RNAi-mediated blocking of ezrin reduces migration of ectopic endometrial cells in endometriosis

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ABSTRACT: Ezrin is a member of the ezrin–radixin–moesin (ERM) family of membrane–cytoskeletal linkage proteins. It is important for maintenance of cell shape, adhesion, migration and division. The overexpression of ezrin in some tumours is associated with increased cell migration that is mediated by the Rho/ROCK family of small GTPases. To investigate the role of ezrin in the migration of ectopic endometrial cells in endometriosis, we conducted real-time quantitative RT–PCR analysis of the eutopic and ectopic endometrium from women with endometriosis compared with those without the disease. RNAi, wound healing assays and western blot analysis of endometriotic cells were also included in this research. We found significantly higher levels of mRNA expression of ezrin (0.42 versus 0.27, P < 0.05), RhoA (0.99 versus 0.74, P < 0.05), RhoC (0.79 versus 0.43, P < 0.005) and ROCK1 (0.68 versus 0.38, P < 0.005) in the ectopic endometrial cells compared with the eutopic endometrial cells in endometriosis. Blocking ezrin with small-interfering RNA reduced the migration of ectopic endometrial cells with decreased expression of RhoA (42.68%), RhoC (58.42%) and ROCK1 (59.88%). Our results indicate that the over-expression of ezrin in endometriosis may play a significant role in the migration of endometrial cells of endometriosis, and the RhoC/Rock pathway may provide a promising treatment target.

Key words: endometriosis / endometrium / cell culture / RNAi

Introduction

Endometriosis is characterized by growth of endometrial tissue (including glands and stroma) outside the uterus. It causes abdominal pain and infertility in 10–15% of women of reproductive age (Klemmt et al., 2007), but the pathogenesis remains elusive. Recent research shows that endometrial cells in endometriosis have increased adhesion, proliferation and migratory capacity compared with normal endometrial cells, and exhibit features of malignant behaviour including proliferation, angiogenesis, abnormal apoptosis and invasion (Abrao et al., 2006; Niggli and Rossy, 2008).

Ezrin is a member of the ezrin–radixin–moesin (ERM) family of membrane–cytoskeletal linkage proteins. It links actin filaments to cell membranes either directly, by binding to cytoplasmic tails of transmembrane proteins or indirectly, via scaffolding proteins attached to transmembrane proteins (Niggli and Rossy, 2008). Previous research demonstrates that ezrin contributes to cytoskeletal processes underlying many cellular functions including cell division, adhesion and migration (Brambilla and Fais, 2009; Federici et al., 2009; Ren et al., 2009). Ezrin may participate in cell adhesion events by interacting with cell-surface adhesion molecules (Granés et al., 2000; Lozupone et al., 2004; van Buul et al., 2004; Yang et al., 2009). In its activated form, ezrin binds to membrane proteins such as CD43, CD44, CD95, ICAM 1-3, syndecan-2 and E-Cadherin by its N-terminal domain (Granés et al., 2000; Morrison et al., 2001; Lozupone et al., 2004; Yang et al., 2009). Its C-terminal domain anchors filaments to the plasma membrane at specific sites. The expression of ezrin is associated with enhanced invasiveness of tumor cells (Weng et al., 2005; Yu et al., 2004) and poor survival in several cancers (Pang et al., 2004; Weng et al., 2005). The suppression of ezrin in several murine and human cancer models inhibits metastasis of cancer cells (Khanna et al., 2001; Pang et al., 2004; Yu et al., 2004; Ilmonen et al., 2005; Weng et al., 2005).

The overexpression of RhoA/RhoC promotes invasiveness in some cancers and plays a role in tumor cell metastasis (Horiuchi et al., 2003; Shikada et al., 2003; Wang et al., 2003; Kondo et al., 2004). Rho GTPase family proteins regulate cell shape, adhesion and metastasis through the Rho/ROCK pathway (Etienne-Manneville and Hall, 2002; Kamai et al., 2003). Recent studies demonstrate the influence of ezrin on cell migration and division through the RhoA/ROCK I pathway.
The reference gene for normalization of the results. The use of the specific primers for each gene was synthesized according to standard procedures in the literature (Friel et al., 2004). Taken together, these findings suggest a possible role for ezrin in the activation of Rho family members. The overexpression of ezrin in tumor cells promotes tumour cell migration through Rho/ROCK pathways, which may contribute to migration of endometriotic cells in endometriosis. To date, the relationship between endometriosis and Ezrin–Rho–ROCK pathway has not extensively investigated.

This study seeks to determine whether ezrin/Rho/ROCK pathways exert regulatory actions on migration of human endometrial cells in endometriosis.

**Materials and Methods**

**Patient selection and sample collection**

We obtained endometrial cells from endometriosis lesions [ectopic endometriosis (EMs)], and homologous eutopic endometrium (eutopic-EMs) tissue in women with stage III–IV endometriosis (according to revised AFS classification). Subjects included 15 women (mean age: 35.8 years, parity: 23–52); 9/15 were in the proliferative phase of the menstrual cycle and 6/15 in the secretory phase. In a control group (non-EMs), we obtained endometrial tissues from 14 women without medical diseases such as hepatic or thyroid disorders from this study. We prepared ezrin siRNA sequences targeted for human ezrin according to previous reports (Barrero-Villar et al., 2009); 5′-UCCACUAUGUGGA AUAA-3′ (forward) and 5′-UUAUAAUUCACAUAGUGGA-3′ (reverse). A non-specific sequence with no homology with the human genome (Jikai, Shanghai, China) was used as a control sequence: 5′-GCGUGACACGUUCGGAG-3′ (forward) and 5′-ACGUCAUGCGAG-3′ (reverse). The endometrial stromal cells were cultured for small-interfering RNA (siRNA) transfection, wound healing assay and western blot analysis. Cells from each patient constituted separate experiments, and we repeated all experiments three times using cells obtained from different patients.

**RNA extraction and real-time quantitative RT–PCR**

We isolated total RNA from tissue samples using Trizol reagent (Invitrogen, CA, USA), according to manufacturer’s instructions. We confirmed purity and quantity of RNA by NanoDrop® ND-100 Spectrophotometer (Thermo Fisher Scientific, Inc., USA). A260/280 of total RNA applied in this study was > 1.8 and < 2.0. cDNA was synthesized from 2 μg of total RNA in 20 μl reaction mixtures, using the PrimeScript™TM RT reagent Kit (Perfect Real-time, Takara, Dalian, China), then all the cDNA samples were used as templates for real-time quantitative PCR. The specific primers for each gene were synthesized according to standard procedures in the literature (Friel et al., 2006; Zhu et al., 2007; Pang et al., 2010; Table I).

Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was chosen as the reference gene for normalization of the results. The 20 μl real-time quantitative reverse transcriptase PCR (QPCR) mixtures contained 1 μl of cDNA, 5 μM of each primer and 2× SYBR® Premix Ex Taq™ (Takara). The reaction systems were carried out as follows: hold for 30 s at 95°C, followed by two-step PCR for 40 cycles of 95°C for 10 s and 60°C for 34 s, then 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. All reactions were performed using the iCycler iQ Real-Time Detection System (Bio-Rad). The threshold cycle (Ct) value was defined as the value when the fluorescence signal increased above the background threshold. A housekeeping gene GAPDH was used as a control sequence: 5′-GCACCCTCAAGGCTGAGAAC-3′ (forward) and 5′-ATGGTAGCTCAGGATGACGTG-3′ (reverse). We calculated relative mRNA expression using the formula: 2(−ΔCt).

**Isolation and culture of human endometrial stromal cells**

We prepared primary endometrial stromal cells in a standard fashion. Briefly, we rinsed endometrial tissues in HBSS to remove blood and debris, before digesting them with collagenase I (1 mg/ml; Gibco, CA, USA) for 1 h on the table concentrator at 37°C, and, purification using serial filtrations. The purified endometrial stromal cells were plated into six-well plates and subcultured twice to eliminate contamination by macrophages and other leukocytes. In addition, the endometrial stromal cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Sijiqing, Hangzhou, China). All cells were incubated at 37°C with 5% CO2 atmosphere and 95% humidity. High purity (>95%) of endometrial stromal cells were confirmed by immunocytochemistry with specific monoclonal antibody vimentin (Fig. 1).

**SiRNA interference and transfection**

We prepared siRNA sequences targeted for human ezrin according to previous reports (Barrero-Villar et al., 2009): 5′-UCCACUAUGUGGA AUAA-3′ (forward) and 5′-UUAUAAUUCACAUAGUGGA-3′ (reverse). A non-specific sequence with no homology with the human genome (Jikai, Shanghai, China) was used as a control sequence: 5′-GCGUGACACGUUCGGAG-3′ (forward) and 5′-ACGUCAUGCGAG-3′ (reverse). The endometrial stromal cells were seeded in a six-well plate at 1.0 × 10^6 cells per well, grown for 24 h and transfected with the constructed siRNA expression vector (100 pmol/well) with Lipofectamine™2000 (Invitrogen) according to the manufacturer’s instructions. The control group was transfected with non-specific control siRNA, while the blank group was transfected with free water. The plasmid–lipofectamine solution was removed and replaced by cell culture medium at 6 h.
after initiation of transfection. The transfection efficiency was measured by parallel transfection with BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen) and analysed by fluorescence microscopy at 24 h after transfection.

**Wound healing assay**

To evaluate the relation between cell migratory ability and expression of ezrin, we conducted a wound healing assay. After transfection and incubation for 24 h, the injury line was made using a pipette tip on cells plated in six-well plates. After rinsing with phosphate-buffered saline (PBS), cells were cultured in a complete medium with 5% FBS. Photographs were taken at time 0, 12, 24 and 48 h after wounding (×40). All experiments were carried out in triplicate.

**Western blot**

Cell lysates were prepared and the expression of proteins was measured with standard techniques (Takahashi et al., 2011). Each protein sample was prepared from cells treated with siRNA at 72 h after transfection. After rinsing with PBS, cells were lysed in 100 μl RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA and 0.1% SDS) containing protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin and aprotinin) on ice for 40 min. Subsequently, we separated 50 μg lysate on a 10% sodium dodecylsulphate polyacrylamide (SDS–PAGE) gel. Proteins were transferred to polyvinylidene fluoride membranes. After each membrane was incubated with various specific primary antibodies (1:500 dilution for Ezrin antibody; Bioworlde, MN, USA; 1:1000 dilution for Rho A, Rho C and ROCK1 antibodies; CST, Billerica, USA), we stripped and re-incubated the blot with a polyclonal antibody against β-actin (1:5000 dilution; ZSGB-BIO, Beijing, China) as an internal positive control. Alkaline phosphatase conjugated secondary antibodies (1:5000 dilution; ZSGB-BIO, Beijing, China) were detected by chemiluminescence with ECL (Thermo, Rockford, USA), using an enhanced chemiluminescence system (Thermo).

**Statistical analysis**

Software SPSS 16.0 was used in the statistical analysis. All data were expressed as the mean ± SD and the statistical significance was calculated by ANOVA with post hoc analysis (Fisher least significant difference (LSD)) or Student’s two-tailed t-test as appropriate. The level of significance was set at \( P < 0.05 \).

The ethics committee of Women’s Hospital, School of Medicine, Zhejiang University, Hangzhou, China approved this study. Written informed consent was obtained from each patient before operation procedure.

**Results**

**Ezrin mRNA expression in patients with EMs**

mRNA expression levels of Ezrin, RhoA, RhoC and ROCK1 were detected in tissue from three groups: ectopic-EMs, eutopic-EMs and non-EMs. The expression of each was significantly higher in ectopic-EMs endometrium than in eutopic-EMs (Fig. 2B) and non-EMs endometrium (Fig. 2B). However, there was no significant difference between the eutopic EMs and non-EMs tissues (Fig. 2B, \( P > 0.05 \)). There were no significant differences between the mRNA expression levels of ezrin, RhoA, RhoC and ROCK1 between the secretory phase and the proliferative phase, (Fig. 2C, \( P > 0.05 \)) in these three groups of endometrial tissues. The amplification plot and dissociation curve are recorded in Fig. 2A.

**Transfection and wound healing assay**

The experimental transfection efficiencies ranged from 80 to 90% (Fig. 3). We determined the migratory potential of primary endometrial stromal cells using a wound healing assay. The wound healing assay showed that endometrial stromal cells transfected with non-specific siRNA or free water, closed the scratch wounds more quickly than cells transfected with specific siRNA of ezrin (Fig. 4A). Furthermore, the migratory ability of endometrial stromal cells transfected with specific siRNA of ezrin was significantly decreased compared with cells transfected with non-specific siRNA and free water (Fig. 4B). Meanwhile, the mobility of cells between control and blank groups showed no significant difference (\( P > 0.05 \)).

**Western blot**

We performed a western blot analysis to assess the effects of ezrin siRNA on ezrin protein expression in primary endometrial stromal cells (Fig. 4C). Transfection with siRNA targeting ezrin markedly down-regulated the expression of ezrin in endometrial stromal cells. The mean interference efficiency of ezrin in the protein level was
Figure 2 The mRNA expression levels of ezrin, RhoA, RhoC, ROCK1 detected by real-time quantitative RT–PCR. (A) The amplification plot and dissociation curve of ezrin, RhoA, RhoC, ROCK1. (a) The amplification plot of ezrin, RhoA, RhoC, ROCK1; (b) the dissociation curve of ezrin, RhoA, RhoC, ROCK1. (B) The comparison of mRNA expression levels of ezrin, RhoA, RhoC, ROCK1 in ectopic-EMs, eutopic-EMs and non-EMs endometrium. *$P < 0.05$, **$P < 0.005$, comparison with the ectopic-EMs group and eutopic-EMs group; #$P < 0.005$, comparison with the ectopic-EMs group and non-EMs group. (C) The comparison of mRNA expression levels of ezrin, RhoA, RhoC, ROCK1 in the proliferative and secretory phases. (a) The mRNA expression levels of ezrin, RhoA, RhoC and ROCK1 in different menstrual cycles in the ectopic-EMs endometrium; (b) the mRNA expression levels of ezrin, RhoA, RhoC and ROCK1 in different menstrual cycles in the eutopic-EMs endometrium; (c) the mRNA expression levels of ezrin, RhoA, RhoC and ROCK1 in different menstrual cycles in non-EMs endometrium.

Figure 3 The detection of transfection efficiency in human endometriotic stromal cells. (A) Morphologic characteristics of endometrial stromal cells under natural light of 24 h after transfection. (B) The same vision of above endometrial stromal cells under red fluorescence. The efficiency of siRNA transfection in endometriotic stromal cells ranged from 80 to 90%.
In addition, the expressions of RhoA/RhoC/ROCK1 in the group transfected with specific siRNA of ezrin were also significantly decreased compared with cells transfected with non-specific siRNA and free water. The mean efficiency of RhoA, RhoC and ROCK1 was 42.68 ± 8, 58.42 ± 14 and 59.88 ± 13%, respectively. The decreased protein level of RhoA had no statistical significant differences (Fig. 4D).

Discussion

Cytoskeletal proteins are known effectors of cell motility. There is evidence that the ectopic endometrial cells were more invasive than normal endometrial cells with the ability to invade the extra-cellular matrix after initial adhesion (Spuijbroek et al., 1992; Ornek et al., 2008). In addition, the metastasis of endometrial cells also includes the cytoskeleton reconstruction and cell—cell and cell—matrix adhesion. In this study, we evaluated the levels of mRNA expression and roles of the cytoskeletal protein, ezrin, in the ectopic and eutopic endometrium in women with advanced endometriosis. Our in vivo data show that mRNA expression levels of ezrin are significantly higher in ectopic endometrial cells compared with eutopic and normal endometrium (Fig. 2). In our in vitro research, down-regulation of ezrin by specific siRNA resulted in reduced migration of endometrial stromal cells (Fig. 4). Ornek et al. (2008) reported that the stroma of ectopic implants had significantly greater expression of ezrin and phospho-ezrin than eutopic and normal endometrium. They also reported more cell protrusions and increased expression of ezrin in membrane invasion assays. They concluded that increased expression of ezrin in women may enable endometrial cells to migrate, and implant outside the uterine cavity. However, they conceded that their studies did not establish whether the changes in ezrin and phospho-ezrin expression were a cause or a consequence of the condition. Our study adds weight to their original observations, although it does not clarify the latter point.

Real-time, quantitative PCR demonstrates that ectopic endometrial cells EMs have the highest mRNA expressions of RhoA, RhoC and ROCK1. RhoA and RhoC are homologous proteins, sharing the same group of effectors with similar functions and actions (Jaffe and Hall, 2005). Both are molecular switches for all extracellular signalling, regulating various biological cellular functions such as the rearrangement of the cytoskeleton and the adhesion and migration of cells. The expressions of Rho/ROCK are also significantly increased in
some tumors, particularly during their progression to more invasive and metastatic phenotypes. The overexpression of RhoA and RhoC disrupts cell–cell junctions, induces migration and movement of tumour cells and promotes focal adhesions between cell and extracellular matrix via ROCK-mediated signal pathways (Sahai and Marshall, 2002a, b). We speculate that increased RhoA, RhoC and ROCK1 in the ectopic endometrium may also contribute to cell migration and invasiveness, which are key characteristics of ectopic endometrial tissues.

Recent studies confirm that RhoA is expressed equally in cancers with metastatic potential, suggesting that the expression of RhoA may not be a primary determinant of metastasis. In addition, when expressed at equivalent levels, RhoC is a better mitogen than RhoA (Clark et al., 2000). However, to the best of our knowledge, there was no report on the relationship between RhoA and RhoC in the migration of ectopic endometrial cells. In our study, down-regulation of ezrin resulted in significant reduced protein expression levels of RhoC and ROCK1, but not RhoA. RhoC is more likely to be associated with enhanced mobility and migration of endometrial stromal cells, and the role of ezrin in the migration of endometrial stromal cells may take effect through the RhoC/ROCK1 pathway rather than the RhoA/ROCK1 pathway.

The coincident endometrial ezrin, RhoA/RhoC and ROCK1 mRNA expression during the proliferative and secretory phases suggested that hormones might not be a core influencing factor. However, according to the former researches on ERM protein signalling in cancers, estrogen has been reported to be an effective regulator of several cytoskeletal components, and it can activate selected members of the ERM family of actin-binding proteins (Simoncini et al., 2006; Giretti et al., 2008). Recently, Flamini et al. (2009) reported that 17β-estradiol and tamoxifen could rapidly activate a estrogen receptor/RhoA/ROCK/moesin signalling pathway in human endometrial Ishikawa cells and in primary cultured stromal cells. The sample size from the proliferative stage in this study were much larger than those from the secretory phase, and the definite relationship between ezrin, RhoA/RhoC and ROCK1 and the menstrual cycle still needs to be further confirmed.

In summary, this study suggests that increased expression of ezrin enhances the migration of ectopic endometrial cells in endometriosis. The interference results suggest that there is a functional link between ezrin and RhoC/ROCK1 signalling pathways in the migration of ectopic endometrial cells. We speculate that increased expression of ezrin in the ectopic endometrium leads to morphological changes in the endometrial cell cytoskeleton, resulting in enhanced cell migration. Furthermore, elevated ezrin levels may amplify activation of RhoC and enhance the expression of RhoC/ROCK1 to promote the adhesion of endometrial cells. Together, these findings support the notion that ezrin and downstream RhoC/ROCK1 signalling regulate the migration of ectopic endometrial cells.

Authors’ roles
The study was designed by R.J.W., the corresponding author who also revised the manuscript critically for important intellectual content. Q.Y.J. wrote the manuscript and performed the real-time RT–PCR, siRNA interference and wound healing assay. J.M.X. performed the western blot analysis. H.G.D. collected the specimen and cultured the ectopic endometrial cells. X.W.F. drafted the figures and J.L. was responsible for the acquisition, analysis and interpretation of data. All authors contributed to the writing of the manuscript.

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Conflict of interest
None declared.

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