cycle were examined. The results are summarized in TABLE 1. The subgroup analysis revealed no significant differences among any of these subgroups. It was then verified that serum FGA concentration was increased in women with endometriosis (median [range]: 397.36 [156.83–902.95] ng/ml) compared with controls in the proliferative phase (288.80 [179.81–542.79] ng/ml; $P < 0.05$; FIGURE 1).

Next, the serum FGA concentrations of patients with stage I–II ($n = 13$), and stage III–IV ($n = 31$) endometriosis were compared with those of healthy controls ($n = 32$). Women with stage III–IV endometriosis had significantly elevated FGA concentrations ($P < 0.05$) compared with those with stage I–II endometriosis (463.46 [371.66–584.05] ng/ml versus 305.19 [292.43–353.29] ng/ml, respectively; $P < 0.05$; FIGURE 1, TABLE 2). No significant difference in serum FGA concentration was found for women with stage I–II endometriosis versus healthy controls (FIGURE 1).

Furthermore, the clinical relevance of serum FGA concentrations in women with endometriosis was analysed (TABLE 2). The results showed that patients with bilateral ovarian lesions had higher serum FGA concentrations than those with unilateral ovarian lesions (464.52 [383.08–654.44] ng/ml versus 348.98 [294.51–494.16] ng/ml, respectively; $P = 0.010$). After 3 years of follow-up, excluding eight patients who did not receive gonadotrophin-releasing hormone analogue (GnRHa) after surgery, the 10 patients who showed disease recurrence had a serum FGA concentration of 491.95 (401.33–594.64) ng/ml, compared with 377.69 (306.13–490.04) ng/ml in patients without recurrence. The difference failed to reach statistical significance ($P = 0.056$). Excluding nine patients who had not attempted to conceive, serum FGA concentrations were not significantly different between infertile and non-infertile patients. There were also no significant differences between the analysed groups based on age, phase of the menstrual cycle, dysmenorrhoea, infertility and whether or not there was a peritoneal lesion.

To determine whether there was also a difference in the expression level of FGA protein at the tissue level, immunohistochemistry was performed to study the location and expression of FGA in 26 matched eutopic and ectopic endometrium samples from endometriosis patients and 22 control endometria. The staining results showed that FGA was expressed in the cytoplasm of stromal cells and glandular epithelial cells (FIGURE 2). The expression of FGA was slightly stronger in stromal cells than glandular epithelial cells. The eutopic endometria and ectopic lesions of endometriosis patients showed mainly positive to strong positive staining, and there was no statistical difference between the two groups (TABLE 3). The majority of control endometria showed weak positive staining. Overall, as shown by staining, FGA expression in control endometria was significantly lower than in eutopic endometrium and ectopic lesions ($P < 0.01$) (TABLE 3). Furthermore, there was no statistical difference in staining intensity between different phases of the menstrual cycle in control endometrium, eutopic endometrium and ectopic lesions. However, the intensity of FGA staining in the eutopic endometrium and ectopic endometrium of patients with stage III–IV endometriosis

TABLE 3 EXPRESSION OF FGA IN ENDOMETRIAL TISSUE

Group		N	Staining intensity (%)			
			–	+	++	+++
CoE		22	4 (18.2%)	15 (68.2%)	3 (13.6%)	0 (0%)
EuE		26	0 (0%)	5 (19.2%)	10 (38.5%)	11 (42.3%)
EcE		26	0 (0%)	5 (19.2%)	12 (46.2%)	9 (34.6%)
EuE	I–II	7	0 (0%)	3 (42.9%)	3 (42.9%)	1 (14.3%)
	III–IV	19	0 (0%)	2 (10.5%)	7 (36.4%)	10 (52.6%)
EcE	I–II	7	0 (0%)	3 (42.9%)	4 (57.1%)	0 (0%)
	III–IV	19	0 (0%)	2 (10.5%)	8 (42.1%)	9 (47.3%)

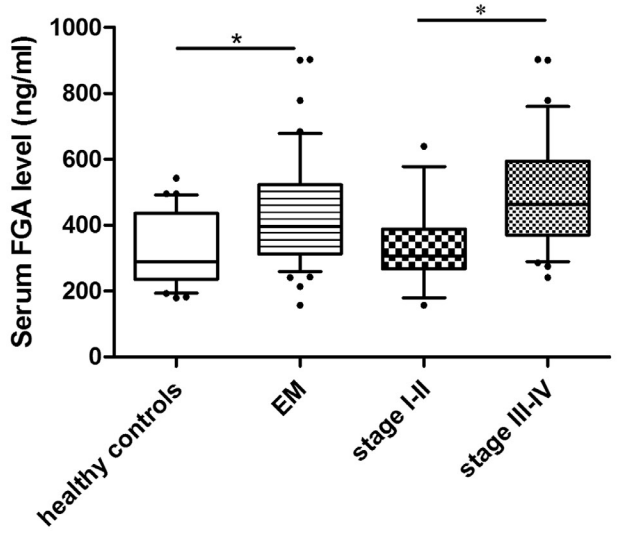
Fibrinogen alpha chain (FGA) expression in control endometria was significantly lower than in patients' eutopic endometrium and ectopic lesions ($P < 0.01$). The FGA expression in the eutopic endometria ($P < 0.05$) and ectopic endometria ($P < 0.05$) of patients with stage III–IV endometriosis was higher than in patients with stage I–II endometriosis. Staining intensity is indicated as negative (–), weakly positive (+), positive (++) or strongly positive (+++).
CoE, control endometrium; EuE: eutopic endometrium; EcE: ectopic endometrium.

A

Serum FGA level of healthy controls

	Number	Median (ng/mL)	Range (ng/mL)
Age			
20–30	11	300.385	164.155–533.698
30–40	11	277.927	173.291–597.066
40–50	10	307.699	161.828–545.135
Cycle phase			
Menstrual	32	261.375	161.828–488.508
Proliferative	32	319.459	197.790–597.066
Secretory	32	288.804	179.809–542.787

B



C

Clinical relevance between serum FGA level and EM

Characteristic	Number	FGA (ng/ml)	P value
Age			
<35	19	382.61	0.878
⩾35	25	401.33	
Menstrual cycle			
Proliferative	23	456.92	0.318
Secretory	21	370.55	
Dysmenorrhea			
Yes	25	372.77	0.132
No	19	437.95	
Infertility			
Yes	8	347.22	0.637
No	27	393.10	
Ovarian lesion			
Unilateral	28	348.98	0.010 *
Bilateral	16	464.52	
Peritoneal lesion			
Yes	24	431.05	0.759
No	20	382.94	
rAFS stage			
I–II	13	305.19	0.003 *
III–IV	31	463.46	
Recurrence			
Yes	10	491.95	0.056
No	26	377.69	

FIGURE 1 Serum concentration of fibrinogen alpha chain (FGA) was significantly higher in women with endometriosis (n = 44; samples taken in the proliferative or secretory phase) than healthy controls (n = 32; three samples per woman, one each during the menstrual, proliferative and secretory phases of the menstrual cycle). Serum FGA concentration stratified in patients according to rAFS stages was significantly higher in stage III–IV (n = 31) than stage I–II (n = 13) endometriosis. rAFS stage: revised American Fertility Society stage. Boxplots display median value, interquartile range and 5th and 95th percentiles. *P < 0.05.

was higher than that of patients with stage I–II endometriosis (both P < 0.05; FIGURE 2, TABLE 3).

Effects of FGA on proliferation and apoptosis in hEM15A cells

To explore the role of FGA in the development of endometriosis, the eutopic endometrial stromal cell line hEM15A was transfected with three siRNA sequences targeting FGA. FIGURE 3A shows the down-regulation of FGA mRNA and protein, according to which the second siRNA (siRNA2) was selected for further investigations. No difference in cell viability (FIGURE 3B) and apoptosis (FIGURE 3C) was observed by CCK-8 assay and flow cytometry in hEM15A cells after transfection by siRNA2 compared with the negative control.

Effects of FGA on adhesion, migration and invasion in hEM15A cells

hEM15A cells display migration capacities that can be attenuated by transfection with siRNA, as determined by the wound healing assay (FIGURE 4A) and the transwell assay in wells without (FIGURE 4B). Transfection with siRNA also markedly decreased the invasive abilities of hEM15A cells in wells with Matrigel (FIGURE 4B). Moreover, in the HUVEC adhesion assay, down-regulation of FGA was able to effectively suppress the adhesion of hEM15A cells to HUVEC (FIGURE 4C).

Effects of FGA on related molecule expression and ultrastructure of hEM15A cells

The regulation of expression of adhesion-related molecules integrin αv

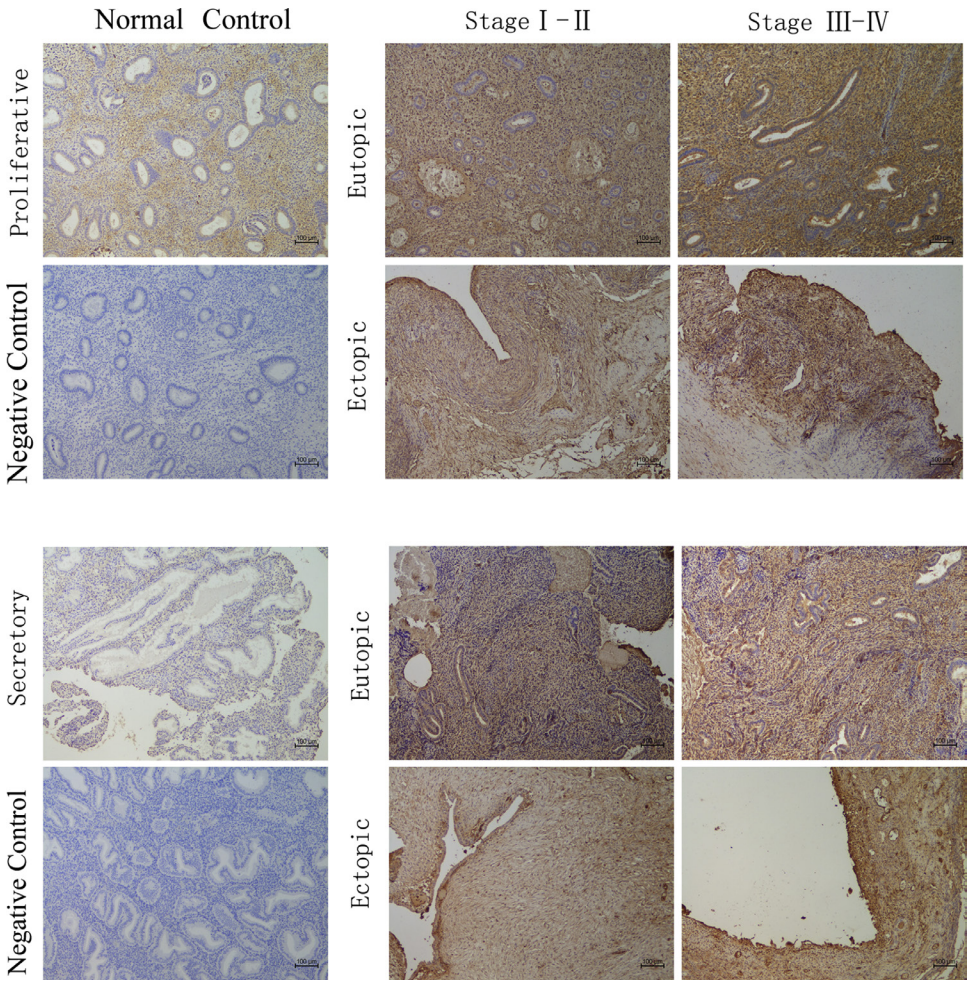


FIGURE 2 Expression of fibrinogen alpha chain (FGA) in control, eutopic and ectopic endometria was examined by immunohistochemistry in proliferative phase and secretory phase endometrial tissue samples collected from 22 control women with tubal infertility without endometriosis and 26 women with endometriosis. Typical examples are shown for each case. For the negative control, the primary antibody was replaced with non-immune rabbit serum.

and β3, migration-related molecules E-cadherin and N-cadherin, and invasion-related molecule MMP-2 by FGA was further investigated. hEM15A cells were transfected with siRNA for 72 h before protein concentrations were measured. Western blot analysis showed that N-cadherin and MMP-2 were down-regulated, and E-cadherin was up-regulated, while concentrations of integrin αν and β3 did not change significantly (FIGURE 5A).

The results of cytoskeleton microfilament staining showed that the microfilaments in the negative control group ran parallel to the long axis of the cells and were neatly arranged. The filopodia between cells were obvious, and the lamellipodia were visible on the cell surfaces. The microfilaments were thickened and increased in siRNA-treated cells, while the filopodia and lamellipodia were notably reduced, further indicating that down-regulation of FGA expression can inhibit the migration ability of endometrial cells (FIGURE 5B).

Using a transmission electron microscope, it was observed that surface pseudopods were more common in the negative control group. Their nuclei were irregularly shaped with a notch, usually lobulated, and the nucleolus was obvious. The surface of the cells treated with siRNA2 had fewer pseudopods (FIGURE 5C).

DISCUSSION

This study has been the first to explore the clinical relevance of FGA in endometriosis and its possible role in pathogenesis. The high serum concentration and endometrial tissue expression of FGA was closely related to the severity of disease. Down-regulation of the expression level of FGA in eutopic endometrial stromal hEM15A cells may attenuate cellar migration and adhesion ability by affecting the cells' stromal phenotype, causing cytoskeletal remodelling and reducing the expression of MMP-2. The present findings provide novel insights into the biological function of FGA in endometriosis.

Accumulating evidence suggests that an up-regulated serum concentration of FGA is involved in various malignant neoplasms, such as breast cancer, gastric cancer, oesophageal squamous cell carcinoma and renal cell cancer (*Bai et al., 2014*; *Shi et al., 2018*; *Tao et al., 2012*; *Yang et al., 2016*). Although endometriosis is a benign disease,

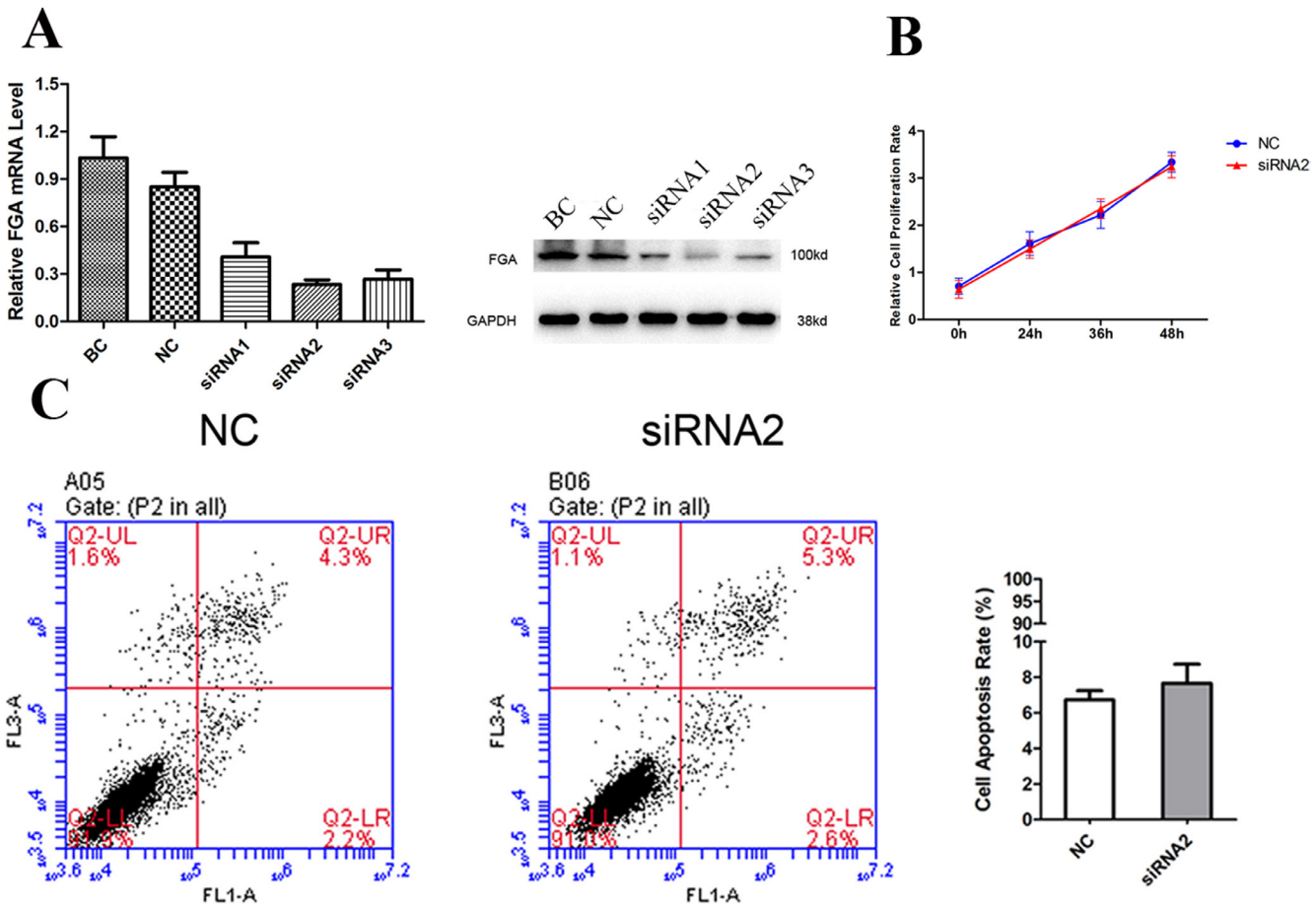


FIGURE 3 The effect on cell viability and apoptosis of fibrinogen alpha chain (FGA) concentration in the hEM15A cell line. (A) Down-regulation of FGA mRNA assessed by real-time polymerase chain reaction (left panel) and protein assessed by western blotting (right panel) after transfection with three short interfering RNA (siRNA) or the negative control (NC). The second siRNA (siRNA2) was selected for further investigations. mRNA expression is shown as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. BC, blank control without siRNA. (B) Cell viability was measured up to 48 h after transfection with short interfering RNA (siRNA2) or the negative control using the Cell Counting Kit-8 (CCK-8) assay. (C) Apoptosis was measured by flow cytometry in hEM15A cells 48 h after transfection with siRNA2. No significant differences were found in cell viability and apoptosis between cells transfected with siRNA2 and negative controls. Values are presented as mean ± SD. Experiments were performed in triplicate.

it exhibits many features similar to those of malignancy. Whether FGA is in involved in the pathophysiology of endometriosis remains unknown. This group's previous study, using surface-enhanced laser desorption/ionization time-of-light-mass spectrometry (SELDI-TOF MS), indicated that FGA was highly expressed in the serum of women with endometriosis . In this study, the expression of serum FGA was further confirmed by ELISA. The results indicated that the serum concentration of FGA was significantly higher in endometriosis patients than in healthy controls, and serum FGA concentrations were significantly higher in women with stage III–IV disease than in those in stage I–II. The findings are consistent with the group's previous study (*Zhao et al., 2015*).

One theory of the pathogenesis of endometriosis states that endometrial cells and tissue fragments travel from the uterine cavity through lymphatic channels and veins to colonize distant ectopic sites. The high serum concentration of FGA in endometriosis patients may be due to the interaction between circulating endometrial cells, endothelial cells and platelets (*Chen et al., 2017*). Moreover, in the current study, 3 years after surgery the FGA concentrations of women with recurrence of endometriosis were apparently higher than in those without disease recurrence; however, the difference failed to reach statistical significance, which might be related to the small sample size remaining after exclusion of data from patients who were not treated with GnRHa after surgery. Further studies are needed to examine whether increased FGA expression is an important promoting factor in the occurrence and progression of endometriosis, which could be an important explanation for the similar behaviour observed in the progression and metastasis of some tumours (*Palumbo et al., 2000*).

FGA protein was subsequently evaluated in ovarian endometriosis, eutopic endometria and normal endometrial tissue samples. FGA was significantly increased in ovarian endometriosis and eutopic endometrium compared with normal endometria. Concentrations of FGA protein was significantly higher in both eutopic and ectopic endometrium in women with stage III–IV than stage I–II endometriosis. This result is consistent with the results of serological tests. The

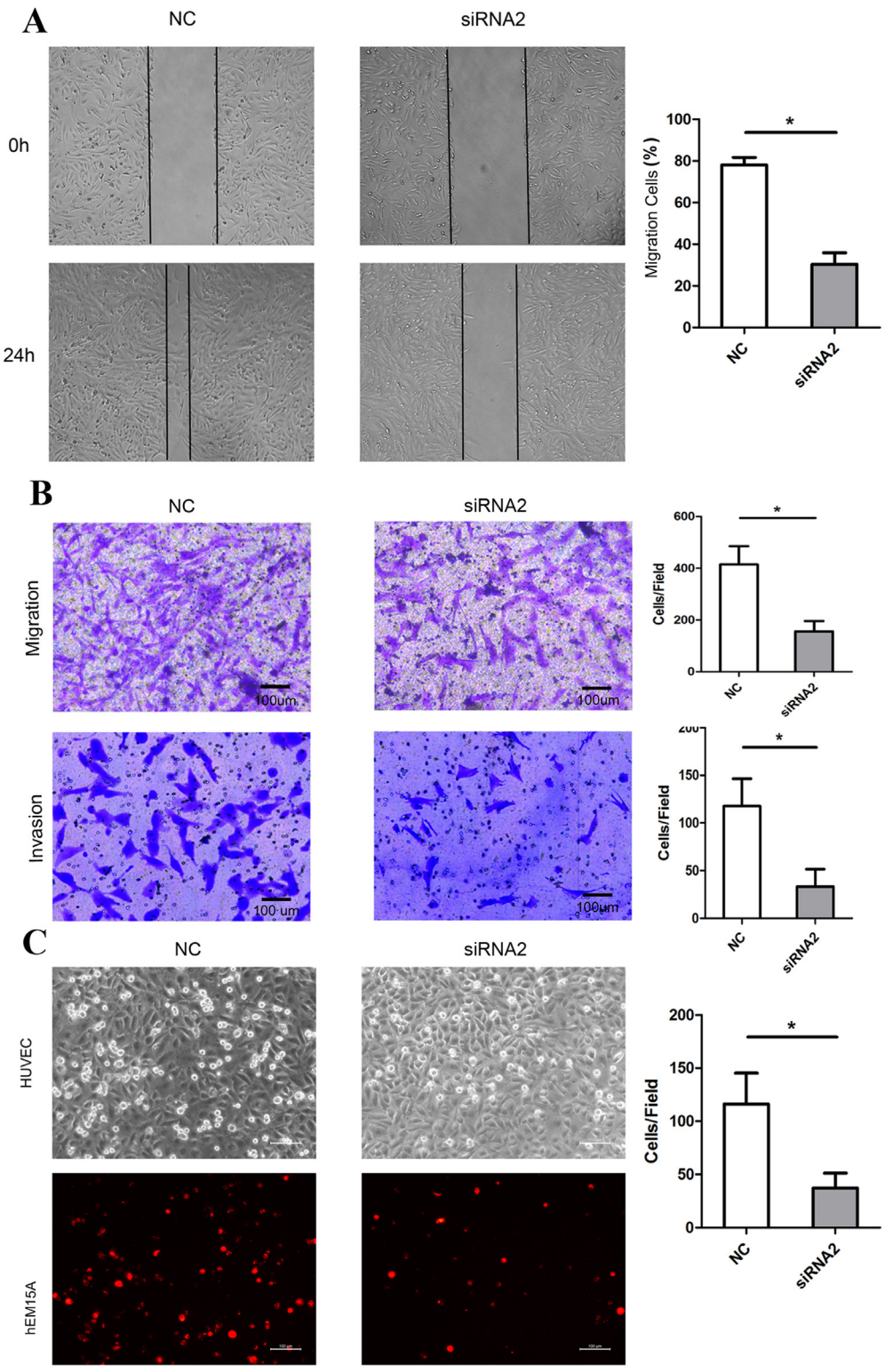


FIGURE 4 Effect of fibrinogen alpha chain (FGA) short interfering RNA (siRNA) on the migratory, invasive and adhesive capacities of hEM15A cells. (A) Migration of hEM15A cells transfected with siRNA compared with cells treated with negative control siRNA (NC) was assessed in wound healing assays. Migration rates were calculated (healing area/wound area × 100) after 24 h incubation with transfected or control cells. Micrographs (left-hand and centre panels) show typical examples. The histograms (right-hand panels) show that migration rates were significantly different ($P < 0.01$) between siRNA transfected cells and controls. (B) Transwell assays were performed to assess the migration and invasion of hEM15A cells transfected with siRNA compared with negative control siRNA. Micrographs show typical examples (left-hand and centre panels). Cell counts of the migration and invasion experiments show that siRNA significantly suppresses migration (upper right-hand panel) and invasion (lower right-hand panel) of hEM15A cells compared with negative controls ($P < 0.05$). (C) Adhesion of hEM15A cells transfected with siRNA compared with negative control siRNA was assessed by a human umbilical vein endothelial cell (HUVEC) adhesion assay. Representative images of hEM15A cells pre-treated with CellTracker adhering to the endothelial monolayer are shown, with HUVEC cells in bright field (upper left and upper middle panels) and hEM15A cells with red fluorescence (lower left and lower middle panels). Histograms of the cell count (right-hand panels) show that siRNA significantly inhibits the adhesion of hEM15A cells to HUVEC compared with negative controls (*$P < 0.05$). Values are presented as mean ± SD. Experiments were performed in triplicate.

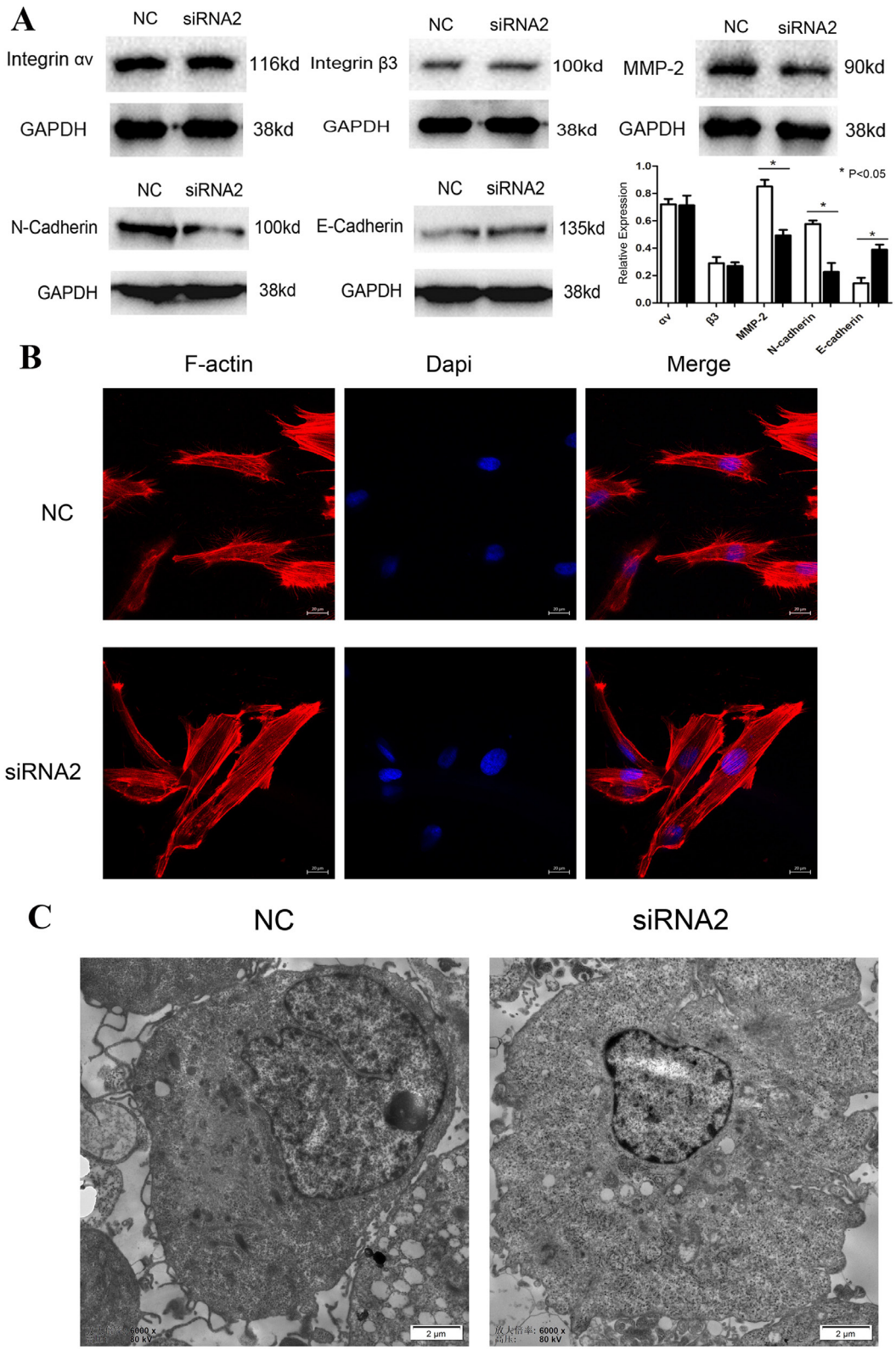


FIGURE 5 Effects of fibrinogen alpha chain (FGA) on related molecule expression and ultrastructure of hEM15A cells. (A) Representative western blots and histogram of densitometric analysis, with values normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH). Cells transfected with short interfering RNA (siRNA) are shown by black bars and negative control siRNA by white bars. N-Cadherin and matrix metalloproteinase-2 (MMP-2) were down-regulated and E-cadherin was up-regulated ($P < 0.05$), whereas integrin αv and β3 did not change significantly 72 h after transfection with siRNA2 compared with the negative control (NC). (B) Phalloidin was used to visualize F-actin and 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The microfilaments were thickened and increased in siRNA-treated cells, while the filopodia and lamellipodia were notably reduced. (C) Representative transmission electron microscope images depict the ultrastructure of hEM15A cells with or without siRNA. The surfaces of the cells treated with siRNA2 showed fewer pseudopods. Experiments were performed in triplicate.

research demonstrates that increased FGA expression is associated with the advanced clinical stages of endometriosis and a higher recurrence rate. It can be supposed that FGA functions play a key role in the pathogenesis of endometriosis in tumour cells as well as in endometriosis.

However, the role of FGA in the development and progress of endometriosis is not clear. Some studies have demonstrated that the aggressive ability of endometriotic cells is one of the distinctive features of the disease, that is, they can attach to, invade and damage other tissues. As stated above, endometriosis is not a malignant disorder, but it has similarities to the behaviour of developing tumours (*Larsen et al., 2011*; *Wang et al., 2011*). Cell migration and invasion are fundamental to a variety of pathological processes, including development, wound healing and immune responses. To migrate, the cell body must modify its shape and stiffness to interact with surrounding tissue structures (*Bruner et al., 1997*; Friedl et al., 2003). Based on the above findings, it was postulated that the abnormal FGA expression might be associated with endometrial tissue adherence, invasion and growth at ectopic sites to form endometriotic lesions.

Primary cells are the main models for studying endometriosis *in vitro*, although their use is limited by their short lifespan, poor homogeneity and need for advanced culture technology. Endometriosis-derived immortalized cell lines avoid the above difficulties and suit basic research and clinical investigations needs. This study used the human endometriosis-derived immortalized eutopic endometrium stromal cell line hEM15A as an in-vitro model (*Chen et al., 2016*). By knocking down FGA expression, this study has shown that down-regulation of FGA attenuated stroma cell migration and invasion in human endometriosis. The study has confirmed the important role of FGA in migration/invasion in human endometriosis, implying that FGA is involved in the progression of endometriosis. Weimar and colleagues reported that the migratory and invasive capacity of human ESCs is increasingly recognized as contributing to the intense tissue remodelling associated with disease progression in endometriosis (*Weimar et al., 2013*). This conclusion is consistent with our findings.

The cytoskeleton is a fibrous protein matrix located on the inner side of the eukaryotic cell membrane and presenting as a network, bundle or band. It is connected to the nucleus, cell membrane and organelles to maintain cell morphology and co-ordinate cell movement (Osorio et al., 2014). Microfilaments in cancer cells are reduced or depolymerized, are not bundled and have fewer connections with cell membranes, resulting in weakened cell rigidity. These rounder cells more easily migrate through tissue, which is a structural marker for evaluating a cell's malignant biological behaviour (*Fife et al., 2014*). In this study, after knocking down the expression of FGA in hEM15A cells, the microfilaments in the cytoskeleton increased and thickened, and the number of filopodia and slab pseudopods was notably reduced, suggesting that down-regulation of FGA may affect the cytoskeleton and pseudopod formation of endometrial cells to inhibit cell migration and invasion.

The initial phase of endometriosis is an invasive event that requires ECM breakdown. ECM undergoes degeneration and regeneration during peritoneal invasion by endometrial tissue, dependent on the action of a class of proteolytic enzymes and MMP systems (*Jana et al., 2016*). Involvement of MMPs in the development of endometriosis has been confirmed. Several studies have shown an increase in the accumulation of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 in eutopic and ectopic endometria that differs from that seen in healthy women (*Ahn et al., 2015*; *Yi et al., 2015*). Among the MMP family, MMP-2 and MMP-9 are important in the formation of endometriosis. In this study, a positive relationship was shown to exist between FGA expression and MMP-2 expression. The up-regulation of FGA expression was associated with the pathogenesis of endometriosis, probably due to the increased expression of MMP-2. This may indicate an involvement of FGA in the migration and invasion process via a regulation of MMP-2 expression. Elevated MMP-2 expression in ectopic endometria in women with endometriosis can disrupt the connection between peritoneal stromal cells and promote ectopic implantation of endometrium in the abdominal cavity (*Xin et al., 2015*). These findings are in agreement with the current results that MMP-2 may play a critical role in the establishment of endometriosis.

It has been reported that integrin $\alpha v\beta 3$ could activate a variety of tyrosinases by binding to the cell adhesion molecules containing RGD sequences, activating phosphorylation-dependent protein kinases of cytoskeletal proteins, to regulate remodelling of the cytoskeleton and affect the cells' ability to migrate and invade (*Bianconi et al., 2016*; *Kim et al., 2003*; Streuli et al., 2016; *Wu et al., 2005*). Integrin $\alpha v\beta 3$ has been reported to regulate MMP-2 in the A549 lung cancer cell line, and it may be responsible for the progression of lung cancer. The results of the current study suggest that FGA may not directly affect the expression of integrin $\beta 3$ and αv. It is speculated that FGA induces changes in adhesion, invasion and migration by binding integrin $\alpha v\beta 3$ to activate certain signalling pathways.

In conclusion, high expression of FGA in endometriosis is closely related to disease severity, but not to age or phase of the menstrual cycle. Abnormally expressed FGA may regulate cellular interstitial phenotype, cytoskeletal remodelling and MMP-2 expression, and affects cell adhesion, migration and invasion, which may play an important role in the occurrence, progression and recurrence of endometriosis.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2019.07.002.

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