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Title: MicroRNA-126-5p downregulates BCAR3 expression to promote cell migration and invasion in endometriosis

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Abstract

Purpose: Endometriosis (EMs) is an estrogen-dependent multifactorial disease. Inhibition of estrogen in endometrial cells contributes to their failure to form lesions in ectopic sites. However, whether reducing or suppressing the inhibitory effect of estrogen results in the establishment of ectopic lesions remains unclear. The *BCAR3* gene induces estrogen resistance in estrogen-dependent breast cancer cells and promotes cell migration, invasion, and epithelial-mesenchymal transition (EMT). However, the expression of *BCAR3* in endometriosis and its effect on endometrial cell function and the anti-estrogen effect of endometriosis have not been reported. These issues are addressed in the present study.

Methods: The study included 32 cases of ectopic endometrium and eutopic endometrium in patients with endometriosis and 31 cases of normal endometrium as controls. The expression of *BCAR3* and microRNA (miR)-126-5p was detected by real-time PCR, immunohistochemistry, and western blotting. The effects of *BCAR3* and miR-126-5p on the morphology and biological behavior of eutopic endometrial cells were verified using lentivirus overexpression and a vector knockdown model, the CCK-8 assay, Transwell experiments, and estrogen intervention experiments using primary cultures of epithelial and stromal cells.

Results: The *BCAR3* gene was highly expressed in ectopic endometrium and the eutopic endometrium of patients with endometriosis, and the expression level was higher in stage III-IV patients than in stage I-II patients. *In vitro* cell experiments showed that miR-126-5p negatively regulated the expression of *BCAR3* and its effect on the migration and invasion of stromal cells. Low expression of miR-126-5p and high expression of *BCAR3* promoted endometriosis stromal cell migration and invasion. Assessment of EMT in endometriosis compared with eutopic endometrium showed that the expression of vimentin was significantly increased and the expression of E-cadherin was significantly decreased in ectopic endometrium. Estrogen promoted EMT in eutopic endometrial epithelial cells and this effect was reversed by estrogen inhibitors. *BCAR3* had no direct effect on EMT and did not act synergistically with estrogen on promoting EMT.

Conclusion: miR-126-5p negatively regulated *BCAR3* expression in eutopic endometriosis, enhanced the migration and invasion of endometrial cells, and

promoted the occurrence of endometriosis. BCAR3 did not induce EMT and had no synergistic effect with estrogen, but its inhibition of anti-estrogen function may provide new insight into the mechanism of local estrogen action in endometriosis.

Keywords: Endometriosis; miR-126-5p; BCAR3; EMT

1. Introduction

Endometriosis (EM) is a common and refractory gynecological disease. Although it is an estrogen-dependent benign disease, its biological behaviors are similar to those of malignant tumors. It is characterized by infiltrated distribution, diverse morphology, local invasion, distant metastasis, and recurrence. There are different theories to explain the pathogenesis of endometriosis, among which the classical theory of "retrograde menstruation" is widely accepted. However, this theory does not explain the common physiological phenomenon of blood reflux in women of reproductive age, which has an incidence of approximately 90%, whereas the incidence of endometriosis is only 10%–15% [1]. This suggests that additional factors contribute to the pathogenesis of endometriosis. Molecular defects that predispose endometrial cells to endometriosis may act synergistically with micro-environmental factors in the pelvis to promote the establishment of endometriotic lesions. Burney [2] and Li [3] showed that the gene expression profile of the eutopic endometrium of endometriosis is significantly different from that of the normal endometrium. Altered expression of genes related to cell adhesion [4] and invasion [5] result in the functional changes of eutopic cells and provide the basic conditions for ectopic implantation. Abnormal autoimmune function and increased estrogen metabolism in ectopic endometrium also play important roles in the occurrence of endometriosis.

The breast cancer anti-estrogen resistance 3 (*BCAR3*) gene, also known as *AND-34* or *NSP2*, belongs to the novel Src homology 2 (SH2) - containing protein (NSP) family [6]. The BCAR3 protein is responsible for the resistance of estrogen-dependent breast cancer cells to anti-estrogen drugs *in vitro*, which is an important cause of the failure of endocrine therapy in estrogen-receptor-positive breast cancer patients. Anti-estrogen in ectopic endometrium is a protective factor against the occurrence of endometriosis, and inhibition of the local anti-estrogenic effect promotes the formation of ectopic lesions[7]. Therefore, the potential role of *BCAR3* as a candidate gene promoting the malignant behaviors of cells is worth

exploring. Abnormal overexpression of *BCAR3* can also induce cytoskeleton rearrangement and promote cell adhesion, migration, and invasion [8]. *BCAR3* is highly expressed in breast cancer, ovarian cancer [9], and most endometrial cancer cells, although the role of *BCAR3* in endometriosis has not been reported to date.

In recent years, microRNAs (miRNAs), which are small RNA molecules that regulate gene expression, have attracted attention for their role in the pathogenesis of many diseases. The miRNAs regulate target gene expression negatively through incomplete complementary pairing with the 3′-untranslated region (3′-UTR) of target genes[10] . A previous study from our group showed that low expression of miR-126-3p in eutopic endometrial stromal cells (ESCs) of patients with endometriosis leads to increased invasiveness [11]. MiR-126-5p and miR-126-3p are derived from the same precursor, pre-mir-126 [12]. They are expressed at the same level in various tissues and cells, have similar variation trends, and play similar functions [13]. The expression of miR-126-5p is low in many cancers, and it can be used as an anti-oncogene to inhibit the migration and invasion of cancer cells [14]. The results of DIANA-microT and Targetscan algorithm showed that *BCAR3* gene was one of the downstream targets of miR-126-5p[15, 16]. Whether miR-126-5p can regulate the invasiveness of ESCs by targeting *BCAR3* remains to be verified.

Epithelial-mesenchymal transition (EMT) is a basic biological process for the maintenance of living tissues and also a key step in the invasion and metastasis of epithelial-derived cancer cells [17] . EMT is characterized by decreased expression of E-cadherin, an epithelial marker, and increased expression of mesenchymal markers such as fibronectin and vimentin [18]. Compared with eutopic endometrium, the proportion of epithelial to stromal cells is inverted in ectopic endometrium, and endometrial epithelial cells (EECs) are often missing in ectopic lesions [19]. Changes of EMT marker proteins have been detected in ectopic epithelial cells showing enhanced migration and invasion[20]. However, the mechanism underlying the function of EMT in endometriosis remains unclear. *BCAR3* was shown to induce EMT in tumor cells [21], and estrogen promotes EMT in endometriosis. Therefore, whether *BCAR3* cooperates with estrogen to promote EMT in endometriosis is worth exploring.

The present study investigated the effect of *BCAR3* on the invasiveness of endometriosis stromal cells and its possible regulatory mechanism to provide a new theoretical basis for the pathogenesis of endometriosis.

2. Materials and methods

2.1 Tissue acquisition

Eutopic endometrium (Eu) and ectopic endometrium (Ec) samples were obtained from 32 patients (mean age, 43.38 ± 5.01 years) who underwent hysterectomy for ovarian endometriosis [according to the revised American Fertility Society classification of endometriosis (rAFS): stage I–II, n=15; stage III–IV, n=17]. Normal endometrium (NE) samples from 31 patients (mean age, 43.74 ± 4.69 years) without endometriosis who were diagnosed with cervical intraepithelial neoplasia during the same period were included as controls (Table S1). All cases were confirmed by postoperative pathologic diagnosis. All patients were premenopausal and had regular menstrual cycles. None of the patients received any hormone therapy in the 6 months prior to surgery. Written informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Boards of Liaoning Cancer Hospital (Shenyang, China). Fresh tissues were divided into three parts: one was fixed in 10% formaldehyde, one was frozen in liquid nitrogen and preserved at –80°C , and the other was placed in DMEM/F12 (1:1) containing 10% FBS and 1% penicillin-streptomycin (Gibco, Gaithersburg, MD, USA) for cell isolation.

2.2 Cell culture

Primary EECs, ESCs, and normal ECSs (NESCs) were isolated from endometrial tissues according to previously described methods. Briefly, all tissue samples were washed with sterile PBS and minced into pieces approximately 1 mm^3 in size, then digested with 0.1% type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 60 min. Undigested tissue was removed with a 100 m sieve. EECs and ESCs were separated using a 40 m sieve. To collect EECs, the 40 m sieve was washed upside down with PBS. Cell suspensions were centrifuged and suspended in phenol red-free DMEM/F12 medium (Gibco) with 10% FBS and 1% penicillin-streptomycin. EECs were cultured for 24 h prior to estrogen (E2, 10-8 M; Sigma), DMSO or ICI182780 (10-7 M; Sigma-Aldrich) stimulation in phenol red-free DMEM/F12 without FBS. HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% FBS.

2.3 Target prediction

Target genes for miR-126-5p was predicted by using the TargetScan and DIANA-microT v5.0 with a prediction threshold of 0.7. Only putative target genes predicted by both of the target prediction tools were accepted.

2.4 RNA extraction and real-time PCR

Total RNA was isolated from cultured cells or tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 500 ng RNA using the Prime Script RT reagent kit (TaKaRa, Otsu, Shiga, Japan). Real-time quantitative PCR (qRT-PCR) of *BCAR3* was carried out with the SYBR® Prime Script™ RT-PCR kit (TaKaRa), using *GAPDH* for normalization. Forward and reverse primers were as follows: 5′-GCGGTGGAACTGAAGGATTC-3′ and 5′-TGGCAGTTTGGGTGTACTGG-3′ for *BCAR3*, 5′-TTTGGAAGACCCAGTTCAGA-3′ and 5′-AGTCCTTCCACGATACCAAAGT-3′ for *GAPDH*. miR-126-5p expression was evaluated using the Mir-X™ miRNA qRT-PCR SYBR® kit (TaKaRa) on a Roche LightCycler480II (Roche Diagnostics GmbH, Mannheim, Germany). *U6* was used for normalization. The forward and reverse primers for *U6* were as follows: 5′-CTCGCTTCGGCAGCACATA-3′, and 5′-CGCTTCACGAATTTGCGTG-3′. The miR-126-5p primer was 5′-TCGTACCGTGAGTAATAATGCG-3′. The 2-ΔΔCt method was used to calculate gene expression levels.

2.5 Immunohistochemistry

The formaldehyde-fixed paraffin-embedded tissue was sliced into 4 μm sections. After blocking with 10% normal goat serum, the sections were incubated with anti-BCAR3 antibody (1:400; Abcam), anti-E-cadherin antibody (1:200; Cell Signaling Technology, Danvers, MA, USA), or anti-vimentin antibody (1:400; Cell Signaling Technology) overnight at 4°C. Then, tissue samples were incubated with biotinylated goat anti-rabbit IgG (Boster Biotechnology, Wuhan, China) for 1 h at room temperature and with diaminobenzidine for 3 min. Negative control was performed by following the same protocol without incubation with primary antibodies. The staining intensity was quantified using H-score=Σ(Pi×I), where Pi represents the percentage of positively stained cells and I represents the coloring intensity, measured by the coloration (brown) of most positive cells: dark brown, 3 points; brown, 2 points; and light brown, 1 point. The mean value of the double-blind test was calculated.

2.6 Western blot analysis

Protein extraction was performed using a whole cell lysis kit (KeyGEN, Nanjing, China) and protein concentrations were measured with the BCA protein assay kit (Beyotime, Shanghai, China). Protein aliquots of 50 μg were subjected to 10% SDS-PAGE, transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA), and blocked with 5% nonfat milk or goat serum. The membranes were then incubated with anti-BCAR3 antibody (1:1000; Abcam, Cambridge, UK), anti-pSrc antibody (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Src antibody (1:1,000; Santa Cruz Biotechnology), anti-E-cadherin antibody (1:1,000; Cell Signaling Technology), anti-vimentin antibody (1:1000; Cell Signaling Technology), anti-fibronectin antibody (1:500; Santa Cruz Biotechnology), or anti-GAPDH antibody (1:1,0000; Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with a peroxide-conjugated secondary antibody (1:2000; Zhongshan Golden Bridge Biotechnology, Beijing, China). Protein bands were imaged using enhanced chemiluminescence reagents (Beyotime).

2.7 Dual luciferase reporter assay

The pmirGLO vectors (Promega, Madison, WI, USA) containing the wild-type (WT)-*BCAR3* 3′-UTR sequence or mutant (Mut)-*BCAR3* 3′-UTR sequence, with a mutated seed region in the miR-126-5p binding site (Fig. 6A), were purchased from Genepharma (Shanghai, China). HEK293T cells were cultured in 24-well plates, then co-transfected with miR-126-5p agomir or negative controls (Genepharma) and WT or Mut reporter vector, using Lipofectamine 2000 (Invitrogen). After 48 h, luciferase activity was measured by the Dual-Glo Luciferase Assay System (Promega). Each experiment was biologically repeated three times and there were three technical replicates in each experimental run.

2.8 Cell transfection and infection

ESCs or normal endometrial stromal cells (NESCs) were seeded in 6-well plates in DMEM/F12 medium without antibiotics until 50% confluent. ESCs were transfected with miR-126-5p agomir or negative control (NC, 100 nM; Genepharma), and NESCs were transfected with miR-126-5p antagomir or NC (100 nM; Genepharma) using Lipofectamine 2000 (Invitrogen). Transfection efficiency was measured by RT-qPCR after 48 h.

Lentivirus packaged human *BCAR3* cDNA, *BCAR3* siRNA, miR-126-5p siRNA, pre-miR-126, and a negative control sequence designed by GeneChem Technology

(Shanghai, China) were used in our study. When stromal cells were 50% confluent or epithelial cells were 70% confluent in 6-well plates, the medium was replaced with phenol red-free DMEM/F12 containing 8 μg/mL polybrene and 10% FBS. The stromal cells or epithelial cells were infected with lentiviral particles at a final concentration of 50 nM or 80 nM. Infection efficiency was measured by RT-qPCR and western blotting at 72 h after transfection.

2.9 Migration and invasion assays

ESCs and NESCs were digested at 48 h after transfection and seeded into the upper chamber (8 μm pore size, 24-well plate; Corning, New York, NY, USA) in 200 μL serum-free DMEM/F12 medium (1×10^5 cells/mL). DMEM/F12 medium with 20% FBS (600 μL) was then added to the lower chamber. After 36 h of incubation, cells on the upper side of each membrane were cleaned with a cotton swab. The membranes were then fixed in 10% formaldehyde for 15 min and stained with 0.2% crystal violet for 30 min. The cells were scored by counting five random high power fields per filter under an inverted microscope. For the invasion assay, the upper chamber was coated with serum-free DMEM/F12 diluted Matrigel (BD, Franklin Lakes, NJ, USA).

2.10 Statistical analysis

Statistical analysis was performed using SPSS 21.0 (IBM, Chicago, IL, USA). Comparison among multiple groups were performed by one-way analysis of variance followed by the Bonferroni (equal variances) or Dunnett's (unequal variances) post-hoc tests. Differences between two groups were tested by Student's *t*-test. The correlation was analyzed using the Pearson's correlation test. Values are expressed as the mean ± SEM, and differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Upregulation of *BCAR3* in eutopic and ectopic endometrium

The expression levels of *BCAR3* in Ec, Eu, and NE were examined by RT-qPCR, western blotting, and immunohistochemistry. As shown in Fig. 1A, we found that BCAR3 staining was seen in cellular nuclei and cytoplasm in both the stromal and epithelial cells of the normal, eutopic and ectopic endometrium. BCAR3 expression was significantly higher in endometriotic epithelial cells (EcECs) than in EECs and NEECs, which had similar expression levels ($P < 0.05$). In stromal cells, BCAR3

protein expression was highest in EcSCs, lower in ESCs, and lowest in NESCs ($P < 0.01$). Western blotting results showed that BCAR3 expression was higher in Ec and in Eu than in NE ($P < 0.01$), and it was higher in patients with stage III/IV endometriosis than in those with stage I/II ($P < 0.05$, Fig. 1B and D). *BCAR3* mRNA level was also higher in Ec and Eu than in NE ($P < 0.05$, Fig. 1C). However, *BCAR3* mRNA expression did not differ between patients with early- and advanced-stage endometriosis (Fig. 1D).

3.2 Correlation analysis of *BCAR3* and miR-126-5p

The expression of miR-126-5p in Ec, Eu, and NE is shown in Fig. 2A. There were significant differences in the expression level of miR-126-5p between Ec (0.36 ± 0.04) and Eu (0.40 ± 0.04), as well as between Eu and NE (1.09 ± 0.09). Correlation analysis showed that miR-126-5p was negatively correlated with *BCAR3* mRNA ($r=-0.417$, $P < 0.01$) and BCAR3 protein expression ($r=-0.669$, $P < 0.01$; Fig. 2B and C).

3.3 The expression of EMT markers in eutopic and ectopic endometrium

Immunohistochemical results showed that vimentin, a mesenchymal marker, was expressed at similar levels in endometriotic stromal cells (EcSCs), ESCs, and NESCs ($P > 0.05$, Fig. 3A), and its expression was higher in EcECs than in EECs and normal endometrial epithelial cells (NEECs) ($P < 0.01$, Fig. 3A), whereas E-cadherin, an epithelial marker, was only expressed in epithelial cells and not in stromal cells. Its expression was significantly lower in EcECs than in EECs ($P < 0.01$, Fig. 3B). E-cadherin expression levels were similar in EECs and NEECs ($P > 0.05$, Fig. 3B).

3.4 Downregulation of miR-126-5p promotes migration and invasion of ESCs.

To evaluate the possible role of miR-126-5p in ESCs, we measured cell migration and invasion using the Transwell assay after transfecting miR-126-5p antagomir into NESCs and miR-126-5p agomir into ESCs. The transfection efficiency was verified by RT-qPCR (Fig. 4A). Compared with negative controls, overexpression of miR-126-5p markedly repressed the migration and invasion of ESCs, whereas downregulation of miR-126-5p promoted migration and invasion of NESCs (Fig. 4B and C).

3.5 *BCAR3* overexpression promotes the proliferation, migration, and invasion of ESCs.

BCAR3 expression was significantly higher in ESCs than in NESCs (BCAR3 mRNA, $P < 0.01$, Fig. 5A; BCAR3 protein, $P < 0.01$, Fig. 5B). To determine whether

BCAR3 plays a role in the migration and invasion of stromal cells, ESCs were infected with si*BCAR3*-lentivirus and NESCs were infected with *BCAR3*-lentivirus ($P < 0.01$, Fig. 5C). As shown in Fig. 5D and E, compared with the control group, cell migration and invasion were markedly suppressed in BCAR3 silenced ESCs and significantly promoted in BCAR3-overexpressing NESCs.

3.6 *BCAR3* is the direct target of miR-126-5p.

Using DIANA-microT and TargetScan algorithms[15, 16], we identified *BCAR3* as a predicted target gene of miR-126-5p (Fig. 6A). Correlation analysis showed that miR-126-5p expression was negatively correlated with BCAR3 mRNA and protein expression. We then constructed WT-BCAR3 3′-UTR and the corresponding Mut-BCAR3 3′-UTR luciferase reporters (Fig. 6B) and performed luciferase activity assays in HEK293T cells. The results showed that miR-126-5p overexpression reduced the luciferase activity of the WT-BCAR3 3′-UTR but not that of the Mut-BCAR3 3′-UTR construct ($P < 0.01$, Fig. 6C). To determine whether BCAR3 was regulated by miR-126-5p, NESCs were transfected with miR-126-5p antagomir and ESCs were transfected with miR-126-5p agomir, and the protein expression of BCAR3 was measured. As shown in Figure 6C, silencing of miR-126-5p increased BCAR3 protein expression in NESCs, whereas overexpression of miR-126-5p decreased BCAR3 protein expression in ESCs.

To further explore whether BCAR3 mediated the invasion-suppressive effects of miR-126-5p, BCAR3 was silenced in miR-126-5p stably silenced NESCs (Fig. 6D). The results showed that the effect of miR-126-5p downregulation on promoting NESC migration and invasion was partially reversed by BCAR3 silencing (Fig. 6E and F). Upregulation of BCAR3 in miR-126-5p stably overexpressed ESCs (Fig. 6G) modestly reversed the repressive effect of miR-126-5p on ESC migration and invasion (Fig. 6H and I).

3.7 Effect of BCAR3 and estrogen on EMT in EECs

As shown in Fig. 7A, EECs infected with BCAR3-lentivirus showed significantly increased BCAR3 expression ($P < 0.05$). Compared with the controls, BCAR3 overexpressing cells showed increased fibronectin expression ($P < 0.05$, Fig. 7B) and mesenchymal-like morphology, characterized by disaggregation of spindle-like cells (Fig. 7C). However, no significant changes in the expression of E-cadherin and vimentin were observed ($P > 0.05$, Fig. 7B).

Compared with control cells, estrogen-treated EECs showed decreased expression of E-cadherin ($P < 0.05$) and increased expression of vimentin ($P < 0.01$), and these changes were abolished by estrogen receptor antagonist ICI182780 (Fig. 7D). In addition, estrogen treatment changed EEC morphology from tightly-arranged round cells to scattered spindle-like cells (Fig. 7F).

EECs treated with estrogen at 24 h after BCAR3-lentivirus infection showed a higher expression of fibronectin (Fig. 7E) and an earlier alteration of cell morphology (Fig. 7F) than those in the estrogen-only intervention group and recombinant BCAR3 group. The expression levels of E-cadherin and vimentin were similar between the estrogen intervention plus recombinant BCAR3 group and the estrogen-only intervention group ($P < 0.05$, Fig. 7E).

4. Discussion

The results of the present study showed that BCAR3 was highly expressed in ectopic and eutopic endometrium of endometriosis, and the expression level of BCAR3 was higher in stage III-IV patients than in stage I-II patients. In addition, the expression pattern of BCAR3 differed between epithelial cells and stromal cells. In epithelial cells, the BCAR3 gene was highly expressed in ectopic EECs, whereas no significant differences in expression were observed between eutopic EECs and normal controls. The expression of BCAR3 in stromal cells was highest in ectopic endometrium, lower in eutopic endometrium, and the lowest in normal endometrium (NE). *In vitro* cell experiments showed that low expression of miR-126-5p and high expression of BCAR3 promoted the migration and invasion of eutopic ESCs in endometriosis, and miR-126-5p negatively regulated the expression of BCAR3 and its effect on the migration and invasion of stromal cells. The effect of BCAR3 on EMT in endometriosis was assessed. Compared with eutopic endometrium, the expression of vimentin was significantly higher and the expression of E-cadherin was significantly lower in ectopic endometrium than in eutopic endometrium. Estrogen promoted EMT in eutopic EECs, and estrogen inhibitors reversed the estrogenic effect. BCAR3 had no effect on EMT and no synergistic effect with estrogen on promoting EMT.

Endometriosis is an estrogen-dependent multifactorial disease. In recent years, studies on the pathogenesis of endometriosis focused on “abnormal endometrium gene expression,” “immune dysfunction” and “stem cell theory.” The enhanced

bioavailability of estrogen in ectopic lesions is an important factor in endometriosis[22, 23]. Delvoux, B. *et al*. [22]and Smuc, T. *et al*.[23] reported that patients with endometriosis have abnormal expression of aromatase and 17 beta-HSD genes, indicating that the synthesis and secretion of estrogen are increased in ectopic lesions . Inhibition of estrogen or anti-estrogen in ectopic endometrium contributes to their failure to form lesions, which is a protective factor against the occurrence of endometriosis. Endometrium apoptosis is caused by immune rejection and the anti-estrogenic effect after blood reflux. Therefore, inhibition of the anti-estrogenic effect of ectopic endometrium may lead to endometriosis, which is rarely reported. BCAR3 can induce antiestrogen drug resistance in estrogen-dependent breast cancer cells, leading to failure of endocrine therapy in estrogen-receptor-positive breast cancer patients [24]. The role of BCAR3 in breast cancer, ovarian cancer, endometrial cancer, and other tumors has been reported. Studies show that [25], compared with control cells, the number of stress fibers transfected with BCAR3 is reduced in breast cancer cells, the arrangement is disordered, and the actin skeleton is reconstructed. Wilson *et al*. reported that BCAR3 alters actin cytoskeletal and adhesion remodeling, and increases the number of plate pseudopods, suggesting that the upregulation of BCAR3 enhances the movement and invasive abilities of cells [26]. In the present study, we showed for the first time that the expression of BCAR3 was abnormally upregulated in eutopic endometrium and ectopic endometrium of endometriosis. In addition, BCAR3 expression was positively correlated with the severity of the disease, and BCAR3 promoted the migration and invasion of eutopic ESCs, suggesting that BCAR3 might be one of the targets of eutopic endometrium of endometriosis. Whether BCAR3 affects the anti-estrogen inhibition in ectopic endometrium needs to be determined. BCAR3 may indirectly promote the effects of estrogen on ectopic endometrium, and the underlying mechanism needs to be explored.

EMT plays an important role in the occurrence of endometriosis [27]. Estrogen can induce EMT in endometriosis and is involved in the development of endometriosis [28, 29]. In adenomyosis, circulating estrogen levels are positively correlated with EMT [30]. Here, we demonstrated that the eutopic endometrium of endometriosis has higher EMT abilities. *In vitro* experiments showed that estrogen decreased the expression of E-cadherin and increased the expression of vimentin and fibronectin in eutopic EECs,

suggesting that estrogen may promote EMT in ectopic endometrium. Cai and Near recently reported that overexpression of BCAR3 alters the growth pattern of mammary epithelial cells from a polygonal cell mass to multi protuberant independent cells, and induces EMT in cells[21, 31]. Based on these findings, we investigated whether the occurrence and development of endometriosis are related to the potential effect of BCAR3 on inhibiting the anti-estrogen effect of ectopic endometrium and acting synergistically with estrogen to promote EMT. However, our results showed that transfection of BCAR3 into eutopic endometrial cells did not upregulate the expression of E-cadherin and vimentin in epithelial cells. The expression of E-cadherin and vimentin in epithelial cells increased in response to treatment with estrogen, whereas the expression levels were the same in the estrogen and BCAR3 plus estrogen groups. This suggested that BCAR3 had no effect on EMT in endometriosis, and the mechanism underlying the role of the BCAR3 gene in endometriosis may not be related to EMT.

In previous work from our group, we showed that the expression of miR-126-3p was low in the eutopic endometrium of endometriosis [11]. miR-126-5p and miR-126-3p are derived from the same precursor and have similar functions. The expression levels of miR-126-5p and miR-126-3p were similar in melanoma [32], lung cancer [33], breast cancer [34], and their changing trends were the same, playing similar roles [35]. *In vitro* experiments showed that the expression of miR-126-5p was also low in endometriosis, and upregulation of miR-126-5p expression in eutopic ESCs could inhibit cell migration and invasion. Conversely, downregulation of miR-126-5p in NESCs could enhance cell migration and invasion. This conclusion is consistent with Musiyenko's report indicating that low expression of miR-126-5p promotes the invasion of prostate cancer cells [36]. The results of luciferase experiments indicated that miR-126-5p could directly bind to the 3′-UTR of BCAR3. Further validation experiments showed that the downregulation of miR-126-5p negatively regulated BCAR3 gene expression and promoted the migration and invasion of endometriotic stromal cells. miR-126-5p was identified as an upstream regulator of BCAR3, suggesting its potential as a molecular marker of endometriosis. This molecular pathway needs to be investigated in-depth. However, this study may have been underpowered given only Han population included since recent evidences showed the divergence in miRNAs expression among different ethnic groups[37, 38].

In conclusion, the downregulation or loss of miR-126-5p expression in eutopic

endometriosis negatively regulated BCAR3 gene expression and promoted endometrial cell migration and invasion, suggesting that it played an important role in the occurrence and development of endometriosis. The BCAR3 gene may be a biomarker of endometriosis in eutopic endometrium. Estrogen promoted EMT in the ectopic endometrium of endometriosis; however, BCAR3 did not have a synergistic effect with estrogen. The effect of BCAR3 on inhibiting anti-estrogenic effects sheds light on the mechanism underlying local estrogen metabolism in endometriosis and provides a new direction for future research.

Declaration of interest

The authors have no conflicts of interest to declare.

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Figure legends

Fig. 1 Expression of BCAR3 mRNA and BCAR3 protein in endometriosis. NE: normal endometrium; Ec: ectopic endometrium of endometriosis; Eu: eutopic endometrium. (A) Immunohistochemistry was used to detect the expression of BCAR3 in NE, Eu, and Ec (200×). (B) BCAR3 protein was quantified by western blotting in NE, Eu, and Ec. (C) Relative expression of BCAR3 mRNA was measured by quantitative real-time PCR (qRT-PCR) in NE, Eu, and Ec. (D) BCAR3 mRNA and protein expression in Eu and Ec from patients with stage I-II or stage III-IV endometriosis; $^{*}P < 0.05$; $^{**}P < 0. .01$.

Fig. 2 Expression of microRNA-126-5p and its correlation with the BCAR3 gene. (A) Relative expression of miR-126-5p was measured by qRT-PCR in normal endometrium (NE), eutopic endometrium (Eu), and ectopic endometriosis (Ec). (B) Correlation analysis was performed by the Pearson's correlation test between miR-126-5p and BCAR3 mRNA from 95 samples ($r=-0.417$, $P < 0.01$, 31 samples from NE, 32 samples from Eu and 32 samples from Ec. (C) correlation analysis between miR-126-5p and BCAR3 protein ($r=-0.669$, $P < 0.01$); $^{*}P < 0.05$; $^{**}P < 0.01$.

Fig. 3 Expression of vimentin and E-cadherin in ectopic and eutopic endometrium of endometriosis. (A) Immunostaining of vimentin in normal endometrium (NE), eutopic endometrium (Eu), and ectopic endometriosis (Ec) (400×). (B) Immunostaining of E-cadherin in EN, Eu, and Ec (400×); $^{*}P < 0.05$; $^{**}P < 0.01$.

Fig. 4 Effects of miR-126-5p on migration and invasion of eutopic stromal cells.(A) Reduction of miR-126-5p expression by transfecting miR-126-5p antagomir in NESCs and ectopic expression of miR-126-5p in ESCs by transfecting miR-126-5p agomir were validated by RT-qPCR. (B-C) The Transwell migration assay showed that miR-126-5p knockdown enhanced migration (B) and invasion (C) of NESCs, whereas miR-126-5p upregulation inhibited migration (B) and invasion (C) of ESCs; $^{*}P < 0.05$; $^{**}P < 0.01$.

Fig. 5 Effects of BCAR3 on migration and invasion of eutopic endometrial stromal cells. (A) Expression of BCAR3 mRNA was measured by RT-qPCR in

normal endometrial stromal cells (NESCs) and eutopic endometrial stromal cells (ESCs). (B) Western blot analysis was used to detect the expression of BCAR3 protein in NESCs and ESCs. (C) Lentivirus containing BCAR3 gene was infected into eutopic endometrial stromal cells (ESCs), and lentivirus containing BCAR3 siRNA was infected in normal endometrial stromal cells (NESCs). Western blot analysis was used to detect the expression level of BCAR3. (D-E) The Transwell assay showed the BCAR3 upregulation enhanced migration (D) and invasion (E) of NESCs, whereas BCAR3 knockdown inhibited migration (D) and invasion (E) of ESCs; $^{*}P < 0.05$; $^{**}P < 0.01$.

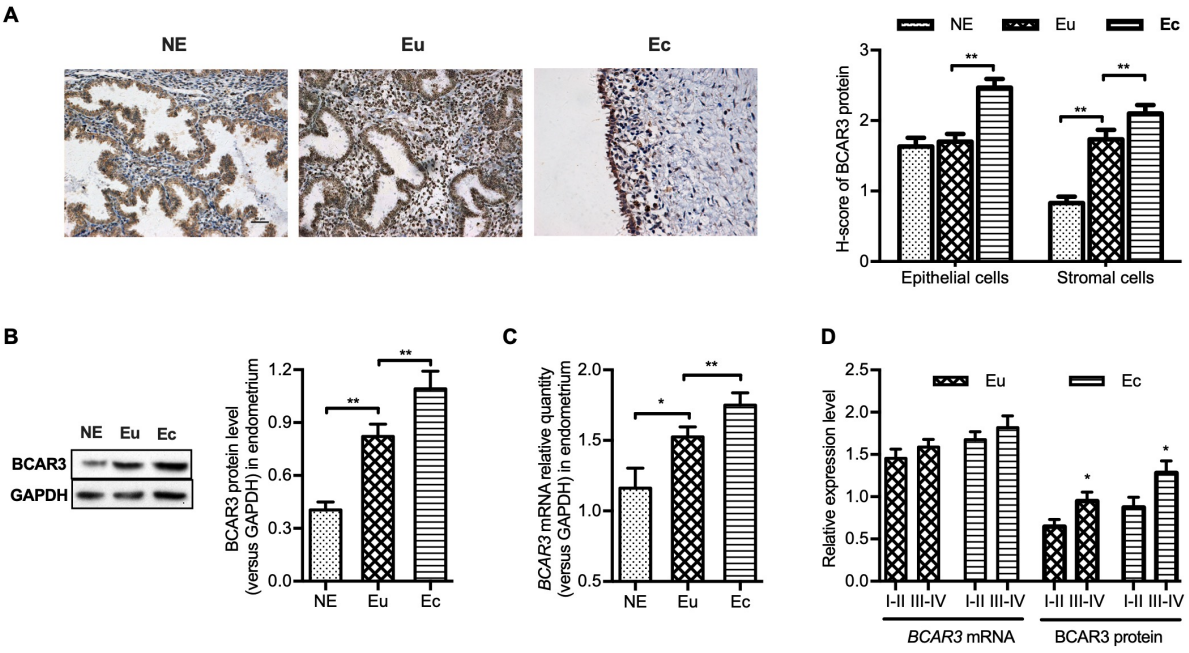
Fig. 6 BCAR3 mediates the effects of miR-126-5p in ESCs. (A) The putative miR-126-5p binding sequences in the BCAR3 3′-UTR; (B) The luciferase reporter assay revealed that miR-126-5p suppressed the luciferase activity of the WT-BCAR3 3′-UTR. (C) miR-126-5p knockdown increased the expression of BCAR3 protein in NESCs and miR-126-5p overexpression decreased the expression of BCAR3 protein in ESCs. (D) Expression of miR-126-5p and BCAR3 in NESCs was explored by RT-qPCR and western blotting after transfection with different vectors. (E-F) BCAR3 silencing reversed the promoting effect of miR-126-5p knockdown on the migration (E) and invasion (F) of NESCs. (G) Expression of miR-126-5p and BCAR3 in ESCs was explored by RT-qPCR and western blotting after transfection with different vectors. (H-I) BCAR3 silencing reversed the promoting effect of miR-126-5p knockdown on the migration (H) and invasion (I) of ESCs. $^{*}P<0.05$;$^{**}P<0.01$.

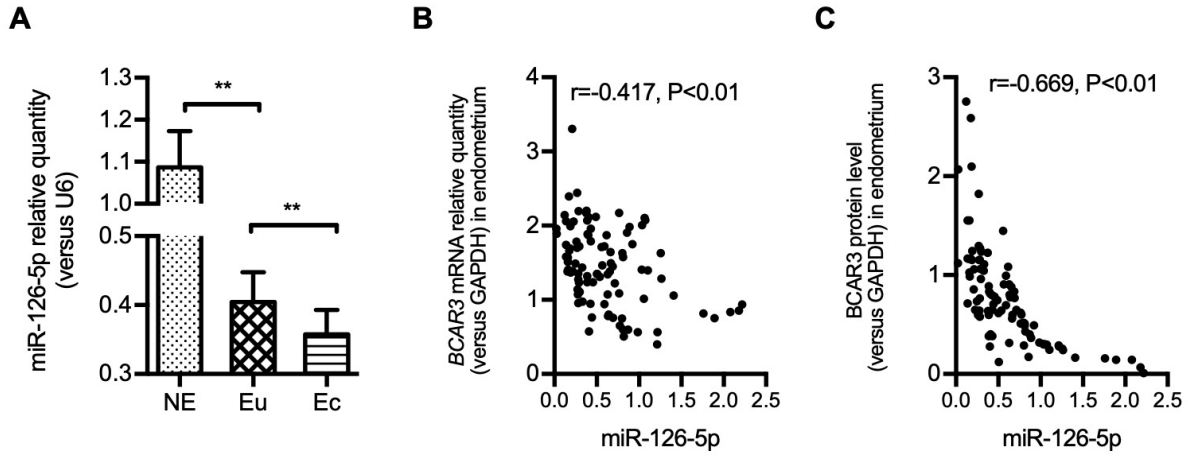
Fig. 7 Epithelial-mesenchymal transition (EMT) of eutopic endometrial cells induced by BCAR3 and estrogen. (A) Expression of BCAR3 protein was examined by western blotting after infecting EECs with BCAR3 lentivirus or negative control lentivirus. (B) Western blot detection of the effect of BCAR3 on the expression of E-cadherin, fibronectin and vimentin in EECs. (C) Cell morphology was observed under a light microscope (200×) at 48- and 96 h after infection. (D) Western blot detection of the expression of E-cadherin, fibronectin and vimentin in response to estrogen (E2, 10-8 M) and estrogen (E2, 10^{-8} M)+ ICI182780 (10^{-7} M) in EECs. (E) Western blot detection of the expression of E-cadherin, fibronectin and vimentin in BCAR3 overexpressed EECs and control EECs with or without estrogen treatment. (F) Cell morphology was observed under a light microscope (200×) with estrogen,

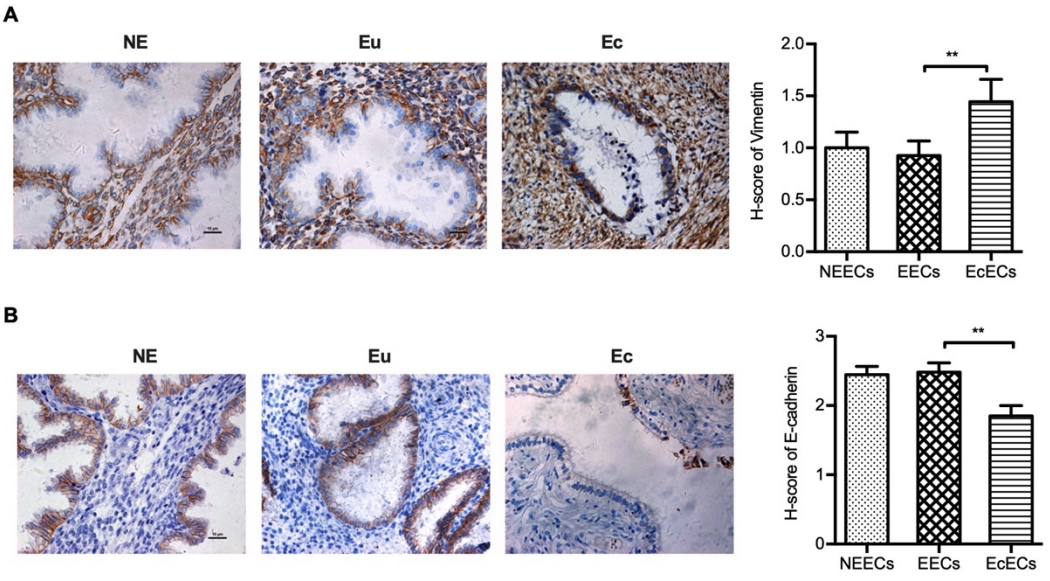
estrogen+ICI180782 or DMSO treatment for 12- and 48 h. $^{*}P < 0.05$;$^{**}P < 0.01$.

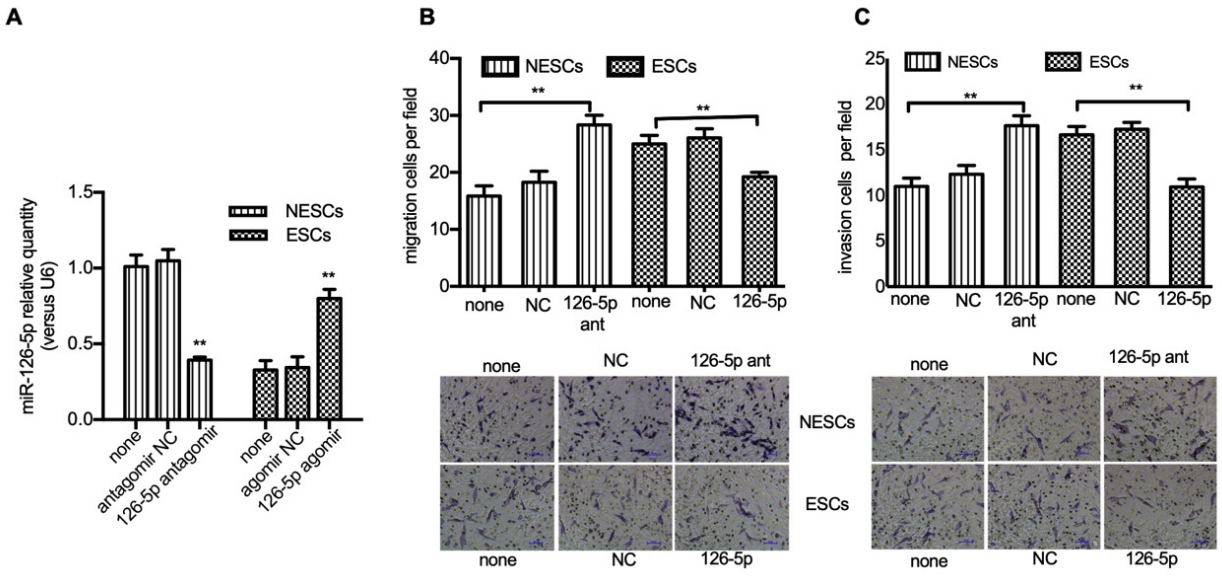
Table. S1 Demographics information of the patients.

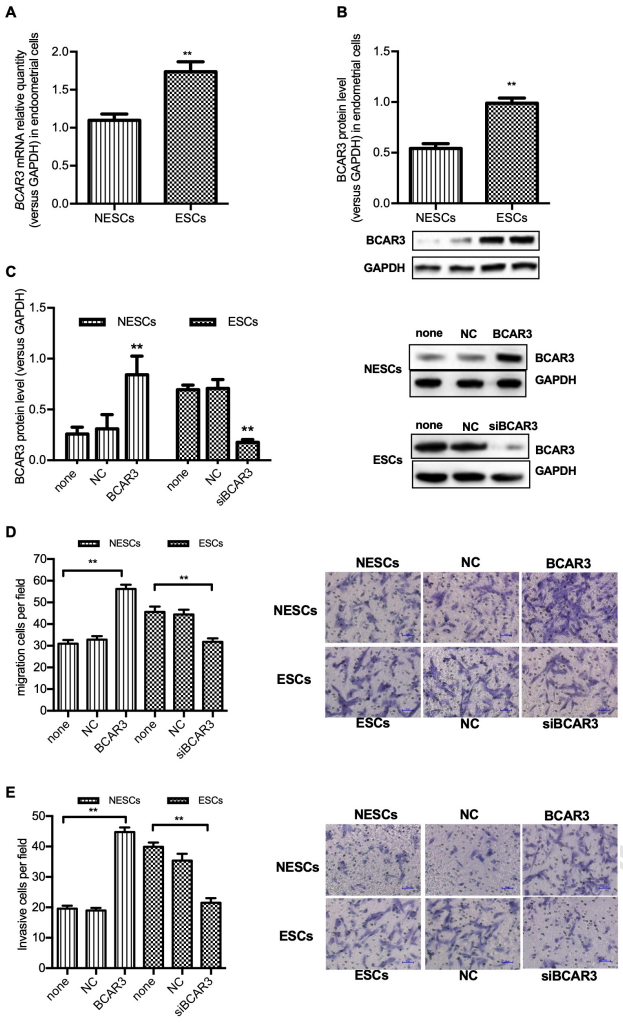
Fig. S2 Western blot detection of the expression of BCAR3 in response to estrogen (E2, 10-8 M) and estrogen (E2, 10^{-8} M)+ ICI182780 (10^{-7} M) in EECs.

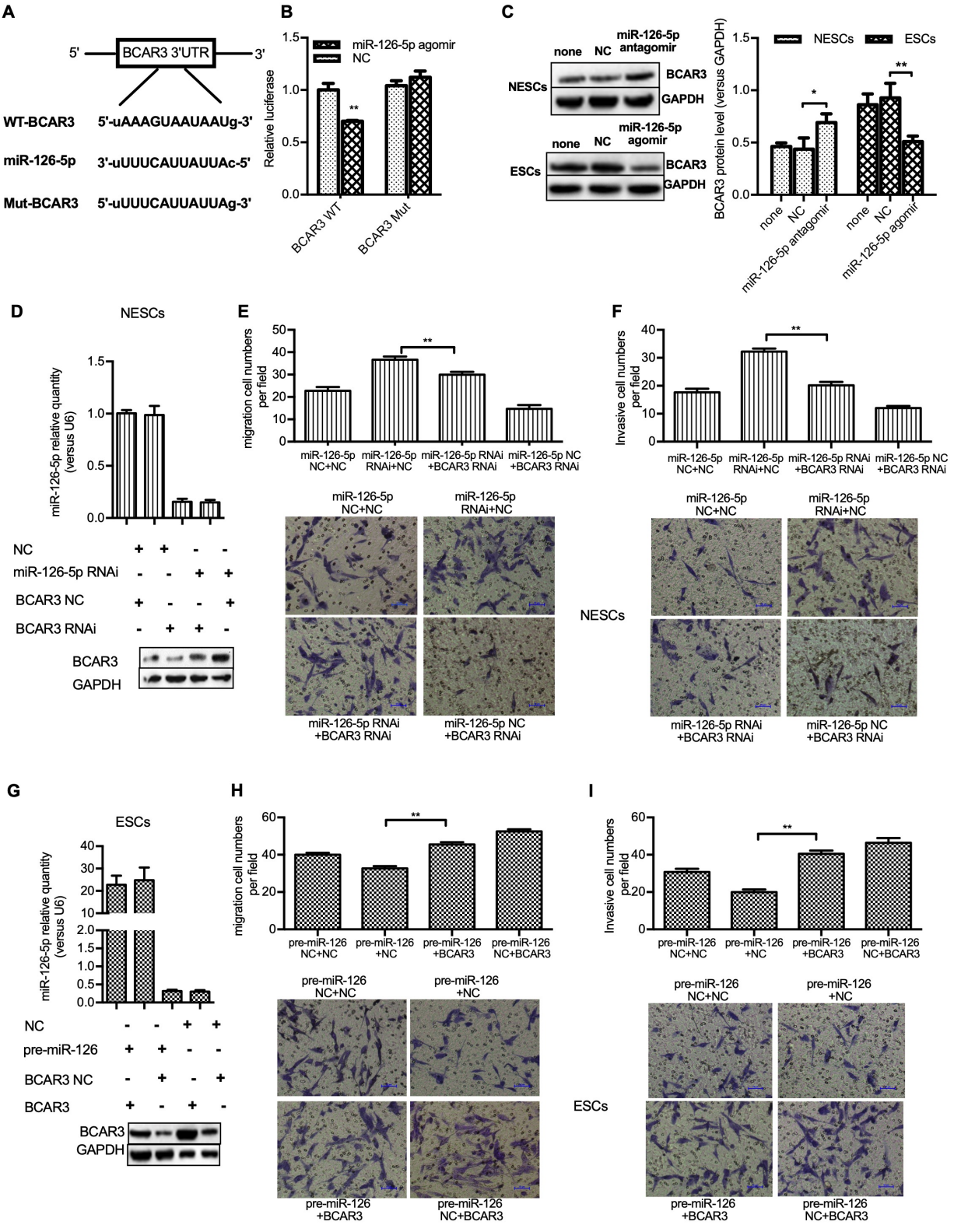











A
5'
BCAR3 3'UTR
3'
WT-BCAR3 5'-uAAAGUAAUAAUg-3'
miR-126-5p 3'-uUUUCAUUAUUAc-5'
Mut-BCAR3 5'-uUUUCAUUAUUAg-3'
B
miR-126-5p agomir
NC
Relative luciferase
BCAR3 WT
BCAR3 Mut
C
miR-126-5p antagomir
none NC
NESCs
BCAR3
GAPDH
miR-126-5p agomir
ESCs
BCAR3 protein level (versus GAPDH)
NESCs ESCs
miR-126-5p antagomir
miR-126-5p agomir
D
NESCs
miR-126-5p relative quantity (versus U6)
NC
miR-126-5p RNAi
BCAR3 NC
BCAR3 RNAi
BCAR3
GAPDH
E
migration cell numbers per field
miR-126-5p NC+NC
miR-126-5p RNAi+NC
miR-126-5p RNAi +BCAR3 RNAi
miR-126-5p NC +BCAR3 RNAi
F
Invasive cell numbers per field
NESCs
G
ESCs
miR-126-5p relative quantity (versus U6)
NC
pre-miR-126
BCAR3 NC
BCAR3
GAPDH
H
migration cell numbers per field
pre-miR-126 NC+NC
pre-miR-126 +NC
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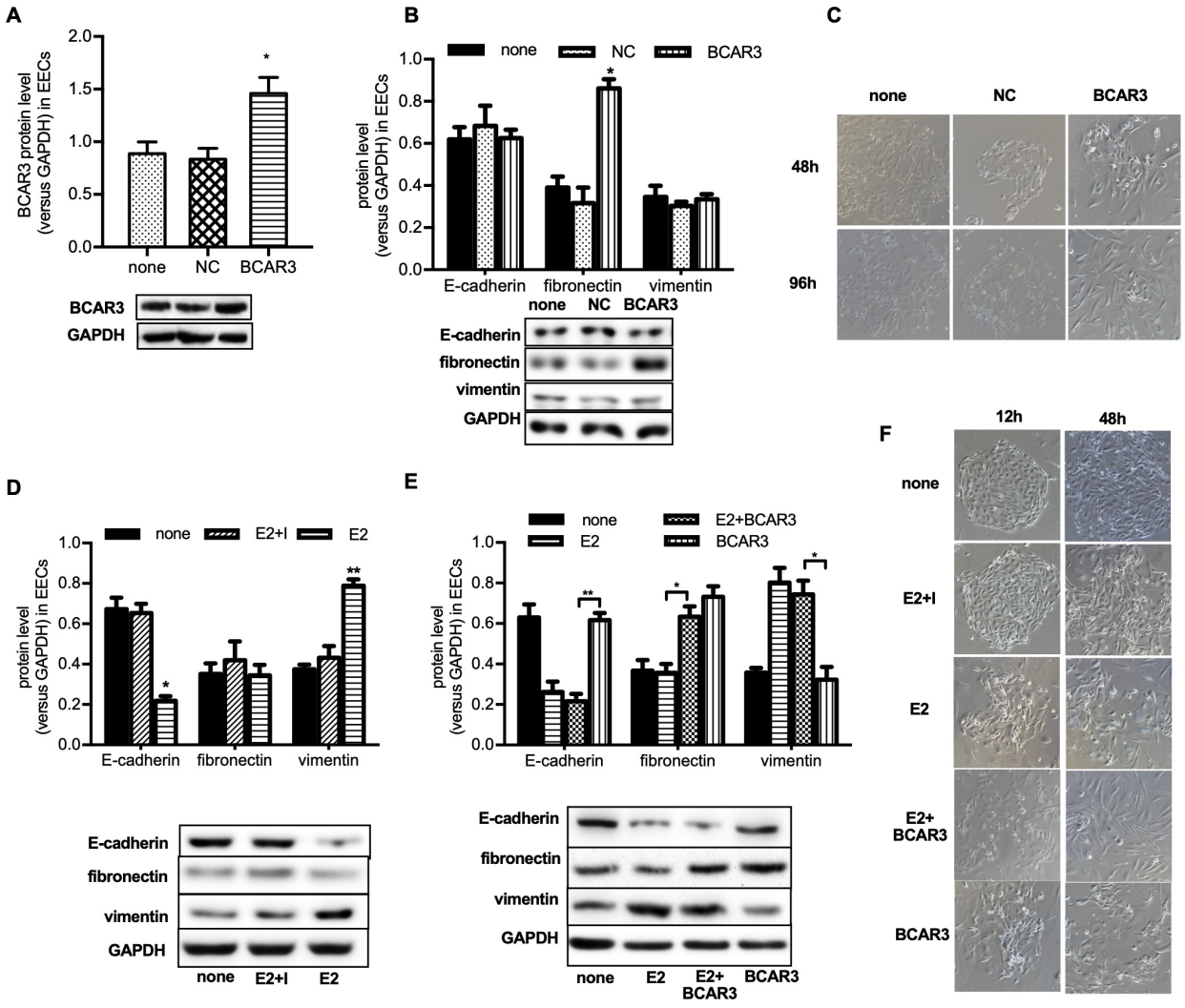
pre-miR-126 NC+BCAR3
I
Invasive cell numbers per field
ESCs



Highlights:

1. Upregulation of BCAR3 in eutopic and ectopic endometrium
2. BCAR3 overexpression promotes the proliferation, migration, and invasion of ESCs.
3. BCAR3 is the direct target of miR-126-5p.
4. Estrogen promoted EMT in the ectopic endometrium of endometriosis
5. BCAR3 did not have a synergistic effect on EMT with estrogen