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 (P4) was able to significantly inhibit estradiol (E2)-stimulated CXCL12 expression in normal and ectopic ESCs. The inhibitory rate of P4 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 E2-induced CXCL12 expression in normal and ectopic ESCs. The ability of P4 to inhibit E2-induced CXCL12 expression and monocyte chemotaxis in normal and ectopic ESCs was significantly attenuated when NCOA2 was silenced. NCOA1 plays a necessary role in E2-induced CXCL12 expression and NCOA2 is required for P4 to inhibit the E2-induced CXCL12 production in normal and ectopic endometrium.

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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 endometrioma 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 were 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, and after passage 0–1, when the ESCs cultured in six-well plates approached 80% confluence, ESCs were cultured in a medium containing 17β-E2 only (10⁻⁸ mol/l; Sigma) or 17β-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. In addition, nuclear proteins were prepared using a kit (Pansomics, 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 according to the manufacturer’s protocol (Amersham Biosciences). Detection of specifically bound proteins was carried out by ECL. The primary antibodies were 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Product (bp)</th>
</tr>
</thead>
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<tr>
<td>NCOA1</td>
<td>TCACCGGCAAGCTACCAT</td>
<td>CAGGCCTTCTCATCTGGTTC</td>
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<td>NCOA2</td>
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<td>CTTGAAAGAAGTGCTAGA</td>
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<tr>
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<td>TTTTGACTGTGGCTACTTG</td>
<td>204</td>
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<tr>
<td>GAPDH</td>
<td>GGAGGTCGACCTGGGCTTCC</td>
<td>GCTGATGATGCTGGCTGTTG</td>
<td>157</td>
</tr>
</tbody>
</table>

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**Monocyte chemotaxis assay**

The ESCs that were isolated from five ectopic and five normal endometria in the secretory phase and human histiociytic 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 μl 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±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 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.

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**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 ± S.E.M. Relative expression was calculated according to the formula (2^{−ΔCT}) (gene/GAPDH). *P<0.05 vs P phase within the same group and ▲, P<0.05 vs normal endometrium within the same menstrual phase.
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 $E_2 + P_4$, 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 $P_4$ signaling in the endometrium may be a causal factor in the development of EMS, and $P_4$ 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 $E_2$-induced CXCL12 production in normal ESCs. In our study, $E_2$ stimulated CXCL12 production from normal and ectopic ESCs in a time-dependent manner. Though the $10^{-8}$ mol/l $P_4$ cannot completely antagonize the $10^{-8}$ mol/l $E_2$-induced CXCL12 production in this study, the $P_4$ was still able to significantly inhibit $E_2$-stimulated CXCL12 expression in both the normal and ectopic ESCs. Our results showed that the inhibitory rate of $P_4$ in ectopic ESCs was significantly lower than that in normal ESCs at 72 and 96 h of $E_2$ stimulation. This suggested the existence of $P_4$ resistance in EMS.

One of the mechanisms of $P_4$ resistance in EMS is the perturbed $P_4$ signal transduction. Once bound with

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 $E_2 + P_4$, when NCOA1 was silenced, the SDF1α expression was significantly lower when compared with those treated with $E_2$ and siRNA negative control. When NCOA2 was silenced, the CXCL12 expression in normal (Fig. 5A) and ectopic (Fig. 5B) ESCs treated with $E_2 + P_4$ showed no significant difference when compared with those treated with $E_2$ and siRNA negative control. In normal ESCs treated with $E_2 + P_4$, the CXCL12 expression after NCOA2 silencing was significantly higher than that after NCOA1 silencing (Fig. 5A). In ectopic ESCs treated with $E_2 + P_4$, the CXCL12 expression showed no significant difference between NCOA1 silencing and NCOA2 silencing (Fig. 5B).

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, $E_2$ only ($10^{-6}$ mol/l) or $E_2$ ($10^{-6}$ mol/l)+ $P_4$ ($10^{-6}$ 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 $E_2$ group.

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.
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 \((10^{-8} \text{ mol/l})\) 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 \(\pm\) S.E.M. \(P < 0.05\) vs siRNA control and \(\Delta\), \(P < 0.05\) vs siSRC2+E2.

The role of SRC1 and SRC2 in SDF1 expression

Figure 4 The effect of NCOA1 or NCOA2 silencing on E2-induced CXCL12 expression in normal and ectopic ESCs. NCOA1 or NCOA2 was silenced whether in normal or ectopic ESCs, though the CXCL12 expression induced by E2 and P4 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

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.
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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**References**


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