

The role of SRC1 and SRC2 in steroid-induced SDF1 expression in normal and ectopic endometrium

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Abstract

To compare the expression patterns of steroid receptor coactivators (SRCs) and steroid-induced stromal cell-derived factor 1 (CXCL12 (SDF1)) in normal and ectopic endometrium and to explore the roles of NCOA1 (SRC1) and NCOA2 (SRC2) in the steroid-induced CXCL12 expression in normal and ectopic endometrial stromal cells (ESCs). The *NCOA1*, *NCOA2*, *NCOA3* (*SRC3*), and *CXCL12* (*SDF1*) α mRNA levels in normal and ectopic endometrium were analyzed by quantitative real-time PCR. Steroid-induced CXCL12 expression was detected by the ELISA method and the chemotactic activity of conditioned supernatant to monocyte was assessed by the Boyden chamber method before and after the silencing of *NCOA1* or *NCOA2* with siRNA in normal and ectopic ESCs. The expression of *NCOA1* and *CXCL12* in ectopic endometrium was significantly greater than that in normal endometrium in the secretory phase. Progesterone (P_4) was able to significantly inhibit estradiol (E_2)-stimulated CXCL12 expression in normal and ectopic ESCs. The inhibitory rate of P_4 in ectopic ESCs at 72 and 96 h was significantly lower than that in normal ESCs. Silencing of *NCOA1* but not *NCOA2* significantly reduced the E_2 -induced CXCL12 expression in normal and ectopic ESCs. The ability of P_4 to inhibit E_2 -induced CXCL12 expression and monocyte chemotaxis in normal and ectopic ESCs was significantly attenuated when *NCOA2* was silenced. *NCOA1* plays a necessary role in E_2 -induced CXCL12 expression and *NCOA2* is required for P_4 to inhibit the E_2 -induced CXCL12 production in normal and ectopic endometrium.

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Introduction

Endometriosis (EMS) is a complex, estrogen-dependent, and inflammatory disease. Estrogen can be synthesized locally in the endometriotic lesions (Rizner 2009). The immunological factors and angiogenesis play a decisive role in the pathogenesis of EMS (Gazvani & Templeton 2006). Stromal cell-derived factor 1 (SDF1, also known as CXCL12) is a member of the CXC chemokine family and is a proliferative and chemotactic factor for endothelial cells, lymphocytes, and hematopoietic progenitor cells (Karin 2010, Liu *et al.* 2011). CXCL12 interacts with CXC chemokine receptor 4 (CXCR4), which is the only physiological receptor for CXCL12. The CXCL12–CXCR4 axis has been shown to have both immune and nonimmune functions, including roles in tissue repair, angiogenesis, invasion, and migration (Cui *et al.* 2011, Domanska *et al.* 2013). There is evidence indicating that these mechanisms are also at play in EMS (Ruiz *et al.* 2010).

CXCL12 has been identified as an estrogen-regulated gene in estrogen receptor (ER)-positive ovarian and breast cancer cells (Hall & Korach 2003). Estradiol (E_2)

enhanced CXCL12 production levels in human endometrial stromal cells (ESCs) in a time- and dose-dependent manner (Tsutsumi *et al.* 2011). Progestins effectively inhibit estrogen-induced CXCL12 expression in human ESCs (Okada *et al.* 2011). The action of the steroid hormone receptors is modulated in part by members of the p160 family of steroid receptor coactivators (SRCs). The SRC family is composed of three distinct but functionally and structurally related members: SRC1/NCOA1, SRC2/TIF2/GRIP1, and SRC3/RAC3/ACTR/pCIP/AIB1/TRAM1 (Oñate *et al.* 1995, Xu *et al.* 2009). The SRC family members enhanced the transcriptional activity of a variety of nuclear receptors, including ER and progesterone receptor, and are expressed in a variety of hormone-responsive tissues including the ectopic endometrium (York & O'Malley 2010, Kumagami *et al.* 2011). In this study, we observed the expression patterns of SRCs and CXCL12 in normal and ectopic endometrium by quantitative real-time PCR (RT-qPCR) and explored the roles of NCOA1 (SRC1) and NCOA2 (SRC2) in the steroid-induced CXCL12 expression in normal and ectopic ESCs employing siRNA-based gene silencing.

Materials and methods

Sources of tissues

Women with or without EMS, who had not received hormones or gonadotropin-releasing hormone agonist therapy for at least 3 months prior to the study, were recruited after they had provided written informed consent under a study protocol approved by The First Affiliated Hospital of Nanjing Medical University. The ectopic endometrial tissues were obtained from ovarian endometriomata in 16 women with EMS (stage III or IV) who have been identified pathologically and staged according to the revised American Society for Reproductive Medicine (1997) classification. Of them, nine women were in the proliferative phase (days 5–14) and seven in the secretory phase (days 15–28). Normal endometria were acquired from ten healthy women with normal menstrual cycles and no history of EMS and have been identified pathologically. Out of the ten healthy women, five were in the proliferative phase and five in the secretory phase. The ectopic and normal ESCs that were isolated and cultured *in vitro* successfully were obtained respectively from five ectopic and five normal endometria that were collected in the secretory phase.

Quantitative real-time PCR

All 16 ectopic and ten normal endometria were analyzed by the RT-qPCR. Primer sequences for *NCOA1*, *NCOA2*, *NCOA3* (*SRC3*), and *CXCL12* were shown in Table 1. RNA was reverse transcribed with random primers and SuperScript II reverse transcriptase according to Invitrogen's protocol. For analysis, qPCRs were conducted using TaqMan or Power SYBR Green PCR Master Mixes (Applied Biosystems), and samples were amplified with the ABI Prism 7700 sequence detector (Applied Biosystems). The relative mRNA of *NCOA1*, *NCOA2*, *NCOA3*, and *CXCL12* was calculated as the ratio of sample gene to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. All samples were analyzed in triplicate.

Steroid hormone treatment and transient transfection with siRNA

The ectopic and normal ESCs that were isolated and cultured *in vitro* successfully were obtained respectively from five ectopic and five normal endometria that were collected in the secretory phase. Human ESCs were purified by the standard enzyme digestion method, as described previously (Ryan *et al.* 1994, Xiu-li *et al.* 2009). After passage 0–1, when the ESCs cultured in six-well plates approached 80% confluence, ESCs were cultured in a medium containing 17 β -E₂ only (10⁻⁸ mol/l; Sigma) or 17 β -E₂ (10⁻⁸ mol/l) +

progesterone (P₄; 10⁻⁶ mol/l; Sigma). The supernatants were collected 24, 48, 72, and 96 h after the steroid hormone treatment to quantify CXCL12 (SDF1 α) expression by the ELISA method. To determine the effect of *NCOA1* or *NCOA2* silencing on steroid-induced CXCL12 expression, 1 day prior to transfection, 2 \times 10⁵ cells were plated into each well of six-well plates and grown overnight in Opti-MEM (Gibco). The next day, cells were transfected with siRNA negative control (Dharmacon Research, Inc., Lafayette, CO, USA), *NCOA1* siRNA (Dharmacon Research, Inc.), or *NCOA2* siRNA (Dharmacon Research, Inc.) and the final concentration was made up to 100 nM per well using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen).

After 4 h, cells were treated with complete medium. Two days later, media were replaced and 17 β -E₂ (10⁻⁸ mol/l) and/or P₄ (10⁻⁶ mol/l) were added. On day 3 after the steroid hormone treatment, the media were collected to quantify CXCL12 expression. In addition, nuclear proteins were prepared using a kit (Panomics, Redwood, CA, USA) at 48 h after transfection and assessed by western blotting to verify the reduction in protein expression by siRNA treatments.

CXCL12 ELISA

CXCL12 in cell culture supernatants was assessed using commercially available sandwich ELISA Kits (Quantikine ELISA Kit, RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's instructions.

Western blotting analyses

At 48 h after transfection with siRNA, nuclear proteins were extracted from ectopic and normal ESCs and were assessed by western blotting to observe the *NCOA1* and *NCOA2* expression. The protein concentration was determined using a Miro-BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE. Nonspecific sites were saturated by incubating the blots in blocking buffer for 1 h at room temperature and probed overnight with primary antibodies. After incubation with the appropriate HRP-conjugated anti-rabbit or anti-mouse antibody (Amersham Biosciences), detection of specifically bound proteins was carried out by ECL+PLUS according to the manufacturer's protocol (Amersham Pharmacia Biotech) using XL-1 Blue film (Kodak). The density of specific bands was quantified using a densitometer image (Image J, Bethesda, MD, USA). The relative band density was calculated as the ratio of sample to poly(ADP-ribose) polymerase (PARP). The primary antibodies are anti-SRC1 (Santa Cruz Biotechnology), anti-SRC2 (Abcam Ltd., Cambridge, UK), and anti-PARP (Abcam Ltd.).

Table 1 Primer sequences used in real-time PCRs.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product (bp)
<i>NCOA1</i>	TCACCGCAAGAGCTACCAT	CAGCGTCCTCATCTGGTTTC	193
<i>NCOA2</i>	GCTGGGAGGACCTGGTAAGA	ATTGACTGAATGCCAATCCT	124
<i>NCOA3</i>	AGTCTAAATGATGGGCAGTCATG	CTGAAGGAAGTGGGTTGAGACA	225
<i>CXCL12</i>	TACAGATGCCCATGCCGATT	TTTTGGCTGTTGTGCTTACTTG	204
<i>GAPDH</i>	GGAGTCCACTGGCGTCTTC	GCTGATGATCTTGAGGCTGTTG	157

Monocyte chemotaxis assay

The ESCs that were isolated from five ectopic and five normal endometria in the secretory phase and human histiocytic cell line (U937, purchased from the Cell Bank of the Chinese Academy of Science, Shanghai, China) were used for the monocyte chemotaxis assay. U937 cells were treated with 1 mM 8-bromo-cAMP for 48 h to induce a monocyte phenotype and upregulate oligopeptide chemoattractant receptors. For the measurements, 24-well plates assembled with Boyden chambers (0.4-mm pore size polycarbonate membranes) were used. ESCs were placed in the lower chamber and transfected with siRNA. After 4 h, cells were treated with complete medium. Two days later, media was replaced and 17β -E₂ (10^{-8} mol/l) and/or P₄ (10^{-6} mol/l) were added. In the upper chamber, 200 μ l suspensions of U937 cells (1×10^5 cells) in PBS were placed. The chambers were then incubated in a humidified CO₂ incubator at 37 °C for 100 min. Nonmigrating cells were removed by washing with PBS and by scraping from the top. The cells migrating across the membrane were fixed, stained, and counted directly. Results are reported as chemotactic index, i.e. cells migrating in response to the test substance divided by cells migrating in siRNA negative control medium.

Statistical analysis

SPSS 11.0 Software was used in the statistical analysis. All data have been checked by the normality assumption and the homogeneity of variances assumption in one-way ANOVA. Each parameter was presented as mean \pm s.e.m. and compared using one-way ANOVA. The level of significance was set at $P < 0.05$.

Results

RT-PCR analyses of NCOA1, NCOA2, NCOA3, and CXCL12 in normal and ectopic endometrium throughout the menstrual cycle

As shown in Fig. 1, the NCOA1 level was significantly higher in ectopic endometrium when compared with normal endometrium in the secretory phase. CXCL12 increased statistically in ectopic endometrium when compared with normal endometrium either in the proliferative phase or in the secretory phase. NCOA1, NCOA2, and CXCL12 showed marked cyclic differences in normal endometrium. There was no periodic variation in the expression of NCOA1, NCOA2, NCOA3, and CXCL12 in ectopic endometrium throughout the menstrual cycle. NCOA3 decreased significantly in ectopic endometrium when compared with normal endometrium either in the proliferative phase or in the secretory phase.

Effect of steroid hormones on CXCL12 expression in ectopic ESCs

Based on the results shown in Fig. 1, we next observed the steroid hormone-induced CXCL12 expression in

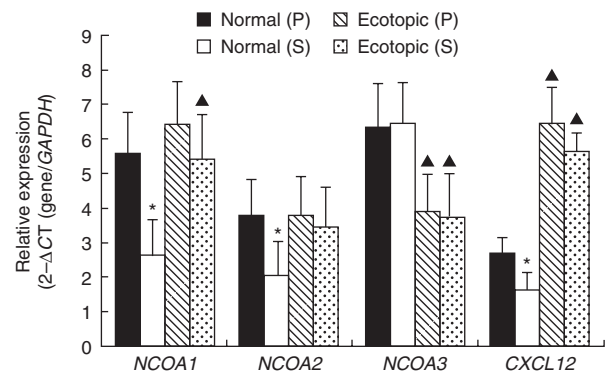


Figure 1 Real-time PCR analyses of NCOA1, NCOA2, NCOA3, and CXCL12 in normal and ectopic endometrium throughout the menstrual cycle. NCOA1, NCOA2, NCOA3, and CXCL12 mRNA of ectopic and normal endometrium obtained from 16 women with EMS and ten normal women without EMS were analyzed by qPCR. Nine women with EMS were in the proliferative (P) phase and seven were in the secretory (S) phase. Five women without EMS were in the P phase and five were in the S phase. Experiments were performed three times for each sample and each in triplicate. The points represent the mean \pm s.e.m. Relative expression was calculated according to the formula $(2^{-\Delta\text{CT}} (\text{gene}/\text{GAPDH}))$. * $P < 0.05$ vs P phase within the same group and \blacktriangle , $P < 0.05$ vs normal endometrium within the same menstrual phase.

ectopic and normal ESCs isolated from five women with EMS and five women without EMS in the secretory phase. CXCL12 in the culture medium was assessed using an ELISA Kit. As shown in Fig. 2, 10^{-8} mol/l E₂ induced a significant increase in CXCL12 production after 48 h of culture in normal ESCs (Fig. 2A) and after 24 h of culture in ectopic ESCs (Fig. 2B) when compared with that in the control, and this effect continued to increase until the end of the study at 96 h. P₄ was able to effectively antagonize E₂-stimulated CXCL12 expression in both the groups. At 48, 72, and 96 h, the inhibitory rates were 27.52, 33.15, and 28.31% respectively in ectopic ESCs and were 31.99, 45.69, and 41.72% respectively in normal ESCs. The inhibitory rate of P₄ in ectopic ESCs was significantly lower than that in normal ESCs at 72 and 96 h.

Effects of SRC silencing on steroid hormone-induced CXCL12 expression in ectopic ESCs

To investigate the roles of NCOA1 and NCOA2 in the steroid-induced CXCL12 expression, negative control siRNA and siRNA targeting NCOA1 or NCOA2 were transfected into normal and ectopic ESCs respectively, which were subsequently treated with E₂ only (10^{-8} mol/l) or E₂ (10^{-8} mol/l) + P₄ (10^{-6} mol/l) for 3 days. As shown in Fig. 3, siRNA effectively reduced NCOA1 and NCOA2 expression at 48 h after transfection in ectopic ESCs. Silencing of NCOA1 but not NCOA2 significantly reduced the E₂-induced CXCL12 expression in normal (Fig. 4A) and ectopic (Fig. 4B) ESCs.

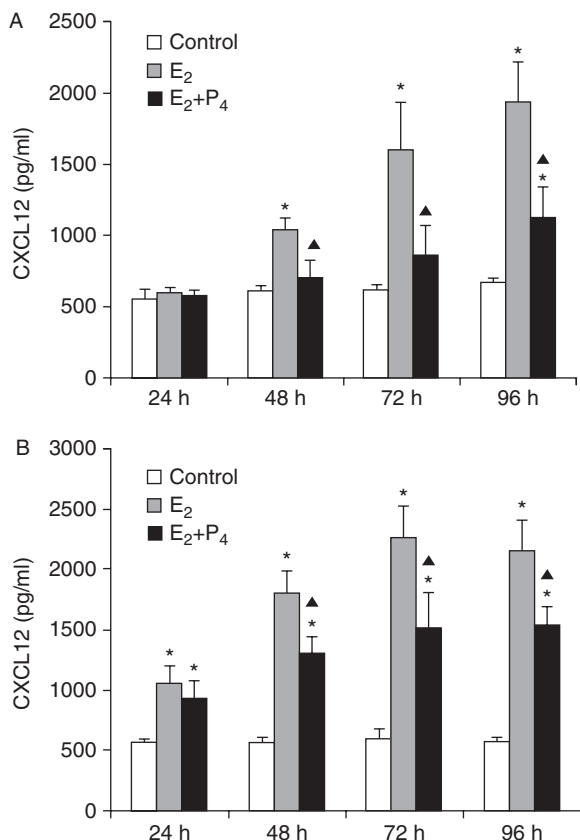


Figure 2 The effects of steroid hormones on CXCL12 expression in normal and ectopic ESCs. Normal (A) and ectopic (B) ESCs were isolated from five women with EMS and five women without EMS in the secretory phase. ESCs were cultured in six-well plates with either control, E₂ only (10⁻⁸ mol/l) or E₂ (10⁻⁸ mol/l)+P₄ (10⁻⁶ mol/l). ELISA measured CXCL12 concentration in the culture medium at different time points. Experiments were performed three times for each sample and each in duplicate. The points represent the mean ± s.e.m. *P<0.05 vs control group and ▲, P<0.05 vs E₂ group.

There existed a significant difference in CXCL12 expression between *NCOA1* silencing and *NCOA2* silencing (Fig. 4A and B).

Both in normal (Fig. 5A) and ectopic (Fig. 5B) ESCs treated with E₂+P₄, when *NCOA1* was silenced, the SDF1 α expression was significantly lower when compared with those treated with E₂ and siRNA negative control. When *NCOA2* was silenced, the CXCL12 expression in normal (Fig. 5A) and ectopic (Fig. 5B) ESCs treated with E₂+P₄ showed no significant difference when compared with those treated with E₂ and siRNA negative control. In normal ESCs treated with E₂+P₄, the CXCL12 expression after *NCOA2* silencing was significantly higher than that after *NCOA1* silencing (Fig. 5A). In ectopic ESCs treated with E₂+P₄, the CXCL12 expression showed no significant difference between *NCOA1* silencing and *NCOA2* silencing (Fig. 5B).

Effects of SRC silencing on the chemotactic activity of conditioned supernatant from steroid-treated ectopic ESCs

Conditioned media from steroid-treated normal and ectopic ESCs that were pre-transfected with siRNA were used for the chemotactic assay in the Boyden chamber. As shown in Fig. 6, whether in normal ESCs (Fig. 6A) or in ectopic ESCs (Fig. 6B) treated with E₂+P₄, silencing of *NCOA1* has no marked influence on the chemotactic activity of conditioned medium when compared with siRNA negative control. But the chemotactic activity of conditioned medium caused by *NCOA2* silencing was significantly higher than that caused by *NCOA1* silencing.

Discussion

EMS is an estrogen-dependent disease associated with pelvic pain and infertility. The estrogen plays an important role in the planting and growth of ectopic endometrium (Burney & Giudice 2012). Loss of P₄ signaling in the endometrium may be a causal factor in the development of EMS, and P₄ resistance is commonly observed in women with this disease (Bulun *et al.* 2010). Tsutsumi *et al.* (2011) reported that medroxyprogesterone acetate almost completely antagonized the E₂-induced CXCL12 production in normal ESCs. In our study, E₂ stimulated CXCL12 production from normal and ectopic ESCs in a time-dependent manner. Though the 10⁻⁶ mol/l P₄ cannot completely antagonize the 10⁻⁸ mol/l E₂-induced CXCL12 production in this study, the P₄ was still able to significantly inhibit E₂-stimulated CXCL12 expression in both the normal and ectopic ESCs. Our results showed that the inhibitory rate of P₄ in ectopic ESCs was significantly lower than that in normal ESCs at 72 and 96 h of E₂ stimulation. This suggested the existence of P₄ resistance in EMS.

One of the mechanisms of P₄ resistance in EMS is the perturbed P₄ signal transduction. Once bound with

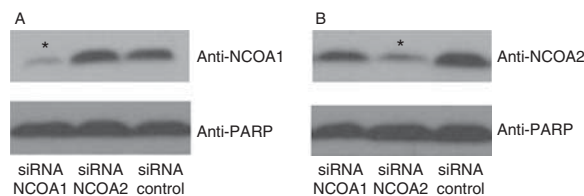


Figure 3 Western blotting image of NCOA1 and NCOA2 expression in ectopic ESCs at 48 h after transfection with siRNA targeting NCOA1 or NCOA2. At 48 h after transfection with siRNA, western blotting was used to assess the NCOA1 (A) and NCOA2 (B) protein levels in ectopic ESCs that were isolated from five women with EMS in the secretory phase. The density of specific bands was quantified using a densitometer image. Experiments were performed three times for each sample. The points represent the mean ± s.e.m. *P<0.05 vs siRNA negative control.

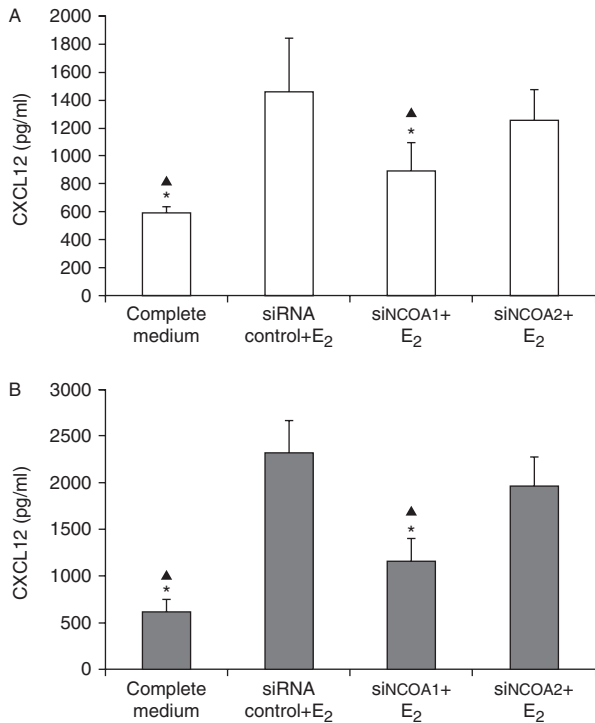


Figure 4 The effect of *NCOA1* or *NCOA2* silencing on E₂-induced CXCL12 expression in normal and ectopic ESCs. siRNA targeting *NCOA1* or *NCOA2* was transfected into normal (A) and ectopic (B) ESCs isolated from five women with EMS and five women without EMS in the secretory phase respectively, which were subsequently treated with E₂ (10⁻⁸ mol/l) for 3 days. CXCL12 in the supernatant was detected by the ELISA method. Experiments were performed three times for each sample and each in duplicate. The points represent the mean ± s.e.m. **P*<0.05 vs siRNA control+E₂ and ▲, *P*<0.05 vs siNCOA2 + E₂.

ligand-bound nuclear receptors, the SRCs SRC1, SRC2, and SRC3 function to bridge histone acetyl- and methyltransferases to specific enhancer/promoter regions (Johnson & O'Malley 2012). Several researchers have reported the expression of SRCs in normal endometrium, ectopic endometrium, and ovarian endometriotic tissue detected by immunohistochemistry and semi-quantitative RT-PCR (Suzuki *et al.* 2010, Kumagami *et al.* 2011). In this study, the expression patterns and levels of *NCOA1*, *NCOA2*, *NCOA3*, and CXCL12 in normal and ectopic endometrium during the menstrual cycle were characterized using qPCR. The *NCOA1* expression in ectopic endometrium was significantly higher than that in normal endometrium in the secretory phase. In nearly all ectopic endometrial glandular and some stromal tissues of ovary, *NCOA1* was colocalized with ER α (ESR1; Kumagami *et al.* 2011). Our study adopted the siRNA-based silencing technique to evaluate the roles of *NCOA1* and *NCOA2* in the steroid hormone-induced expression of CXCL12. The results showed that silencing of *NCOA1* but not *NCOA2* significantly reduced the E₂-induced CXCL12

expression. These suggest that *NCOA1* plays a necessary role in E₂-induced CXCL12 expression.

We observed that there was no cyclic difference in the expression of the three SRCs in ectopic endometrium throughout the whole menstrual period. Our results are similar to the previous immunohistochemical data (Kumagami *et al.* 2011). The ability of P₄ to inhibit E₂-induced CXCL12 expression and monocyte chemotaxis was greatly attenuated compared with E₂ stimulation alone when *NCOA2* was silenced whether in normal ESCs or in ectopic ESCs, though the CXCL12 expression showed no significant difference between *NCOA1* silencing and *NCOA2* silencing in ectopic ESCs treated with E₂ + P₄. These suggest that *NCOA2* is required for P₄ to inhibit the E₂-induced CXCL12 expression in normal and ectopic endometrium.

Though SRC1 and SRC2 belong to the same family, we found that they play different roles in E₂- and P₄-induced CXCL12 expression in normal and ectopic ESCs. *NCOA1* is more important for the E₂-induced CXCL12 expression and *NCOA2* is more important for the activity of P₄ to inhibit the E₂-induced CXCL12 expression. At the same time, we also observed that expression of *NCOA2*

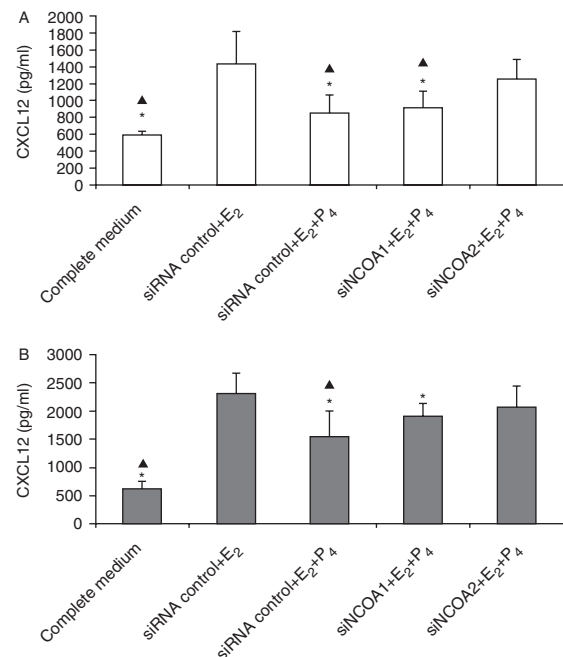


Figure 5 The effect of *NCOA1* or *NCOA2* silencing on the inhibitory activity of P₄ against E₂-induced CXCL12 expression in normal and ectopic ESCs. siRNA targeting *NCOA1* or *NCOA2* was transfected into normal (A) and ectopic (B) ESCs isolated from five women with EMS and five women without EMS in the secretory phase respectively, which were subsequently treated with E₂ only (10⁻⁸ mol/l) or E₂ (10⁻⁸ mol/l) + P₄ (10⁻⁶ mol/l) for 3 days. CXCL12 in the supernatant was detected by the ELISA method. Experiments were performed three times for each sample and each in duplicate. The points represent the mean ± s.e.m. **P*<0.05 vs siRNA control+E₂ and ▲, *P*<0.05 vs siNCOA2 + E₂ + P₄.

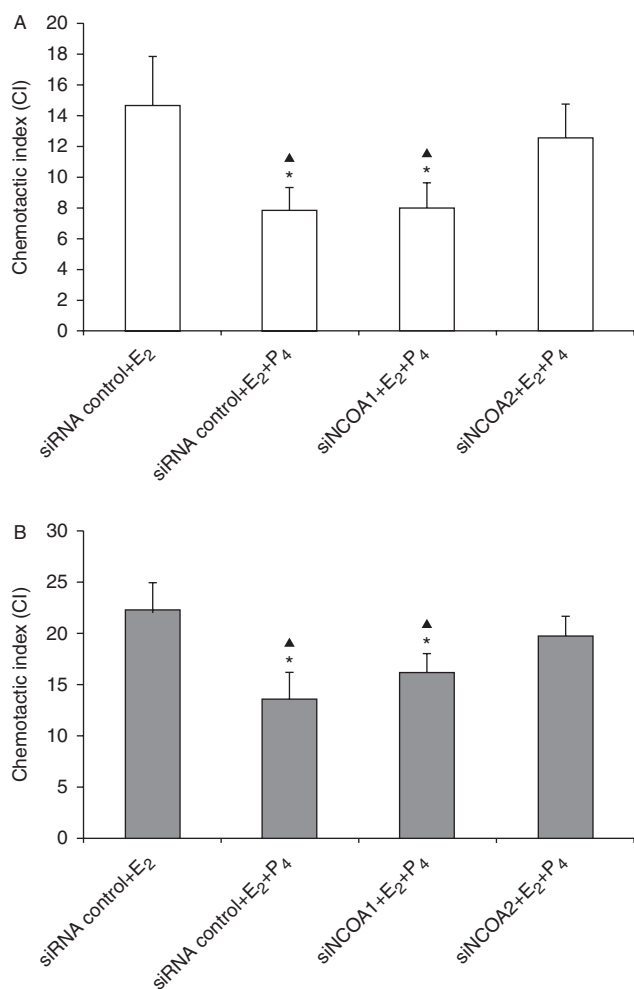


Figure 6 The effect of *NCOA1* or *NCOA2* silencing on the chemotactic activity of conditioned medium from steroid-treated normal and ectopic ESCs. siRNA targeting *NCOA1* or *NCOA2* was transfected into normal (A) and ectopic (B) ESCs isolated from five women with EMS and five women without EMS in the secretory phase respectively, which were subsequently treated with E₂ (10⁻⁸ mol/l) or E₂+P₄ (10⁻⁶ mol/l) for 3 days. Boyden chambers were used to detect the monocyte chemotaxis of the conditioned medium. Results are reported as chemotactic index (CI), i.e. cells migrating in response to the test substance divided by cells migrating in siRNA negative control medium. Each point represents the mean ± S.E.M. **P*<0.05 vs siRNA control+E₂ and ▲, *P*<0.05 vs siRNA control+E₂+P₄.

mRNA was lower in normal endometrium than in ectopic endometrium in the secretory phase. This is in contrast to the inhibitory activity of P₄ in EMS. In fact, there was no significant difference in the *NCOA2* mRNA level between the normal and ectopic endometrium in the secretory phase. But the expression of *NCOA1* mRNA in ectopic endometrium was significantly higher than that in normal endometrium in the secretory phase. On the other hand, aberrant aromatase activity and defective E₂ metabolism induced the local high E₂ environment in EMS (Bulun *et al.* 2002). These may be the reason for the contradiction between *NCOA2*

expression and P₄ resistance in EMS. Considering the different role of *NCOA1* and *NCOA2* in steroid-induced CXCL12 expression in ectopic ESCs, we prospect that it may be helpful in improving the P₄ resistance in EMS by regulating the *NCOA1* and *NCOA2* expression.

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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