CD82 gene suppression in endometrial stromal cells leads to increase of the cell invasiveness in the endometriotic milieu

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Abstract

Tetraspanin CD82 is a wide-spectrum tumor metastasis suppressor that inhibits motility and invasiveness of cancer cells. Endometriosis is a benign gynecological disorder, but appears malignant behaviors including invasion, ectopic implantation and recurrence. This study is to elucidate the role of CD82 expression regulation in the pathogenesis of endometriosis. The short interfering RNA silence was established to analyze the roles of CD82, chemokine CCL2, and its receptor CCR2 in the invasiveness of endometrial stromal cells (ESCs). We have found that the mRNA and protein levels of CD82 in the primary normal ESCs from endometrium without endometriosis are significantly higher than that of the primary ESCs from eutopic endometrium and ectopic tissue. CD82 inhibits the invasiveness of ESCs by downregulating CCL2 secretion and CCR2 expression via mitogen-activated protein kinase (MAPK) and integrinβ1 signal pathway, and in turn upregulating the expression of TIMP1 and TIMP2 in an autocrine manner. The combination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with 17β-estradiol can promote the invasion of ESCs via suppressing CD82 expression and stimulating CCL2 secretion and CCR2 expression, and the enhanced interaction of CCL2–CCR2 recruits more macrophages into the ectopic milieu in a paracrine manner, which further downregulates CD82 expression in the ectopic ESCs. Our study has demonstrated for the first time that the abnormal lower CD82 expression in ESCs induced by TCDD and estrogen may be an important molecular basis of endometriosis pathogenesis through enhancing the CCL2 secretion and CCR2 expression and the invasion of ESCs via MAPK and integrinβ1 signal pathway.

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Introduction

Endometriosis is a very frequent benign gynecological disorder in fertile women, but the pathogenesis still remains controversial despite extensive research; however, Sampson’s theory of implantation of endometrial cells and fragments refluxed during the menstrual period is generally accepted among these hypotheses (Sampson 1925). Retrograde menstruation is, however, a physiological process that takes places almost in all menstruation cycles, and a growing body of evidence suggests that the primary defect in endometriosis can be located in the eutopic endometrium. Abnormalities inherent to the eutopic endometrium that are not found in the endometrium of women without endometriosis might therefore contribute to ectopic growth outside the uterine cavity (Ulukus et al. 2006). Different characteristics of eutopic endometrium of women with endometriosis, such as aberrant production of cytokine, growth, adhesion, and angiogenic factors as well as specific cancer-related molecules, are believed to contribute to the occurrence and maintenance of this disease.

The CD82 metastasis suppressor gene is implicated in biological processes of tumor invasion, metastases, growth of metastatic tumors, cell motility, and adhesion (Takaoka et al. 1998, Yang et al. 2001). The growing evidence shows that CD82 inhibits cell motility through regulating the associated protein such as integrin (Mannion et al. 1996, Sugiura & Berditchevski 1999, Sridhar & Miranti 2006), epidermal growth factor receptor (EGFR; Odintsova et al. 2000), and Duffy antigen receptor for chemokines (DARC; Bandyopadhyay et al. 2006). The expression of CD82 is involved in decidual transformation from human endometrial stromal cells (ESCs; Gellersen et al. 2007). Moreover, our previous work has confirmed that CD82 in decidual stromal cells controls the trophoblasts invasiveness by paracrine signals, such as...
HB-EGF, IL1, and LIF, which also can induce CD82 in ESCs for controlling trophoblasts invasion (Gonzalez et al. 2011). Since similarity between cancer and endometriosis is valid, it appears feasible to hypothesize that the regulation of CD82 expression occurs in the eutopic endometrium that predisposes to invasion, implantation, adhesion, survival, and growth of ESC in the ectopic milieu.

An increased number of active macrophages have been found in peritoneal fluid of patients with endometriosis. A series of research has shown that chemokines produced in the endometriotic milieu may contribute to a feed-forward cascade of events, which accentuates the recruitment of leukocytes into the peritoneal cavity of patients with endometriosis (Akoum et al. 2000). CCL2 is a specific factor that chemoattracts and activates monocytes and macrophages that is a major ligand of receptor CCR2. The monocyte/macrophage system is considered to play an important role in the maintenance of humoral and cell-mediated immunity. It has been reported that the activity of CCL2 is elevated in the peritoneal cavity of patients with endometriosis (Akoum et al. 1996a,b). Several investigators reported a relationship of CCL2 levels in serum (Akoum et al. 1996a,b, Kim et al. 2008) or peritoneal fluid (Arici et al. 1997, Kim et al. 2008) to endometriosis, but the published results are conflicting. Moreover, Garcia-Velasco et al. (1999) have found that integrin β1 can stimulate the CCL2 secretion during the process of ESCs adhesion to extracellular matrix (ECM), therefore, it can be speculated that CD82 may regulate the biological function of ESCs by the effect of integrin β1 on CCL2 secretion.

The initial phase of endometriosis is an invasion event that requires ECM breakdown and repair of tissues, such as an increased activity of these enzymes (MMP1, MMP2, and MMP9; Bruner-Tran et al. 2002, Wu et al. 2005). Indeed, MMPs and TIMPs levels have been correlated to the development and progression of endometriosis (Wu et al. 2005, Kang et al. 2008). In addition, integrins mediate the cell–cell and cell–matrix interaction, and regulate various cellular functions including motility, migration, death, metastasis, and proliferation (Hynes 1992), and are also related to the progression of uterine adenomyosis (Klemmt et al. 2007).

Recently, evidence has begun to accumulate that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure promotes occurrence of endometriosis (Birnbaum & Cummings 2002, Rier & Foster 2003). Research work on primates has shown that exposure to TCDD is associated with an increased prevalence and severity of endometriosis (Rier & Foster 2002). Within either the endocrine or immune system, exposure to TCDD affects individual cell behavior by initially binding to the aryl hydrocarbon receptor (AhR) that rapidly forms a heterodimeric complex with AhR nuclear translocator (ARNT; Mimura & Fujii-Kuriyama 2003). The TCDD/AhR/ARNT complex associates with dioxin response elements to act as a signal transducer and transcription factor for target genes, including cytochromes P450 1A1 (CYP1A1), P450 1B1 (CYP1B1) (Kress & Greenlee 1997), and the upregulation of these genes is involved in cell proliferation, differentiation and inflammation. Since endometriosis is an estrogen-dependent disease (Rizner 2009), and the inflammatory milieu in the peritoneal cavity of women with endometriosis has been extensively characterized, altered metabolism of estradiol by TCDD or other dioxin-like halogenated aromatic hydrocarbons, and pro-inflammatory effects of TCDD may be involved in the pathogenesis of endometriosis. Our previous research has demonstrated that the combination of 17β-estradiol (E2) with TCDD upregulates CXC1R1 expression in ESCs, and promotes secretion of IL8, a ligand of CXC1R1, in co-culture of ESC–HPMC (HMrSV5, a human peritoneal mesothelial cell line) cells (Shi et al. 2006). Moreover, combination of E2 with TCDD increases the secretion of RANTES and MIP-1z, promotes the invasiveness of ESCs and increases the expression of MMP2 and MMP9 in ESCs (Yu et al. 2008).

In this study, we first evaluated the expression of CD82 in the primary ESCs from the normal endometrium, or eutopic and ectopic ESCs with endometriosis, and then observed the effects of TCDD and E2 on CD82 expression and invasiveness of the ESCs. To better understand the role of CD82 in the progression of endometriosis, we investigated the effects of CD82 expression on CCL2 secretion, CCR2 and the invasion-related molecules expression, and their potential pro-invasion activity to ESCs.

Materials and methods

Tissue collection, cell isolation, and culture

All tissue samples were obtained with informed consent in accordance with the requirements of the research ethics committee in Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. Samples of endometriotic peritoneal surface lesion (n=6) and ovarian lesion (n=6) were obtained from women age 21–49 years undergoing laparoscopy for pain or other benign indications. The patients with endometriosis were classified according to the revised American Fertility Society (AFS) classification: five in Stage 1 and seven in Stage 2. Endometrial tissues were obtained from fertile women (age 22–48 years) with (n=12) or without (n=6) endometriosis as control. The samples were obtained by pipeelle biopsy during diagnostic laparoscopy or by uterine curettage for benign indications. The absence of visible endometriosis as the control was confirmed by the surgeon.
performing the operation. None of the women had received hormonal medication in the 3 months before the surgical procedure. All the samples were obtained in the proliferative phase of the cycle, which was confirmed histologically according to established criteria.

All the tissues were collected under sterile conditions and transported to the laboratory on ice in DMEM/F-12 (Gibco) with 10% FCS (HyClone, Logan, UT, USA). The endometriotic tissue was dissected away from the adjacent tissue, and diagnosis was confirmed by histological examination. The endometriotic and endometrial tissues were digested with collagenase type IV (0.1%; Sigma) for 30 min at 37°C with constant agitation for recovering ESCs. The tissue pieces were filtrated through sterile gauzes (pore diameter sizes: 200 mesh) to remove debris. Following gentle centrifugation, the supernatant was discarded, and the cells were resuspended in DMEM/F-12. The ESCs were separated from epithelial cells by passing them over sterile gauzes (pore diameter sizes: 400 mesh). The filtrated suspension was layered over Ficoll, and centrifuged at 800 g for 20 min to further remove leukocytes and erythrocytes, and the middle layer was collected and then washed with D-Hanks solution. The ESCs were placed in a culture flask, and allowed to adhere for 20 min. The adherent stromal cells were cultured as monolayer in flasks with DMEM/F-12 containing 10% FCS and 20 mmol/l HEPES and incubated in 5% CO2 at 37°C. This method supplied a 95% purity of ESCs.

Human monocyte U937 cell line (purchased from Bank of Cell, Chinese Academy of Sciences, Shanghai, China) was maintained in RPMI 1640 medium (Life Technologies) with 10% bovine calf serum and 20 mmol/l HEPES at 37°C in a humidified, CO2-controlled (5%) incubator.

Quantitative real-time PCR

The total RNA was extracted from the normal (n=6), eutopic (n=6), and ectopic (n=6) ESCs with TRI reagent (Molecular Research Center, USA). The cDNA was generated with oligo (dT) 18 primers using Revert Aid First, Strand cDNA Synthesis Kit (Fementas Life Science, Glen Burnie, MD, USA). Triplicate samples containing cDNA prepared as mentioned-above, Taqman universal PCR master mix (Applied Biosystems, Foster City, CA, USA), specific primers and fluorescent dye-labeled Taqman MGB probes for CD82 and GAPDH were mixed, and analyzed on an ABI7000 thermal cycler (Applied Biosystems). The primers were designed and synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). The primer pairs for cDNA amplification were as follows: 5'-CTG GGG CTC TG TTT GCT TTC-3' (forward) and 5'-GAG GGG CCA AAA GGG TCA TCA TCT-3' (reverse) for human CD82; 5'-GCC CCA UGG UGA AGC UCA GUC A-3' (forward) and 5'-GAG GGG CCA AAA GGG TCA TCA TCT-3' (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cycling conditions consisted of a denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, a 60 s annealing step at 62°C, and finally a holding temperature of 15°C. To determine the amount of gene product present in the sample, cycle time (Ct) was determined. The average Ct value was calculated from triplicate wells for each sample with each primer set. Most duplicate samples varied by <0.5 Ct. The relative gene expression for individual cDNA samples was determined by calculating ΔCt values (ΔCt) by subtraction of the Ct value for GAPDH primers from the Ct value for CD82 primers. The relative fold expression of CD82 was determined compared with the control. The experiments were carried out in triplicate.

CD82 silence in ESCs

For short interfering RNA (siRNA) transfection, ESCs from eutopic endometrium with endometriosis (n=6) were seeded in 96-well plates. When cells had reached confluency, medium was changed to OPTIMEM (Invitrogen). The siRNA oligonucleotides targeting CD82 (set of three oligonucleotides; Stealth Select RNAi; Invitrogen) and Lipofectamine 2000 (Invitrogen) were mixed in OPTIMEM, and then added to the cells at room temperature with non-targeting siRNA oligonucleotides as negative control, without any treatment group as blank control. After 6 h incubation, the cells were incubated in DMEM for further 72 h in 5% CO2 at 37°C until the successful gene knockdown was confirmed by in-cell Western and western blot. The sequences for three dsRNA oligonucleotides were as follows: (CD82-HSS105652) 5'-AUC AGG AGC AGG AAA GGA AAG UAC A-3' (forward) and 5'-UGU ACU UUG CUU UCC UGC UCC UGA U-3' (reverse); (CD82-HSS105653) 5'-UGC CCA UGU UGA AGA AGA GGG C-3' (forward) and 5'-GCC CUC UUC UAC UUC AAC AUG GCC A-3' (reverse); and (CD82-HSS105654) 5'-UCU CGA AUG AGC UCA GUC ACG AUG C-3' (forward) and 5'-GCA UCG UGA AGC UCA UUC GAG A-3' (reverse). The results showed that the silencing efficiency of the first one is best, so in all subsequent experiments, we used this siRNA to silence CD82 expression in eutopic ESCs, with non-targeting siRNA as control.

Treatment in vitro with E2 and TCDD

After starvation for 12 h, the ESCs (1×10^5 cells/well; FCS of cultured media was the charcoal stripped FCS) from women with (n=6) or without endometriosis (both the sample number of eutopic and ectopic was 6) were treated, respectively, with TCDD (Sigma) or E2
(Sigma) at concentrations ranging from $10^{-12}$ to $10^{-7}$ M for 48 h, to observe the effect of TCDD or E_{2} on CD82 expression in ESCs. In the subsequent investigation, ESCs were treated with TCDD at $10^{-9}$ M, E_{2} at $10^{-8}$ M, or the combination of TCDD with E_{2} for 48 h, respectively, with vehicle dimethyl sulfoxide (DMSO) as control. Each experiment was carried out in triplicate, and repeated three times.

**Contact co-culture of two sorts of cells**

The eutopic ESCs ($n=6$) from endometrium with endometriosis were cultured in 24-well plates at a concentration of $1 \times 10^{5}$ cells/well until adhering to the plastic. The media was removed, and then the U937 cells ($2 \times 10^5, 1 \times 10^5,$ or $5 \times 10^5$ cells/well) were applied. The proportion of ESCs and U937 cells was 5:1, 1:1, and 1:5 respectively. The cells were cultured in a final volume of 200 μl fresh DMEM/F-12 with 10% FCS for 48 h. The ESCs of $1 \times 10^5$ cells/well cultured alone were used as controls. Then, we discarded the suspended U937 cells and co-cultured supernatant, and used in-cell Western to analysis the CD82 expression. Each experiment was carried out in triplicate, and repeated three times.

**Treatment in vitro with CCR2 antagonist RS102895 and anti-CCL2 neutralizing antibody**

The primary ESCs ($n=6$) or CD82-silenced ESCs ($n=6$) from normal endometrium were treated with various concentration of RS102895 (a CCR2 antagonist, 0–500 ng/ml, Sigma) or anti-CCL2 neutralizing antibody (0–5 μg/ml, R&D Systems, Abingdon, UK), 0.1% DMSO was used as control. After 24, 48, and 72 h of culture, the cells were detected by Matrigel invasion assay. In the subsequent investigation, these ESCs and CD82-silenced ESCs were treated with RS102895 at 100 ng/ml or anti-CCL2 neutralizing antibody at 1 μg/ml for 48 h. Each experiment was carried out in triplicate, and repeated three times.

**Matrigel invasion assay**

The invasion of the ESCs ($n=6$) or CD82-silenced ESCs ($n=6$) from normal endometrium across Matrigel was evaluated objectively in an invasion chamber, based on our previous procedure (Yu et al, 2008). Briefly, the cell inserts (8 μm pore size, 6.5 mm diameter; Corning, New York, NY, USA) coated with 15–25 μl Matrigel were placed in a 24-well plate. The primary ESCs or siRNA-transfected ESCs of $2 \times 10^4$ were plated in the upper chamber (the media contained 1% charcoal stripped FCS). TCDD or/and E_{2}, MAPK inhibitor U0126 (30 μM), anti-integrinβ1 neutralizing antibody (1 μg/10^6 cells, R&D Systems), RS102895, or anti-CCL2 neutralizing antibody were added respectively. The lower chamber (the media contained 5% charcoal stripped FCS) was filled with 800 ml medium. The cells were then incubated at 37°C for 48 h. The inserts were removed, washed in PBS and the non-invading cells together with the Matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10 min at room temperature and stained with hematoxylin. The result was observed under Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan). The cells that had migrated to the lower surfaces were counted at a magnification of ×200. At same time, we seeded the same cells with Matrigel invasion assay in a 96-well plate, and detected the protein concentration by BCA Protein Assay (Beyotime Institute of Biotechnology, China). The invasion index of each group was calculated as the ratio of the number of cells migrated to the lower surfaces to the protein concentration. Each experiment was carried out in triplicate, and repeated three times.

**In-cell Western**

According to the description by Egorina et al. (2006), we used a newly set up assay called in-cell Western to determine the in-cell protein level of CD82, CCR2, MMP2, MMP9, TIMP1, TIMP2, integrinβ1, and integrinβ3. Further details are given in the Supplementary Materials and methods, and the precision analysis of the in-cell Western has been provided in Supplementary Figure 1 (see section on supplementary data given at the end of this article). The procedure was as follows: normal ESCs ($n=6$) or siRNA-transfected normal ESCs ($n=6$) in 96-well plate were incubated with or without U0126 (30 μmol/l; Cell Signaling Technology, Danvers, MA, USA), and anti-integrinβ1 neutralizing antibody (1 μg/10^6 cells; R&D Systems) for another 24 h, or treated with TCDD or E_{2} or combination of TCDD and E_{2} for 48 h, respectively, with vehicle as control. Then cells were immediately fixed with 4% formaldehyde in PBS for 20 min at room temperature. After washing with 0.1% Triton, the cells were blocked by adding 150 μl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 90 min at room temperature. The cells were incubated with mouse anti-human CD82 (20 μg/ml, SC-15572; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-human CCR2 (1:80; Abcam, Cambridge, MA, USA) or mouse anti-human MMP2 (20 μg/ml, R&D Systems), MMP9 (20 μg/ml, R&D Systems), TIMP1 (15 μg/ml, R&D Systems) or TIMP2 (15 μg/ml, R&D Systems) or integrinβ1 (10 μg/ml, R&D Systems), or integrinβ3 (10 μg/ml, R&D Systems) antibody. To assess the housekeeping protein actin, rabbit anti-human actin (Santa Cruz Biotechnology) was added to each well at the same time as an internal control. After overnight treatment
at 4 °C, the wells were incubated with corresponding second IRDye 700DX-conjugated affinity purified (red fluorescence) anti-mouse and IRDye 800DX-conjugated affinity purified (green fluorescence) anti-rabbit. However, for the CCR2 detection group, the wells were incubated with corresponding second IRDye 700DX-conjugated affinity purified (red fluorescence) anti-rabbit and IRDye 800DX-conjugated affinity purified (green fluorescence) anti-goat, fluorescence antibody recommended by the manufacturer (Rockland, Inc., Gilbertsville, PA, USA). This procedure must be carried out in the dark. Images of target gene were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences). The expression level of the correspondent molecules was calculated as the ratio of the intensity of target gene to actin. The experiments were carried out in triplicate, and repeated three times.

**Western blot analysis**

Total protein extracted from primary cultured ESCs from the endometriotic (n=6) or endometrial tissues (both the sample number of eutopic and normal groups was 6) was prepared using RIPA buffer. Then 30 μg protein was loaded onto 10% polyacrylamide-SDS gels. The resolved protein was transferred onto polyvinylidine difluoride membranes (Bio-Rad), and incubated with a 1:500 dilution of mouse anti-human CD82 monoclonal antibody (SC-15572, Santa Cruz Biotechnology) and a 1:1000 dilution of mouse anti-integrin β-actin monoclonal antibody (Santa Cruz Biotechnology) in PBS containing 0.05% Tween-20 and 5% FCS respectively. After an extensive washing, the bound primary antibodies were detected by a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), respectively, with a chemiluminescent detection system. The experiments were repeated three times.

**ELISA for determination of CCL2**

ESCs or siRNA-transfected ESCs (2×10⁵ cells/well) from normal endometrium in 24-well plates were treated with TCDD or/and E₂, or treated with U0126 (30 μmol/l) and anti-integrinβ1 neutralizing antibody for 48 h, respectively, and then the culture supernatant was harvested, centrifuged to remove cellular debris, and store at –80 °C until being assayed by ELISA. The CCL2 concentration in the supernatant was quantified by ELISA kits (R&D Systems) according to the manufacturer’s instruction. At the same time, we detected the protein concentration of each group, and the CCL2 level of each group was calculated as the ratio of the CCL2 concentration of supernatant to the protein concentration. Each experiment was carried out in triplicate.

**Statistical analysis**

All values were shown as the mean ± S.E.M. Data were analyzed by one-way ANOVA and least significant difference (equal variances assumed) or Tamhane’s test (equal variances not assumed) was used post hoc for multiple comparisons with Statistical Package for the Social Sciences software version 11.5 (SPSS Inc., Chicago, IL, USA). Differences were considered as statistically significant at P<0.05.

**Results**

**The expression of CD82 is decreased in primary ESCs from patients with endometriosis**

To clarify the relationship of CD82 expression in endometriosis, we collected the endometriotic and endometrial tissues from women with or without endometriosis, and then detected the mRNA and protein levels of CD82 in ESCs by quantitative real-time PCR, western blot and in-cell Western. As shown, the mRNA level of CD82 in the normal ESCs without endometriosis (n=6) is 2.824-fold (P<0.05) and 11.636-fold (P<0.01) higher than that of the eutopic (n=6) and ectopic ESCs (n=6) with endometriosis, respectively (Fig. 1a). Consistent with transcription level, the normal ESCs show a significant higher CD82 protein expression than that of eutopic ESCs (P<0.05), and the latter is further higher significantly than that of the ectopic ESCs (P<0.05 or P<0.01) by western blot and in-cell Western (Fig. 1b and c). These results above suggest that low expression of CD82 in the eutopic and ectopic ESCs may be involved in the occurrence and development of endometriosis.

**The combination of TCDD with E₂ or co-culture with U937 downregulates CD82 expression in ESCs**

TCDD alone can significantly inhibit expression of CD82 in the ESCs from endometrium with or without endometriosis, especially the concentration 10⁻⁹ M (P<0.05 or P<0.01; Fig. 2a), but E₂ alone increases the expression of CD82 in normal ESCs and eutopic ESCs, especially the concentration 10⁻⁸ M (P<0.05; Fig. 2b). Either TCDD or E₂ alone shows no obvious effect on the CD82 expression in the ectopic ESCs. Interestingly, the combination of TCDD with E₂ has a further inhibition on the CD82 expression in the ESCs from endometrium with or without endometriosis (P<0.05; Fig. 2c), but the expression of CD82 in the ectopic ESCs has not significantly changed. The results indicate that the combination of TCDD with estrogen may downregulate the expression of CD82 in the eutopic ESCs with endometriosis.

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CD82 suppression in the endometriotic milieu

Figure 1 The expression of CD82 is decreased in the primary ESCs from eutopic endometrium and endometriotic tissues.

(a) CD82 mRNA and protein levels in human ESCs were analyzed by quantitative real-time PCR (a), western blot (b), or in-cell Western (c). The relative fold mRNA and protein levels of CD82 in ESCs were compared between the eutopic ESCs, ectopic ESCs, and normal ESCs. CD82 (red) and actin (green). Results were highly reproducible in three independent experiments. In this study, normal, ESCs from endometrium without endometriosis; eutopic and ectopic, ESCs from eutopic endometrium and ectopic tissue with endometriosis respectively. Error bars depict the S.E.M. *P < 0.05 and **P < 0.01 compared with the control. #P < 0.05 and ##P < 0.01 compared with the eutopic.

To further explore the possible roles of the abnormal lower CD82 expression in the endometriotic milieu, we established the contact co-culture of U937 cells and ectopic ESCs from endometrium with endometriosis. U937 cells are monocytes, which may differentiate into macrophages in tissues or in response to stimuli. First, we observed, respectively, ESCs and U937 cells by an Olympus BX51 fluorescence microscope (Olympus) before and after the co-culture (Supplementary Figure 2a, see section on supplementary data given at the end of this article). Moreover, we used immuno-cytochemical staining to identify the cells after supernatants of co-culture were discarded (Supplementary Figure 2b, see section on supplementary data given at the end of this article). As shown in Fig. 2d, U937 can inhibit CD82 expression in the ESCs in a dosage-dependent manner (P < 0.05 or P < 0.01), which suggests that CD82 expression in the retrogradated endometrial cells into the peritoneal cavity is further decreased owing to interaction with the increased active macrophages in peritoneal fluid with endometriosis.

Silencing of CD82 enhances the invasion of ESCs

To test the effects of CD82 on the invasion of ESCs, we silenced the CD82 expression of the primary normal ESCs (n=6) by siRNA transfection (P<0.01; Fig. 3a and b), and then a matrigel-based transwell was carried out. The silenced ESCs were added to the upper chamber, the number of cells migrating to the lower surface was counted in 72 h of incubation. Meanwhile, the total protein concentration of ESCs in each group was analyzed. The invasion index of each group was calculated as the ratio of the number of cells migrated to the lower surfaces to the protein concentration. As shown in Fig. 3c, the CD82 silence in ESCs can significantly enhance the invasiveness of ESCs compared with the si-negative control (P<0.01).

Thereafter, we further investigated invasiveness of the silenced ESCs without endometriosis after treated with TCDD or/and E2 for another 48 h. The results show that TCDD or/and E2 also increase the invasion of ESCs (P<0.05 or P<0.01; Fig. 3d), which echoes a previous result in eutopic ESCs from women with endometriosis (Yu et al. 2008). Therefore, combination of TCDD with E2 presents a synergistic role with CD82 silence in the upregulation of ESC invasiveness (P<0.01; Fig. 3d).

CD82 suppresses ESCs invasion by inhibiting the CCL2 secretion and CCR2 expression

To clarify whether CD82 regulates CCL2 secretion and CCR2 expression in ESCs, ELISA and in-cell Western were used to analyze CCL2 secretion and CCR2 expression in ESCs. The results show that CD82 can significantly inhibit CCL2 secretion (P<0.05; Fig. 4a) and CCR2 expression in the normal ESCs (P<0.01; Fig. 4b) from patients without endometriosis (n=6). We further evaluated the invasiveness of ESCs treated with various concentrations of RS102895 or anti-CCL2 neutralizing antibody. It is shown in Fig. 4c and d that both RS102895 and anti-CCL2 neutralizing antibody can decrease the invasiveness of ESCs (P<0.05 or P<0.01), the optimal concentration is 100 ng/ml and 1 µg/ml respectively.

The results above indicate that CD82 may inhibit the invasion of ESCs through downregulating CCL2 production and CCR2 expression to some extent. Therefore, it can be speculated that the decreased expression of CD82 in ectopic ESCs may recruit more macrophages into the peritoneal cavity via upregulating CCL2 secretion, which in turn leads to the further decline of CD82 expression in the ESCs, and such a vicious circle in the endometriotic milieu.

To elucidate the regulatory mechanism of CD82 expression on ESCs invasion, we evaluated the invasion of the CD82-silenced ESCs after treated with RS102895 or anti-CCL2 neutralizing antibody. The results show that both RS102895 and anti-CCL2 neutralizing antibody can abolish completely the increased invasion of the CD82-silenced ESCs (P<0.01; Fig. 4e).
CD82 inhibits invasion of ESCs by downregulating CCL2 secretion and CCR2 expression via MAPK and integrinβ1 signal pathway

Alterations of MMPs, TIMPs and integrins in eutopic endometrium are important factors in the development of endometriosis (Yoshimura 2002, Collette et al. 2006, Yu et al. 2008). Moreover, the expression of integrins was intimately associated with the function of CD82. Our previous work has confirmed that DSCs-expressed CD82 controls the invasiveness of trophoblasts through suppressing the integrinβ1/MAPK/ERK1/2 signal pathway (Li et al. 2010). Therefore, we next investigated the protein levels of the invasion-relative molecules and integrins in the CD82-silenced ESCs from patients without endometriosis (n=6). It has been clearly demonstrated in Fig. 5a that CD82 silence can obviously inhibit the expression of TIMP1 (P<0.01) and TIMP2 (P<0.01) and enhance the expression of integrinβ1 (P<0.01) and integrinαvβ3 (P<0.05), but has no

| Figure 2 | The combination of TCDD with 17β-estradiol or co-culture with U937 downregulates CD82 expression in ESCs. (a–c) ESCs were treated, respectively, with different concentrations of TCDD (from 10^{-12} to 10^{-8} M), 17β-estradiol (from 10^{-11} to 10^{-7} M) or the combination of 10^{-8} M TCDD and 10^{-8} M 17β-estradiol for 48 h, with vehicle as controls. In-cell Western indicates that TCDD alone can significantly decrease, and 17β-estradiol alone can increase the expression of CD82 in ESCs, but 17β-estradiol can coordinate with TCDD in downregulating CD82 expression. (d) ESCs (1×10^5 cell/well) from eutopic endometrium were co-cultured with U937 of 2×10^4, 1×10^5, or 5×10^5 cell/well for 48 h, respectively, with ESCs cultured alone as control. Thereafter, in-cell Western was used to detect the CD82 expression in ESCs. CD82 (red) and actin (green). Results were highly reproducible in three independent experiments. Error bars depict the S.E.M.* P<0.05 and ** P<0.01 compared with the normal ESCs control or ESCs without co-culture. # P<0.05 and ## P<0.01 compared with the eutopic ESCs control. ∆ P<0.05 compared with TCDD treatment.
effect on the expression of MMP2 and MMP9 in the ESCs. These results indicate that the decreased CD82 expression in ESCs may lead to not only the abnormal increase in invasion through suppressing the TIMP1 and TIMP2 expression but also the abnormal enhanced adhesion of ESCs to ECMs through increasing the expression of integrin β1 and integrin αvβ3, which is attributed to the onset and development of endometriosis.

To explore the molecular mechanisms of CCL2 and CCR2 expression regulated by CD82 in ESCs, we used U0126 or anti-integrinβ1 neutralizing antibody to treat the CD82-silenced ESCs for another 24 h. We have found U0126 and anti-integrinβ1 neutralizing antibody can abolish completely the stimulating effects on CCL2 secretion and CCR2 expression induced by CD82 silence (Fig. 5b).

To understand the relationship of CCL2/CCR2 to TIMPs, we treated the CD82-silenced ESCs with U0126 or anti-integrinβ1 neutralizing antibody for another 24 h, or anti-CCL2 neutralizing antibody or RS102895 for another 48 h. As shown in Fig. 5c, all the treatments can significantly promote TIMP1 and TIMP2 production, and completely reverse the inhibition effects on TIMP1 and TIMP2 induced by CD82 silence. Therefore, CCL2/CCR2 interaction downregulates the expression of TIMP1 and TIMP2 in an autocrine manner, which controls the ESC invasion.

Moreover, we tested whether MAPK and integrin β1 signal pathway was involved in the regulation of ESCs invasion by CD82. As shown, either U0126 or anti-integrinβ1 neutralizing antibody can abolish completely the stimulating effects on CCL2 secretion and CCR2 expression induced by CD82 silence (Fig. 5b).

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anti-integrin β1 neutralizing antibody can also abolish completely the pro-invasion effect on ESCs resulted from CD82 silence ($P<0.01$; Fig. 5d).

Thus, our research above supports the idea that CD82 inhibits ESCs invasion by the downregulation of CCL2 secretion, CCR2 expression, and the upregulation of TIMP1 and TIMP2 expression via MAPK and integrin β1 signal pathway.

The combination of TCDD with E2 stimulates CCL2 secretion, CCR2 expression and invasion of ESCs by downregulating CD82 expression

To further test the effect of TCDD or/and E2 on CCL2 secretion and CCR2 expression via CD82, we treated normal ESCs ($n=6$) or the CD82-silenced ESCs ($n=6$) with TCDD, E2 or the combination of both. The results show that TCDD alone or combined with E2 stimulates CCL2 secretion and CCR2 expression in ESCs ($P<0.01$), and presents the synergistic action with CD82 silence (Fig. 6a and b).

Moreover, our results show that CCR2 blocker, RS102895, or anti-CCL2 neutralizing antibody abolishes completely the increased invasiveness induced by the combination of TCDD with E2 ($P<0.01$; Fig. 6c).

From the results above, it may be concluded that the combination of TCDD with E2 stimulates the ESCs invasion through downregulating CD82 expression and upregulating CCL2 secretion and CCR2 expression, which eventually leads to the occurrence and progression of endometriosis.

Discussion

Endometriosis is considered to be a pathological disorder caused by interaction of multiple molecules including steroid exposure, immunological disturbances, genetic predisposition, and environmental toxin exposure (Osteen et al. 2003). The adherence and invasion of the retrograded endometrial cells into the peritoneum is a key step for the early stage of endometriosis, and the retrograded ESCs are responsible for the adherence and implantation of endometrioma to peritoneum in the early stage of endometriosis (Witz et al. 2001). It has been suggested that several
CD82 inhibits the invasion of ESCs by downregulating CCL2 secretion and CCR2 expression via MAPK and integrinβ1 signal pathway. (a) CD82 upregulates TIMP1 and TIMP2 expression, downregulates integrinβ1 and integrinανβ3 expression in ESCs, but has no influence on MMP2 and MMP9 expression. In this study, MMP2, MMP9, TIMP1, TIMP2, integrinβ1, and integrinανβ3 are shown in red and actin is shown in green. (b) After the silence, ESCs were treated with U0126 or anti-integrinβ1 neutralizing antibody for another 24 h, and then the CCL2 secretion and CCR2 expression were detected by ELISA and in-cell Western, respectively, and the invasion of ESCs (d) by invasion assay. The results show that U0126 or anti-integrinβ1 neutralizing antibody can abolish completely the increase of CCL2 secretion, CCR2 expression and invasion of ESCs induced by CD82 silence. CCR2 (green) and actin (red). (c) We treated CD82-silenced ESCs with U0126 or anti-integrinβ1 neutralizing antibody for another 24 h, or anti-CCL2 neutralizing antibody or RS102895 for another 48 h, and then TIMP1 and TIMP2 expression were evaluated by in-cell Western. TIMP1 and TIMP2 (red) and actin (green). The CCL2 level was calculated as the ratio of the secretion of CCL2 in supernatant by ELISA to the protein concentration of total cells. The cell invasiveness was calculated as the ratio of the cells migrated to the lower chamber to the protein concentration of total cells. Results were highly reproducible in three independent experiments. In this study, control, the non-targeting siRNA oligonucleotides; silence, CD82 is knocked down. Error bars depict the S.E.M.* P < 0.05 and ** P < 0.01 compared with the negative control. ## P < 0.01 compared with CD82 silence.
tumor relative genes are involved in susceptibility to endometriosis. In view of that, we first evaluated the mRNA and protein levels of CD82 in primary ESCs from endometrium with or without endometriosis or the ectopic ESCs. We have found that the expression of metastasis suppressor CD82 in the eutopic and ectopic ESCs with endometriosis is significantly decreased, especially for the ectopic ESCs, which suggests that the abnormal lower CD82 in the eutopic and ectopic ESCs may induce abnormal increase of its invasion, initiating the invasion and implantation of the shed endometrium to peritoneum.

To analyze the potential mechanisms of CD82 reduction in the eutopic and ectopic tissue, we detected the CD82 expression in the eutopic and ectopic ESCs after treatment with TCDD or/and E2. TCDD has been viewed as a toxic compound with both estrogenic and anti-estrogenic activity (Grochowalski et al. 2001). In this study, we have demonstrated that E2 alone increases and TCDD alone decreases CD82 expression, respectively, while CD82 in the eutopic ESCs is reduced further after the treatment with the combination of TCDD with E2. Hence, TCDD and estrogen have the synergistic effect on CD82 expression. Moreover, the expression of CD82 is decreased when the eutopic ESCs were co-cultured with macrophages, which suggests that the abnormal decrease of CD82 expression in the ectopic ESCs is a direct response to the specific microenvironment in the ectopic milieu.

In agreement with our previous studies (Shi et al. 2006, Yu et al. 2008), the immuno-inflammatory microenvironments mediated by numerous cytokines and growth factors, especially for chemokine, are now believed to play an important role in the progression of endometriosis. However, previous studies about CCL2 focused mainly on its chemotactic effect on monocyte–macrophages. In this study, we investigated the role and regulating mechanism of metastasis suppressor CD82 in the onset and development of endometriosis, and analyzed whether TCDD, E2, CCL2, and receptor CCR2 participated in the CD82-mediated progression. We have demonstrated that the ESC-expressed CD82 inhibits the invasion of ESCs themselves. Interestingly, our observation has shown that CD82 in ESCs downregulates the CCL2 secretion and CCR2 expression of ESCs, and CCR2 antagonist, RS102895, or anti-CCL2 neutralizing antibody abolishes the increased invasiveness of ESCs induced by CD82 silence. Thus, it can be concluded that the abnormal decreased CD82 expression in the ectopic ESCS may lead to invasiveness increase of ESCs through stimulating the CCR2 expression and CCL2 secretion.

This study has shown that CD82 promotes the expression of TIMP1 and TIMP2 in ESCs via inhibiting CCL2 and CCR2 production, but does not change

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**Figure 6** The combination of TCDD with 17β-estradiol stimulates CCL2 secretion, CCR2 expression and invasion of ESCs by downregulating CD82 expression. (a and b) After CD82 was silenced in normal ESCs (n=6), the ESCs were treated with TCDD, 17β-estradiol or the combination of both for another 48 h, and then CCL2 secretion and CCR2 expression of ESCs were detected by ELISA and in-cell Western respectively. TCDD not only stimulates CCL2 secretion and CCR2 expression in ESCs, but also recovers the increase of CCL2 secretion and CCR2 expression of ESCs induced by CD82 silence. CCR2 (green) and actin (red). (c) The ESCs were treated with TCDD, 17β-estradiol, or the combination of both with RS102895 or/and anti-CCL2 neutralizing antibody for 48 h respectively. Thereafter, the invasion of ESCs was detected by Matrigel invasion assay. The results indicate that either RS102895 or anti-CCL2 neutralizing antibody abolishes completely the invasion enhancement of ESCs induced by the combination of TCDD and 17β-estradiol. The CCL2 level was calculated as the ratio of the secretion of CCL2 in supernatant by ELISA to the protein concentration of total cells. Meanwhile, the cell invasiveness was calculated by the ratio of the cells migrated to the lower chamber to the protein concentration of total cells. These pictures are representatives of three individual experiments. Error bars depict the s.e.m. *P<0.05 and **P<0.01 compared with the negative control. #P<0.05 compared with CD82 silence. „P<0.05 compared with E2 treatment group.
MMP2 and MMP9 expression. Indeed, the invasion-relative molecules, such as MMPs and their inhibitor TIMPs, are correlated to onset and progression of endometriosis, which suggests that the CD82-regulated TIMP1 and TIMP2 expression decrease may upregulate the ESCs invasion, which plays roles in pathogenesis of endometriosis.

Meanwhile, we have found that CD82 can inhibit integrinβ1 and integrinβ3 expression in ESCs. Cell adhesion molecules, such as integrins and cadherins, have been shown to be involved in the shedding of endometrium during menstruation and in the adhesion of endometrial cells to the peritoneum (Lessey et al. 1994, Lessey & Young 1997). A recent study has shown that interaction between galectin-3 and integrinβ3 promotes endometrial cell proliferation and adhesion (Lei et al. 2009). The adhesion of endometrial stromal cells to various ECM proteins induces an upregulation in CCL2 gene expression and protein secretion by integrinβ1 (Garcia-Velasco et al. 1999).

In our study, abnormal decrease of CD82 expression in the eutopic and ectopic ESCs from endometrium with endometriosis may promote the adhesion and invasion of ESCs, stimulates CCL2 secretion, and results in the development of endometriosis through upregulating integrinβ1 and integrinβ3 expression via MAPK and integrinβ1 signal pathway, which is consistent with other studies about CCL2 (Garcia-Velasco et al. 1999, He et al. 2007).

Interestingly, in our Matrigel invasion test, the increased invasiveness of ESCs induced by TCDD or combination with E2 can be reversed completely by RS102895 or anti-CCL2 neutralizing antibody. On the other hand, TCDD alone or combined with E2 can downregulate CD82 expression, and increase CCL2 secretion and CCR2 expression in ESCs.

In conclusion, based on our results, a hypothetical model may be proposed to illustrate the complicated pathogenesis of endometriosis (Fig. 7). The endometrium with the abnormal repressed CD82 expression owing to inherent defects or the combined effect of TCDD and estrogen regurgitates into the peritoneal cavity, which upregulates the CCL2 secretion and CCR2 expression, invasion, and adhesion of ESCs through MAPK and integrinβ1 signal pathway. In this progression, downregulation of TIMP1 and TIMP2 as effective molecules is involved in the invasiveness increase of ESCs. On the other hand, after more macrophages are recruited into the ectopic milieu by the increased CCL2 secretion derived of ESCs, the CD82 levels are further downregulated, which leads to a vicious circle, and eventually the onset and development of endometriosis. Meanwhile, TCDD and estrogen can coordinate to evoke and aggravate the inflammatory progression by stimulating other pro-inflammatory cytokine secretion, such as RANTES and MIP-1α in the endometriotic milieu (Yu et al. 2008).

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-10-0165.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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