Effect of estradiol on proliferation and differentiation of side population stem/progenitor cells from murine endometrium

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Abstract

**Background:** In our previous study, endometrium side population cells (SP cells) were isolated from postpartum murine uterus, and characterized by a heterogeneous population of stem/progenitor cells. In this study, we investigated the effect of estrogen on the proliferation and differentiation of SP cells.

**Methods:** SP and non-SP cells of postpartum murine endometrium were isolated by DNA dye Hoechst 33342. The expression of estrogen receptor 1 (ESR1) was measured by reverse transcription polymerase chain reaction (RT-PCR), Real-time PCR, Western blot, immunofluorescence and immunohistochemistry. The proliferation and differentiation of SP cells treated with different concentrations [10(-8) M-10(-6) M] of estradiol (E2) and E2+ICI182780 (Faslodex, inhibitor of ESR1) were measured by 3-(4, 5-dimethylthiazoly1-2)-2,5-diphenyltetrazolium bromide(MTT) and clonogenic assays.

**Results:** (1) SP cells expressed ESR1 at a higher level than non-SP cells. (2) The level of E2 in the serum and the expression of ESR1 in the uterus of postpartum murine changed in the same manner with the ratio of SP cells to total uterus cells at a different postpartum time point. ESR1, as ABCG2 is also predominantly located in the stroma and the glandular epithelium of the uterus. (3) 10(-6) M E2 notably promoted the proliferation of SP cells after treatment for 24 h. This effect could be inhibited by ICI182780. E2 at the concentration of 10(-7) M or 10(-8) M was sent to impair the large cloning efficiency (CE) of SP cells.

**Conclusions:** The effect of estrogen on the proliferation and differentiation of endometrium SP cells via ESR1 was observed and it was in a concentration dependent fashion. Clearly, more work is needed to understand the in vivo effect of E2 at the physiological concentration on the differentiation of SP cells.

Background

It has been proposed that human endometrium contain a population of stem cells which are responsible for their remarkable regenerative ability [1,2]. Side population cells (SP cells) have been shown in many adult tissues, and the phenotypes of SP cells might represent common molecular features for a wide variety of stem cells [1-3]. In a previous study, SP cells were isolated from the endometrium of postpartum murine uterus and these SP cells were characterized by a heterogeneous population of stem/progenitor cells [3]. Estrogen is an important hormone for repairing postpartum uterus endometrium repair. Estrogen receptor (ESR) has two isoforms: ESR1 and ESR2. Although both ESR1 and ESR2 are present in the endometrium, ESR1 seems to be the primary mediator of the estrogenic action in these tissues [4]. Some investigators found that ESR1 amplification and over-expression is likely to have a growth stimulatory effect on endometrium-derived cancer cells [5]. It is important to know how SP cells participate in the repair of cyclical and postpartum endometrium, and the effect of estrogen (via ESR1) in this procedure. Meanwhile, research on the proliferation and differentiation of
endometrium SP cells, as well as the effect of steroid hormones, will add knowledge to our understanding of pathophysiology of endometriosis.

The objectives of the present study were: 1) To evaluate the potential of the in vivo effect of estrogen on the proliferation and differentiation of SP cells during endometrium repairing by investigation of the serum estradiol level and the expression of ESR1 in murine uterus at different postpartum stages. 2) To observe directly the in vitro effect of estradiol on the proliferation and differentiation of cultured SP cells treated with different concentrations of E2 and ICI182780 (inhibitor of ESR1).

**Methods**

**Animals**

Female ICR mice [Institute of Cancer Research (ICR)], aged 6-8 weeks, were used. ICR mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Sixty mice were divided into six groups based on their postpartum day (Day 1, 7, 14, 18, 21, 28) to detect serum estradiol (E2) level and the expression of estrogen receptor 1 (ESR1) in postpartum endometrium. Another 60 ICR mice were used at postpartum Day 18 to isolate endometrium side population (SP) cells. Animal studies were conducted according to the protocols approved by the Animal Care and Use Committee of Nanjing Medical University.

**Cell preparation**

Endometrium SP cells were isolated and cultured using pancreatic enzyme, collagenase, as well as mechanical separation [3]. Cells were suspended at a concentration of 1 x 10^6 cells/ml and were then incubated in 5 μg/ml Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO). Suspensions were analyzed and sorted using a FACSVantage SE cell sorter (Becton Dickinson, Franklin Lakes, NJ) with a 350 nm UV diode laser. Hoechst 33342 fluorescence was measured at both 402 - 446 nm for Hoechst blue and 640 nm for Hoechst red.

**Immunocytochemistry**

The freshly sorted SP cells were collected and re-suspended to a final concentration of 1 x 10^6/ml. An aliquot of 0.2 ml of the suspension was used for each cell smear. Cells were cytopspun onto plus-coated slides, air dried, and fixed in acetone for 10 min at 4°C. The sections were incubated with anti-ESR1 pAb (1:50 dilution, Santa Cruz, CA) as a negative control. Sections were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:200 dilution, Santa Cruz, CA) or normal rabbit IgG (1:100 dilution, Santa Cruz, CA) as a negative control. Sections were counterstained with Harris’s hematoxylin for 35 seconds.

**RT-PCR and real-time PCR**

Total RNA was extracted from the pellets of sorted SP and main population (MP, non-SP) cells using a Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) and was reverse-transcribed into cDNA with a reverse transcription kit (Takara Bio Inc., Shiga, Japan) as per manufacturer instruction. The total RNA and cDNA of postpartum mouse uterus were prepared as the same way. β-actin was used as a housekeeping gene. PCR was performed using the following primers: β-actin primers, 5’-CCG TAA AGA CCT CTA TGC C-3’ and 5’-CTC AGT AAC AGT CCG CCT A-3’ for a 278-bp fragment; ESR1 primers, 5’-GCA CAG GAT GCT AGC CTT GTC TC-3’ and 5’-CCA GCT TGC AGG TTC ATT GTG-3’ for a 98-bp fragment. Cycling conditions for both β-actin and ESR1 were 94°C for 5 minutes, 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute, 35 cycles. The PCR products were separated on 2% agarose gels for analysis.

**Western blot**

The different postpartum days (Day1, 7, 14, 18, 21, 28) of mouse uteri were homogenized on ice with lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% [w/v] 3-[[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 2% [w/v] dithiotheriol (DTT), 2% [v/v] immobilized pH gradient [IPG] buffer, pH 3-10) in the presence of 1% (v/v) protease inhibitors cocktail kit (Pierce Biotechnology, Rockford, Illinois, USA). After centrifugation at 40000 g at 4°C for 1 hour, protein extracts in the supernatants were exposed to citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven for 10 minutes for antigen retrieve. The sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 minutes and blocked with 10% normal goat serum (Zhongshan Biotechnology Co. Ltd., Beijing, China) for 2 hours. It was allowed to react for 12 hours with the primary rabbit anti-ESR1 antibody (1:50 dilution, Santa Cruz, CA) or normal rabbit IgG (1:100 dilution, Santa Cruz, CA) as a negative control. Sections were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:200 dilution, Santa Cruz Biotechnology Inc, CA) at room temperature for 1 hour and the reactivity was visualized with peroxidase-substrate solution (diaminobenzidine, DAB) until the desired stain intensity developed. Sections were counterstained with Harris’s hematoxylin for 35 seconds.
collected and stored at -80°C until use. Aliquots of 50 μg protein extracts from each sample were loaded and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resulting proteins were then transferred onto a nitrocellulose membrane. After treatment with blocking solution (5% non-fat milk powder in Tris-buffered saline [TBS; pH 7.4]) for 2 hours, the membranes were incubated with rabbit polyclonal antibodies of ESR1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), or β-tubulin (1:5000, Abcam, Cambridge Science Park, Cambridge, UK) at 4°C overnight. The membrane was then incubated with HRP-conjugated anti-rabbit secondary antibodies (1:1000, Zhongshan Biotechnology Co. Ltd., Beijing, China), or anti-α-tubulin (1:500, Abcam, Cambridge, UK), or anti-α-tubulin (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), or β-tubulin (1:5000, Abcam, Cambridge Science Park, Cambridge, UK) at 4°C overnight. The membrane was then incubated with HRP-conjugated anti-rabbit secondary antibodies (1:1000, Zhongshan Biotechnology Co. Ltd., Beijing, China) for 1 hour at 37°C, and examined by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). The membranes were then scanned, and the signal intensity of each band was quantified using AlphaEaseFC (Fluorchem 5500) software (Alpha Innotech Corp., CA). Relative protein levels in each sample were normalized to the β-tubulin level in order to standardize the loading variations.

Serum preparation and Hormone assays
Mice of different postpartum time (day1, 7, 14, 18, 21, 28) were anesthetized by 1% pentobarbital sodium and approximately 1 ml blood was withdrawn from the right ventricle. The blood samples were kept at room temperature for 2 hours, centrifuged at 1500 g for 10 minutes, and the sera were stored at -80°C until use. Concentrations of estradiol in the sera and conditioned medium of cultured SP cells were measured via a sensitive (<0.02 ng/mL) and reproducible (total coefficient of variation [CV], <10%) radioimmunoassay (RIA) with a measurement range from 0.1 to 20 ng/mL. The RIA kits were obtained from the Beifang Biotechnique Institute (Beijing, China).

Clonogenic assay
SP cells were seeded in triplicate at 500 cells/cm² in flat-bottomed, 6-well culture plates. For the first 2 days, the culture medium was phenol red-free DMEM/F-12 (Gibco, USA) containing 5% FBS, 100 IU/ml penicillin, and 10 mg/ml streptomycin. Starting from Day 3, the cells were treated with different concentrations (10⁻⁸ - 10⁻⁶M) of 17βE₂ or 17βE₂ in the presence of ICI182780 (inhibitor of ESR1, 10⁻⁶M), Cells were cultured for 15 days in 5% CO₂: 95% air. The culture medium was changed every 2 days. Colonies were monitored microscopically daily to ensure that they were derived from single cells. The dishes for clone analysis were fixed in 4% paraformaldehyde in PBS for 30 minutes, stained with Harris’s hematoxylin for 5 minutes, then washed in running tap water for 20 minutes and dried. Clusters of cells were considered colonies when they were visible macroscopically and contained more than 50 cells. Colonies more than 70 cells were considered large clones. Colonies were counted and images were recorded. The cloning efficiency (CE) was determined from the formula: CE (%) = (number of colonies/number of cells seeded) × 100%.

Cell proliferation and viability assays
The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Briefly, SP cells were plated at a density of 3 × 10⁴cells/well in 96-well plates, cultured for 2 days in phenol red-free serum medium and then exposed with different concentrations (10⁻⁸ - 10⁻⁶M) of 17βE₂ in the presence or absence of ICI182780 (10⁻⁶M) for 24 hours. The cells were then incubated with 0.5 mg/ml MTT for 4 hours at 37°C. The media was carefully removed and 150 μl of DMSO were added to each well for 15 minutes in order to solubilize the dark blue formazan crystals formed in intact cells. The absorbance was measured at 570 nm with the microplate reader (Bio-Tek ELX800, USA).

Statistics
Each experiment was repeated at least three times. All data were presented as mean ± SD. One-way analysis of variance was used to compare the mRNA and protein levels. Chi-square analysis was used to compare the rates of SP cell large clones. A value of P < 0.05 was considered statistically significant.

Results
Variation of SP cells proportions along with postpartum days
Fluorescence activated cell sorter (FACS) analysis showed that along with the changing of postpartum time (Day1, 3, 7, 14, 18, 21, 28, 60) the proportion of SP cells increased significantly and then gradually decreased after Day 18 (P < 0.05) (Figure 1A).

Variation trend of serum E2 level along with postpartum days
Serum estradiol level was high at D1, and then remarkably decreased at Day 7 (Figure 1B). The E₂ level, on the other hand, had a gradual increase from Day 7 to Day 18 after which it showed a decreasing trend (P < 0.05) (Figure 1B). This trend is representative of the change in SP cell proportion. These findings suggest that estradiol may play a role in the regulation of SP cells, which participate in the regeneration of postpartum mice endometrium.
Expression of ESR1 in mouse endometrium at different postpartum days

Analysis with real-time PCR showed increased ESR1 expression within the first 14 days of postpartum, with the highest peak at Day 14 ($P < 0.05$) (Figure 1C). This trend was similar to the trend of SP cell ratio in the endometrium of postpartum mice. Western blot showed the same result as PCR analysis. Compared with other days of postpartum, ESR1 obviously expressed at highest level at Day 14 ($P < 0.05$) (Figure 1D).

Immunohistochemistry showed the same variation as Real-time PCR analyses. Luminal epithelium at Day 1 expressed low level of ESR1, whereas stroma and glandular epithelium at Day 7 showed mid level of ESR1 expression. Stroma at Day 14 expressed high level of ESR1. From Day 18 to Day 28, expression of ESR1 decreased gradually. At Day 18, significant amounts of ESR1 were expressed in the Glandular epithelium. ESR1 expression was seen to decrease significantly at Day 21 and Day 28 (Figure 2).

Preferential expression of ESR1 in SP cells when compared with non-SP cells

We also examined the basal expression of ESR1 in SP cells and non-SP cells. RT-PCR analyses showed that the expression of ESR1 mRNA was higher in SP cells than that in non-SP cells ($P < 0.05$) (Figure 3B, C). Immunocytochemical analysis showed that 75.18 ± 5.47% of SP cells expressed ESR1, and only 32.28 ± 4.3% of non-SP cells expressed ESR1 (Figure 3A). These findings suggested that a large number of SP cells were able to respond to estrogen (target cells of estrogen).

Cell proliferation of SP cells modulated by 17βE2

To study the proliferative effects of 17βE2, SP cells were treated with different concentrations ($10^{-8} - 10^{-6}$M) of...
17βE₂ in the presence or absence of ICI182780 (10⁻⁶M). 17βE₂ at the dose of 10⁻⁶ M induced a significant increase in SP cell numbers (P < 0.05), even though E₂ at lower doses (10⁻⁷, 10⁻⁸ M) could not significantly change SP cell numbers (P > 0.05) (Figure 4A). The stimulated proliferation was blocked by ICI182780 (P < 0.05) indicating the effect was specifically mediated through estrogen receptors.

**Variation of large CE of SP cells after 17βE₂ treatment**

Total CE of SP cells in all of 17βE₂ treated groups did not significantly change. The CEs of large colonies were as follows: 0.073% ± 0.046% (n = 3) for 10⁻⁶ M 17βE₂, 0.04% ± 0.031% (n = 3) for 10⁻⁷ M 17βE₂, 0.027% ± 0.011% (n = 3) for 10⁻⁸ M 17βE₂, 0.126% ± 0.042 (n = 3) for 10⁻⁶ M 17βE₂+ICI182780, 0.073% ± 0.023% (N = 3) for 10⁻⁷ M 17βE₂+ICI182780, and 0.113% ± 0.03%
(n = 3) for $10^{-8}$ M $17\beta$E$_2$+ICI182780. There was a significant decrease in CE of large colonies of SP cells in groups treated with $10^{-7}$ M and $10^{-8}$ M of $17\beta$E$_2$. ICI182780 was seen to block this effect (P < 0.05) (Figure 4C). The size distribution of these SP cells colonies was also examined by scoring 344 colonies from nine samples and sorting them into numerical order. Figure 4B shows that colonies could be categorized into two groups: small colonies (<70 cells) or large colonies (>70 cells).

**Discussion**

Adult stem cells have tissue-specific function as they are known to repair and maintain their corresponding tissues [3]. The concept that endometrial regeneration is mediated by endometrial stem/progenitor cells was proposed many years ago [7,8]. It is widely believed that endometrial restoration after menses suggests a need for estrogen-primed proliferation. In the previous study, it was shown that SP cells of postpartum murine endometrium were a heterogeneous population of endometrial stem/progenitor cells [3]. In the mouse, the normal estrus cycle recovers several days after the lactation is over, which is approximately 21-24 days after parturition. In this study, an initial increase was seen in the percentage of SP cells in postpartum murine endometrium. This increase was observed until Day 18, after which a gradual decrease was recorded. It was suggested that this phenotype might correlate with endocrinological hormonal changes after parturition. Uterus is an important target organ of estrogen. Estrogen receptor (ER) has two isoforms: ESR1 and ESR2. ESR1 is the main isoform of ER in humans and rodents. Although ESR1 and ESR2 were both expressed in endometrium, when compared with the wild type, it was seen that the uterus of the ESR1 knockout mice is immature with...
fewer glands. Even with the administration of exogenous E2, there was no thickening of the endometrium or the muscular layer in ESR1 knockout mice. This is indicative of the primary function of ESR1 in the uterus [9,10]. Estrogen regulates endometrial cell survival, viability and mitogenic activity via ESR1 [11,12]. Decreased expression of ESR2 has previously been seen in many estrogen-dependent tumors [13]. ESR2 acts as a modulator of ESR1 and has an anti-proliferative and pro-apoptotic role [14]. In this study, it was found that the expression of ESR1 was greater in SP cells, when compared with the expression in non-SP cells. Since ESR1 is the dominant isoform in the uterus, it was assumed that estrogen might regulate SP cells via the ESR1 function of regenerating the endometrium.

The E2 level in serum and the ESR1 expression in uterus showed the same trend with the proportion of SP cells and total uterus cells varying in different postpartum days (Day 1 to Day 28). It was found that the E2 level in serum gradually increased from postpartum Day 7, and then declined after Day 18. However, the ESR1 expression at both the gene level and the protein level peaked at postpartum Day 14. It was reported by Kang et al. [15] that after treatment with 17-ethinyl estradiol (EE) (doses of 3.0 and 10.0 μg/kg/day), ESR1 expression decreased in the uterine luminal and glandular epithelium, as well as in the stroma and the uterine smooth muscle cells. The data suggested that the increased endogenous E2 level could negatively regulate ESR1 expression in uterus to some extent, which could have
resulted in the discord of time-course of E2 level and ESR1 expression peaks.

In this study, it was found that ESR1 in mouse uterus at postpartum Day 14 was predominantly expressed in endometrium stroma, while it was highly expressed in glandular epithelium at postpartum Day 18. In previous studies, it was found that the ATP-binding cassette superfamily G member 2 (ABCG2) was mainly expressed in postpartum mouse endometrium stroma. ABCG2 was also expressed in a small part of vascular endothelial cells and glandular epithelium [3]. ABCG2 is specially expressed in SP cells. Estrogen promotes endometrium cell mitosis, which is an important factor for the development and functional maintenance of female reproductive system. In uterus, E2 promotes endothelial progenitor cell (EPC) differentiation, migration, proliferation, and apoptosis inhibition [16-18]. The enhanced biological activities in EPC by E2 are blocked by the specific ESR1 antagonist ICI182780, which indicates that the effect of estrogen on EPCs is via functional ESR1 in EPCs. In this study, we found that 10^{-6} M E2 could significantly promote proliferation of SP cells in vitro and this effect could be blocked by ICI182780 (P < 0.05). Three other concentrations of E2 at a lower level showed concentration dependent tendency of stimulation of SP cell proliferation although they did not reach statistical significance. Therefore, E2 leads to the promotion of SP cells via ESR1 by participating in the regeneration of the endometrium.

Stem cells are able to differentiate, self-renew and replace themselves, into committed progenitors. These committed progenitors are differentiated transit amplifying (TA) cells, which rapidly proliferate and finally differentiate to produce a large number of terminally differentiated functional cells with no capacity for proliferation. It is possible that the large colonies are initiated by putative stem/progenitor cells and the small colonies are initiated by putative TA cells [2]. In this study, it was found that CE of large clones treated by 10^{-7} M and 10^{-8} M E2 notably decreased and this effect could be blocked by ICI182780 (P < 0.05). With E2 concentration gradually decreasing from 10^{-6} M to 10^{-8} M, CE of large clones also showed decreasing trend even though there was not a statistically significant difference recorded (P > 0.05). These results indicated that E2 at the circumambient concentration of physiological range could promote the committed differentiation of SP cells in vitro. The physiological serum concentration of 17beta E2 in postpartum mice is about 10^{-8} M. Deasy et al., [17] when studying Duchenne Muscular Dystrophy (DMD), found that the female muscle-derived stem cells (MDSCs) regenerated skeletal muscle more efficiently than those of the males. It was therefore concluded that E2 at high concentrations promoted proliferation of SP cells of postpartum mouse endometrial, whereas E2 at physiological concentrations could promote differentiation of SP cells.

In clinical practice, many patients become infertile due to declining endometrial regeneration capability. The common reasons include abortion, infection and endocrinical dysfunction. Conversely, abnormal endometrial hyperplasia could lead to endometriosis, functional uterine bleeding and endometrial carcinoma [19-22]. The current study advanced the understanding of the role of SP cells in repairing postpartum endometrium and in the pathological mechanism of abnormal endometrial proliferation, as well as the accumulated materials for experimental and clinical applications of endometrial SP cells.

Conclusions

In conclusion, evidence provided in the present study indicated that E2 promoted SP cells via ESR1, which in turn plays a role in the regeneration of the endometrium. It was observed that E2 at high concentrations promoted proliferation of SP cells of postpartum mouse endometrial, while E2 at physiological concentrations could lead to the promotion of differentiation of SP cells. Further studies will be required to elucidate the role of SP cells in repairing postpartum endometrium and in the pathological mechanism of abnormal endometrial proliferation.

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Authors' contributions

JX and FFH carried out the main experiments and wrote the first draft of manuscript. YGC and JYL, teachers of JX and FFH, designed the study and revised manuscript. YGC investigated data and proofread the final manuscript. JL, CYJ, LG XQQ and YDM participated in some experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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