Altered expression of epithelial mesenchymal transition and pluripotent associated markers by sex steroid hormones in human embryonic stem cells

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Abstract. Embryonic stem (ES) cells are pluripotent stem cells derived from a developmental stage of pre-implanted embryos. The present study investigated the effect of female sex steroid hormones on the characteristics of human ES cells by using a feeder-free culture protocol. In a feeder-free condition without sex hormones, human ES cells assumed the form of tightly packed cells that grow in a monolayer. The cells had clean and defined edges with no evidence of differentiation and expressed several markers specific for undifferentiated ES cells including POU class 5 homeobox 1 (POU5F1), sex determining region Y-box 2 (SOX2) and NANOG homeobox (NANOG). It was then investigated if female sex steroid hormones including 17β-estradiol (E2) and progesterone (P4) altered the protein expression of epithelial-mesenchymal transition (EMT) associated markers in addition to pluripotency markers including POU5F1, SOX2 and NANOG in human ES cells. The protein expression levels of N-cadherin, Snail and Slug were increased while E-cadherin expression was decreased by treatment of E2 or P4, and the expression levels of POU5F1, SOX2 and NANOG were decreased by the treatment of E2 or P4. When E2 and P4 were treated in combination with an estrogen receptor inhibitor (ICI 182,780) and progesterone receptor inhibitor (RU486) respectively, their effects on EMT and pluripotency of ES cells were restored to control levels. The results suggested that E2 and P4 may regulate EMT and pluripotency of human ES cells by mediating their receptors. The present study may aid in the understanding of the role of sex steroid hormones in the cellular biology of human ES cells.

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Abbreviations: ES, embryonic stem; E2, 17β-estradiol; EMT, epithelial-mesenchymal transition; P4, progesterone; PR, progesterone receptor; MEF, mouse embryonic fibroblast; ECM, extracellular matrix; CM, conditioned medium

Key words: sex steroid hormones, human embryonic stem cells, pluripotency, epithelial-mesenchymal transition

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of a blastocyst of preimplantation embryos (1). They have pluripotency because they are capable to differentiate into all of the three germ layers: Ectoderm, endoderm, and mesoderm. Particular transcription factors and molecular markers are known to regulate pluripotency of ES cells and empower them ability to differentiate into cells of the three germ layers (2,3). POU5F1, SOX2, and NANOG proteins may modulate the pluripotency of ES cells by adjusting how ES cells differentiate to any germ layer (4). Specifically, POU5F1 is known to suppress neural ectodermal differentiation and promote mesodermal differentiation. On the other hand, SOX2 inhibits mesodermal differentiation and promotes neural ectodermal differentiation (5). NANOG also helps the actions of pluripotent factors such as POU5F1 and SOX2 to regulate the target genes that play an important role in the maintenance of ES cell pluripotency (6). Using these established markers to regulate the pluripotency of stem cells may enable to anticipate how stem cell-specific characteristics are changed by various factors (7).

Estrogen and progesterone are among the primary female sex hormones which are involved in the menstrual cycle, pregnancy, and embryogenesis of humans and other species (8,9). The placenta synthesizes increased levels of female hormones during pregnancy (10,11). Extraordinarily, high levels of these hormones enable the uterus and placenta to improve vascularization and transfer of oxygen and nutrients, and support the developing fetus via their receptors (12,13). They also regulate differentiation in human embryonic stem cells (hESCs). Estrogen compounds, estradiol (E2) and estriol (E3), have effects on endodermal and mesodermal differentiation of human embryoid bodies (14). In another study, the effects of E2 on differentiation of neural cells were investigated. E2 promoted differentiation of hESCs into

dopamine neurons via cross-talk between insulin-like growth factors-1 and estrogen receptor β (15). Moreover, pregnancy hormones, human chorionic gonadotropin and progesterone, induced hESCs proliferation and differentiation into neuroectodermal rosettes. hESCs expressed P4 receptor A, and treatment of hESCs colonies with P4 induced neurulation, as demonstrated by the expression of nestin and the formation of columnar neuroectodermal cells that organize into neural tubelike rosettes. Suppression of P4 signaling by treating with the P4-receptor antagonist RU-486 inhibited the differentiation of hESC colonies into EB's and rosettes (16). These results demonstrate that these hormones influence on the differentiation of hESCs.

In addition, they play an important role in the epithelial-mesenchymal transition (EMT) process (17). EMT is a multiple step of conserved cellular programs that enable polarized immotile epithelial cells to transform to motile mesenchymal cells. EMT and its reverse process, MET (mesenchymal-epithelial transition), are critical for embryonic development and the formation of various tissues or organs (18). During development, the EMT program has been observed in a variety of tissue remodeling events, including mesoderm formation, neural crest development, heart valve development, and secondary palate formation (19,20). During EMT, epithelial cells lose apical-basal polarity and cell junctions like tight junctions to acquire a mesenchymal phenotype (21). Adherens and gap junctions are also dissolved, and cell surface proteins such as E-cadherin and epithelial-specific integrins that mediate epithelial connections to neighboring cells and the basement membrane, respectively, are replaced by N-cadherin and mesenchymal integrins. Inside the cells, cytokeratins, the epithelial intermediate filaments, are replaced by Vimentin (22,23). Consequently, mesenchymal cells devoid of cell-cell contacts, which are derived from epithelial cells via EMT, move into and invade surrounding stroma. As a highly coordinated and specific series of events, EMT is modulated by diverse regulators including SNAIL/SLUG (SNAI1/SNAI2), Twist, and Six1 and signaling pathways (23).

In this study, we investigated whether female sex steroid hormones influence EMT and maintenance of pluripotent propensity of human ES cells using a new culture method including conditioned medium (CM) that can maintain undifferentiated state of ES cells without the mouse feeder cells. Generally, mouse feeder cells have been used to help the growth and maintenance of human ES cells, however, there may be a risk of contamination of culture media by viruses or other macromolecules, which already exists in the mouse cells and can transfer to the ES cells (24).

Our findings demonstrated that hormone treatment leads to the process of EMT through the up- or down-regulation of cellular markers and signaling proteins. In addition, its treatment altered protein expression of transcriptional factors such as POU5F1, SOX2, and NANOG, which are known to help maintain pluripotent ability of ES cells. From this study, we confirmed that E2 and P4 could affect the EMT in human ES cells and ES cell differentiation due to the loss of pluripotency. It is hoped that this study will provide new insights for the relationships between female sex steroid hormones and embryo developmental process.

Materials and methods

Reagents and human ES cells. 17β-Estradiol (E2), progesterone (P4), fulvestrant (ICI 182,780), and mifepristone (RU486, a progesterone receptor (PR) antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in dimethyl sulfoxide (DMSO; Junsei Chemical Co., Ltd., Tokyo, Japan). The prepared solution was stored at room temperature. H9 human ES cells were obtained from Dr Jaejin Cho (Department of Dental Regenerative Biotechnology, Seoul National University, Seoul, Republic of Korea). The use of human ES cells in this study was approved by IRB Committee of Chungbuk National University (CBNU-201407-ETC-064-01).

Preparation of human ES cells medium. A culture medium for human ES cells is composed of Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12; Gibco, Grand Island, New York, USA) supplemented with 20% knockout serum replacement (KSR; Gibco), 0.25 M Peni/Strep (A&E Scientific, Logan, UT, USA), 0.1 mM nonessential amino acids (Gibco), and 0.1 mM 2-mercaptoethanol. For a prolonged culture, 4 ng/ml recombinant human fibroblast growth factor-basic (bFGF; R&D Systems, Inc., Minneapolis, MN, USA) was required to be added in the medium just before use.

Preparation of CM. Mouse embryonic fibroblast (MEF) feeder cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F12; Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA), 1% Peni/Strep (A&E Scientific), 2% Antibiotic-Antimycotic (GIBCO Invitrogen, Grand Island, New York, USA), 1% glutamax (Gibco), 1% nonessential amino acids (Gibco), and 0.1% 2-mercaptoethanol in a humidified atmosphere of 5% CO_2 containing air. When MEF feeder cells reached approximately 80% of confluent growth, the MEF medium was removed, and the cells were washed twice with 1X PBS. Fresh medium for human ES cells was added to the MEF dishes, and CM was collected every day. MEF cells can be used for up to 7 days to produce CM. The CM collected from the MEF culture dishes after 24 h was sterilized with a 0.2-μm filter and was stored at -20˚C before adding bFGF for later use.

Preparation of Matrigel-coated plates. Matrigel (BD Biosciences, Bedford, MA, USA) was slowly thawed on ice at 4˚C for at least 1 h to prevent the formation of gel clot. To prepare the working solution, Matrigel (BD Biosciences) stock solution was diluted to 1:4 in cold CM prepared beforehand. Each bottom of a 35 mm culture plate was coated with 1 ml of Matrigel working solution. Next, the working solution was removed, and the Matrigel-coated plates were incubated for 1-2 h at room temperature, or overnight at 4˚C until the plate is completely dried.

Culture of human ES cells in a feeder-free condition. A sharpened tip was made by heating a sterile pasteur pipette with the alcohol lamp. The morphology of human ES cells was checked under the microscope. Advised shape of ES cell

colonies is round, and the color is recommended to be dark. When cells begin to differentiate, they look like dim because they grow being overlapped so that the light of the microscope is difficult to permeate the cells. It should be careful that the cell surface is not clean or uneven. When the suitable number of colonies is determined, the culture medium for human ES cells was completely removed and washed with CM twice. The ES cell colonies were separated into small numbers about 20-30 using a pasteur pipette tip with a sharpened edge. After collecting the colonies using a 200 μl pipette, they were moved to the conical tube containing approximately 1 ml of CM. After adding an appropriate amount of CM, the cells were dissociated into small clusters (50-100 cells) by gentle pipetting. Human ES cells were seeded into each well of Matrigel-coated plate and were feed with 2-2.5 ml of CM supplemented with 4 ng/ml bFGF per well every day.

RNA extraction and cDNA synthesis. mRNA expression of pluripotency-related markers was identified in human ES cells cultured in a conventional medium as well as a CM containing E2 and P4. Human ES cells were treated with E2 (10^{-8} M) and P4 (10^{-6} M) for 24 h. RNA was extracted with the Cell Lysis & RT Kit for qPCR (Toyobo Co., Ltd., Osaka, Japan) with a following protocol recommended by the manufacturer. Briefly, 50 μl lysis solution with gDNA remover was added to plates, and the solution was mixed gently using a 1 ml pipette and incubated at room temperature for 5 min. Next, 10 μl stop solution with RNase inhibitor was added to wells, and the solution was mixed with tapping hands for 30 sec and incubated at room temperature for 1.5 min. When the RNA extraction is completely prepared, 8 μl 5X RT Master Mix was add to 24 μl nuclease-free water in a tube. 32 μl reaction mixture was transferred into a PCR plate, and 8 μl of the RNA lysate was added. The solution was mixed gently and span down. And then, the mixture was incubated as follows: for 15 min at 37˚C, 5 min at 50˚C, and 5 min at 98˚C.

Polymerase chain reaction (PCR). PCR amplification was performed for 30 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 58˚C, and extension for 30 sec at 72˚C. The composition of the reaction product is composed of cDNA template, Taq polymerase (Intron Biotechnology, Seoul, Korea), dNTP, 10x PCR buffer (Intron Biotechnology), and forward and reverse primers (Table I). The PCR products were loaded onto a 1.5% agarose gel pretreated with EtBr, and the band sizes were compared with 100-bp ladders. The bands were analyzed with Gel Doc 2000 software (Bio-Rad Laboratories Inc, Hercules, CA, USA). GAPDH was used as an endogenous control for normalization.

Western blot assay. Human ES cells were treated with E2 (10^{-8} M), P4 (10^{-6} M), ICI 182,780 (10^{-6} M) or RU486 (10^{-8} M) for 48 h. Cells were harvested with RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich), 0.5% deoxycholic acid (Sigma-Aldrich), and 0.1% SDS. Western blot analysis was performed as previously described (25). Briefly, protein concentration was determined by a bicinchoninic acid (Sigma-Aldrich) assay. Total cell proteins (40 μg) were separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, Laboratories, Inc., Berkeley, CA, USA). The membrane was then incubated with E-cadherin, N-cadherin, Snail, Slug, POU5F1, SOX2, and NANOG. Mouse monoclonal antibodies against GAPDH (1A10), N-cadherin (5D5), Slug (1G7), POU5F1 (C-10), SOX2 (E-4), NANOG (H-2) (1:12,000, 1:1,000, 1:1,000, 1:1,000, 1:1,000 and 1:1,000 dilution, respectively; Abcam, Cambridge, UK), and rabbit polyclonal antibodies against E-cadherin (1:2,50 dilution; Abcam) and Snail (1:1,000; Abcam) were overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) G or anti-rabbit IgG (1:3,000, 1:2,500 dilution, respectively; Thermo Fisher Scientific, Inc., Rockford, IL, USA) was used as a secondary antibody. Immunoreactive bands were detected using a West Q chemiluminescent substrate Kit Plus (GenDEPOT, Barker, TX, USA). Densities of target protein bands were quantified using Gel Doc 2000 (BioRad Laboratories, Inc.).

Statistics analysis. All experiments were repeated at least 3 times, and data are presented as the mean ± SD. Statistical analysis was conducted by using one-way ANOVA test, followed by Dunnett's multiple comparison test or Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Maintenance of undifferentiation of ES cells in a feeder-free condition. Undifferentiated human ES cells usually form round colonies with clear margins. The morphology of ES cell colony from conventional culture seen in Fig. 1A (Control) displayed the propensity of small, tightly packed cells that grow in a monolayer. The colony looked clean, defined edges, with little or no differentiation. Different cell features were identified because they were tightly packed with each other suggesting close cell membrane contact. Cells were grown to overlapping, so it seems like dark. As we expected, however, the colony of human ES cells cultured in a feeder-free condition showed a round and well-defined edge (Fig. 1A, Feeder-Free), which is similar to that of ES cell colony from conventional culture. The surface of the colony looks like bumpy, because feeder-free medium did not contain MEF cells, which support ES cells. In Fig. 1B, RT-PCR data showed that human ES cells cultured in a conventional method expressed mRNAs of undifferentiated ES cell-markers such as *POU5F1, SOX2,* and *NANOG* genes. By western blotting, protein expression levels of POU5F1, SOX2, and NANOG were similar to mRNA expression, as shown in Fig. 1C. Specifically, we also observed that the protein expression levels of POU5F1, SOX2, and NANOG were constantly maintained in a conventional medium as well as a feeder-free CM after the 10th passage of human ES cell culture as in the initial passage (Fig. 1C).

Sex steroid hormones affected EMT of ES cells through their cognate receptors. To investigate whether sex hormones influence on EMT of human ES cells, E2, P4, and inhibitors of their cognate receptors (estrogen receptor inhibitor; ICI 182,780 and progesterone receptor inhibitor; RU486) were treated to human ES cells individually or by mixture. As shown in Figs. 2 and 3, E2 (10^{-8} M) and P4 (10^{-6} M) treatment induced

Table I. Oligonucleotide primer sequences of genes for RT-PCR.

Gene	Primer sequences (5'→3')	Expected size
GAPDH	F: 5'-ATGTTCGTCATGGGTGTGAACCA-3' R: 5'-TGGCAGGTTTTTCTAGACGGCAG-3'	356 bp
POU5F1	F: 5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3' R: 5'AAGGGCCGCAGCTTACACATGTTC-3'	245 bp
SOX2	F: 5'-ACACCAATCCCATCCACACT-3' R: 5'-GCAAACTTCCTGCAAAGCTC-3'	224 bp
NANOG	F: 5'-TGCAAATGTCTTCTGCTGAGAT-3' R: 5'-GTTCAGGATGTTGGAGAGTTC-3'	286 bp

F, forward sequence; R, reverse sequence.

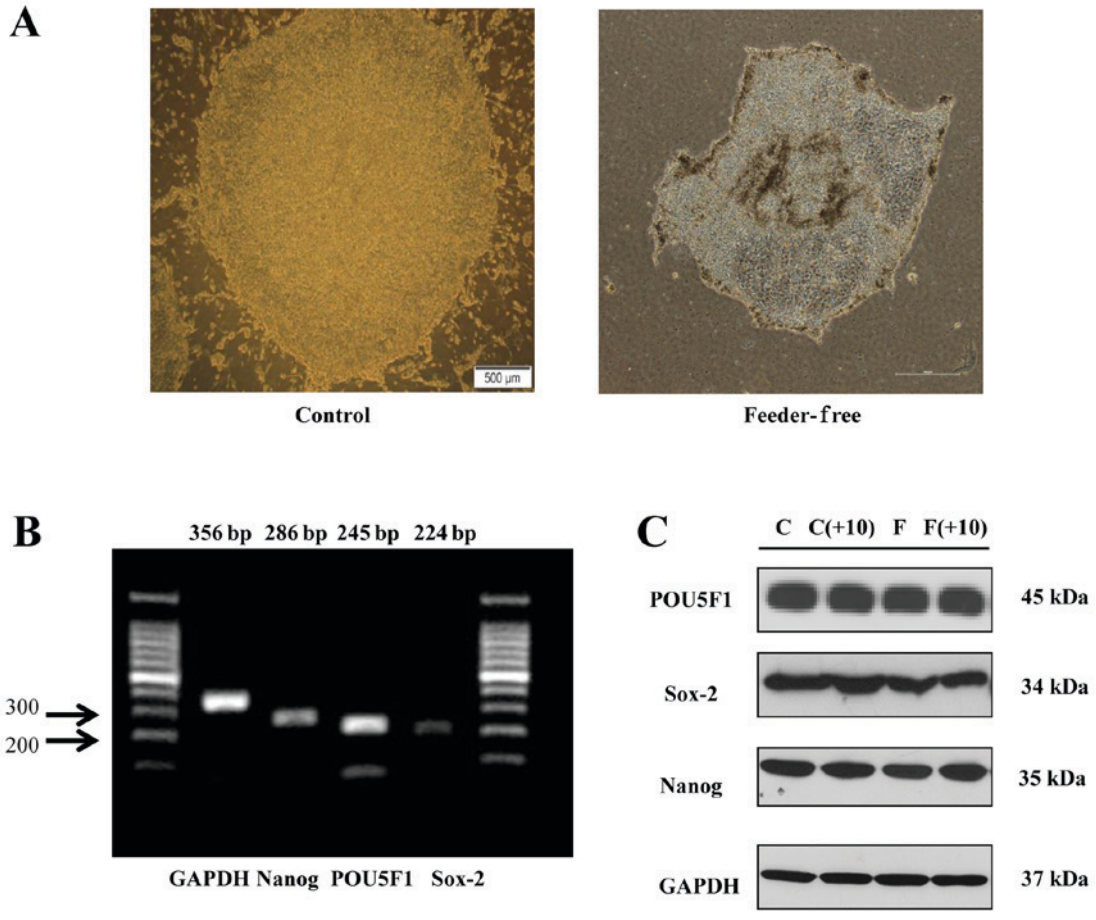

Figure 1. Characterization of H9 human ES cells cultured in conventional and feeder-free conditioned media. (A) The cellular morphology of human ES cell colony was captured using an optical microscope under 4x magnification. The left colony is from human ES cells cultured in a conventional medium, and the right colony is from human ES cells cultured in a feeder-free conditioned medium. A scale bar represents 500 μm in length. (B) mRNA expression of pluripotent markers such as *POU5F1*, *SOX2*, and *NANOG* in human ES cells cultured in a conventional medium was confirmed by RT-PCR. (C) Protein expression of POU5F1, SOX2, and NANOG in human ES cells cultured in a conventional medium (C) as well as a feeder-free CM (F) at initial passage (C and F) and 10th passage [(C+10) and (F+10)] was identified by western blotting. *GAPDH* mRNA and protein were used as a control to identify the normal mRNA and protein expression in human ES cells, respectively.

the EMT process in human ES cells. The protein expression of an epithelial cell marker, E-cadherin, was reduced by E2 and P4. In contrast, the protein expression of a mesenchymal cell marker, N-cadherin, was increased by E2 and P4 as shown in Figs. 2 and 3. When ES cells were co-treated with E2 in the presence of ICI 182,780, the protein expression levels of E-cadherin and N-cadherin were distinctly restored to the control levels as seen in Fig. 2. In addition, P4 distinctly regulated these proteins, and the expression levels of E-cadherin and N-cadherin were restored to the control levels in the

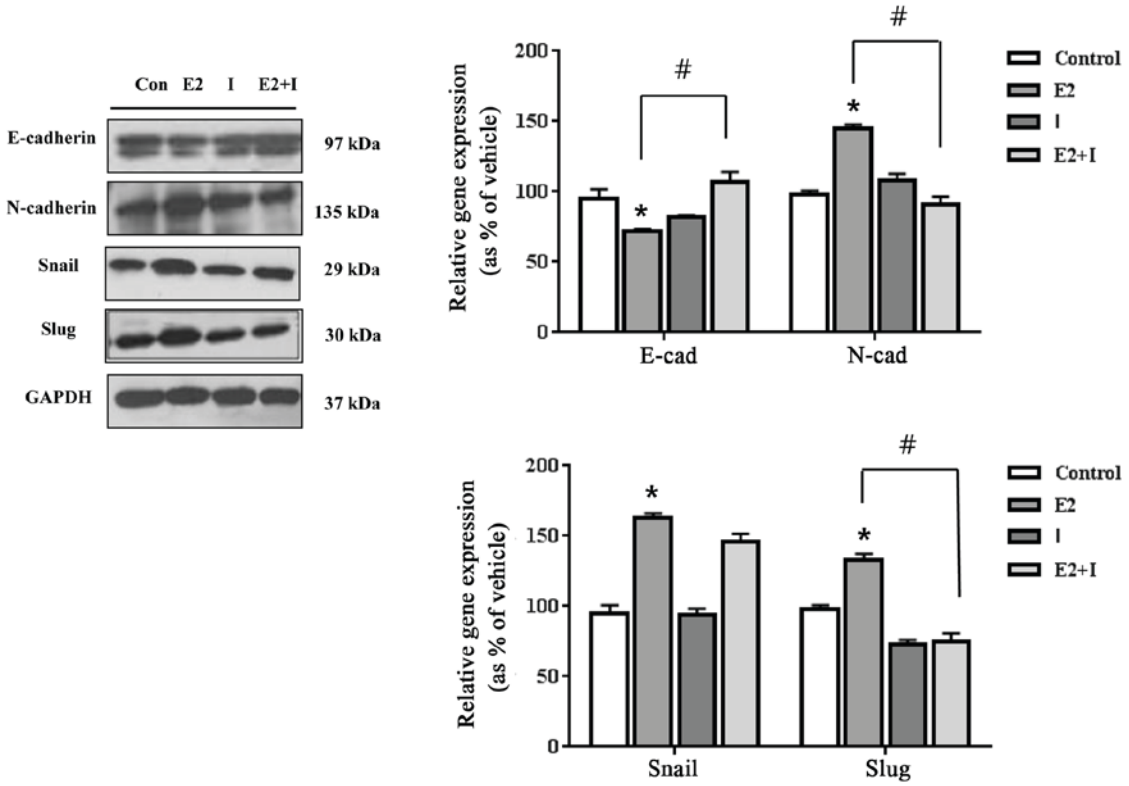

Figure 2. Effects of E2 and its receptor inhibitor on the protein expression of EMT specific markers. After the treatment of E2 (10^{-8} M), ICI 182,780 (10^{-6} M), and E2 (10^{-8} M)+ICI 182,780 (10^{-6} M) in human ES cells for 48 h, total proteins were extracted, and bands corresponding to E-cadherin and N-cadherin were detected by western blotting and quantified using Gel Doc 2000. *Significant elevation or reduction in gene expressions by each treatment comparing with control (P<0.05 in Dunnett's multiple comparison test). #Significant elevation or reduction in gene expressions by treatment of E2+ICI 182,780 (I) comparing with treatment of E2 (P<0.05 in Student's t test).

presence of RU486 (Fig. 3). These results suggest that E2 and P4 may induce EMT by altering protein expression of E-cadherin and N-cadherin via their receptors.

In addition to cell specific markers, the alteration of transcription factors that control EMT was further investigated. E2 increased protein expression of Snail, an E-cadherin repressor, and Slug, an EMT regulator gene, while ICI 182 780 reversed E2-induced increases via an ER-dependent manner (Fig. 2). It was of interest that P4 treatment increased Snail only in these cells as seen in Fig. 3. However, when ES cells were co-treated with P4 and RU486, the altered expression levels of Snail or Slug by P4 were restored to the control levels (Fig. 3). As a result, these results indicate that E2 and P4 appear to induce the EMT process of ES cells through their cognate receptors.

Effect of sex steroid hormones on mRNA and protein expressions of cell pluripotency-related genes. POU5F1, SOX2 and NANOG are essential transcription factors that maintain the self-renewal and pluripotency of ES cells (5). Through RT-PCR and western blot analyses, we investigated whether the female sex steroid hormones could affect the expression of these pluripotency-related genes in human ES cells. When ES cells were treated with E2 (10^{-8} M), mRNA expression of *POU5F1* and *NANOG* genes was decreased compared to the control (Fig. 1). When ES cells were treated with P4 (10^{-6} M), mRNA expression of *NANOG* gene was decreased compared to the control (Fig. 1). However, mRNA expression level of *SOX2* gene was not altered by E2 or P4 treatment (Fig. 4A). For the protein expression of these genes, the expression levels of *SOX2* and *NANOG* genes were shown to be reduced by E2 and P4 compared to the control level (Fig. 4B and C). When ES cells were co-treated with E2 or P4 in combination with each inhibitor, the altered expression levels of SOX2 or NANOG proteins were recovered to the control levels (Fig. 4B and C). These results indicated that E2 and P4 may reduce the pluripotency of human ES cells by inhibiting the expression of pluripotency-associated markers such as *POU5F1, SOX2*, and *NANOG* genes in transcriptional or translational levels.

Discussion

Pluripotency is the main propensity of ES cells, and maintenance of pluripotency requires a balance between survival, proliferation, and self-renewal mechanisms (26). In fact, the precise mechanism that regulates pluripotency of stem cells remains largely unknown. Recently, through the *in vitro* and *in vivo* studies, several genetic regulators have been identified to play important roles in regulating the pluripotency in human and mouse ES cells. They are closely related with extracellular signaling factors, transcription factors, cell-cycle regulators, microRNAs, and DNA methylation (27,28). Investigations for these regulators might be useful to understand basic cellular biology and to exploit human ES cells as a promising source for cell therapy to treat patients with degenerative diseases (29,30).

Mouse feeder cells have been used to help the growth and maintenance of human ES cells, but they must be

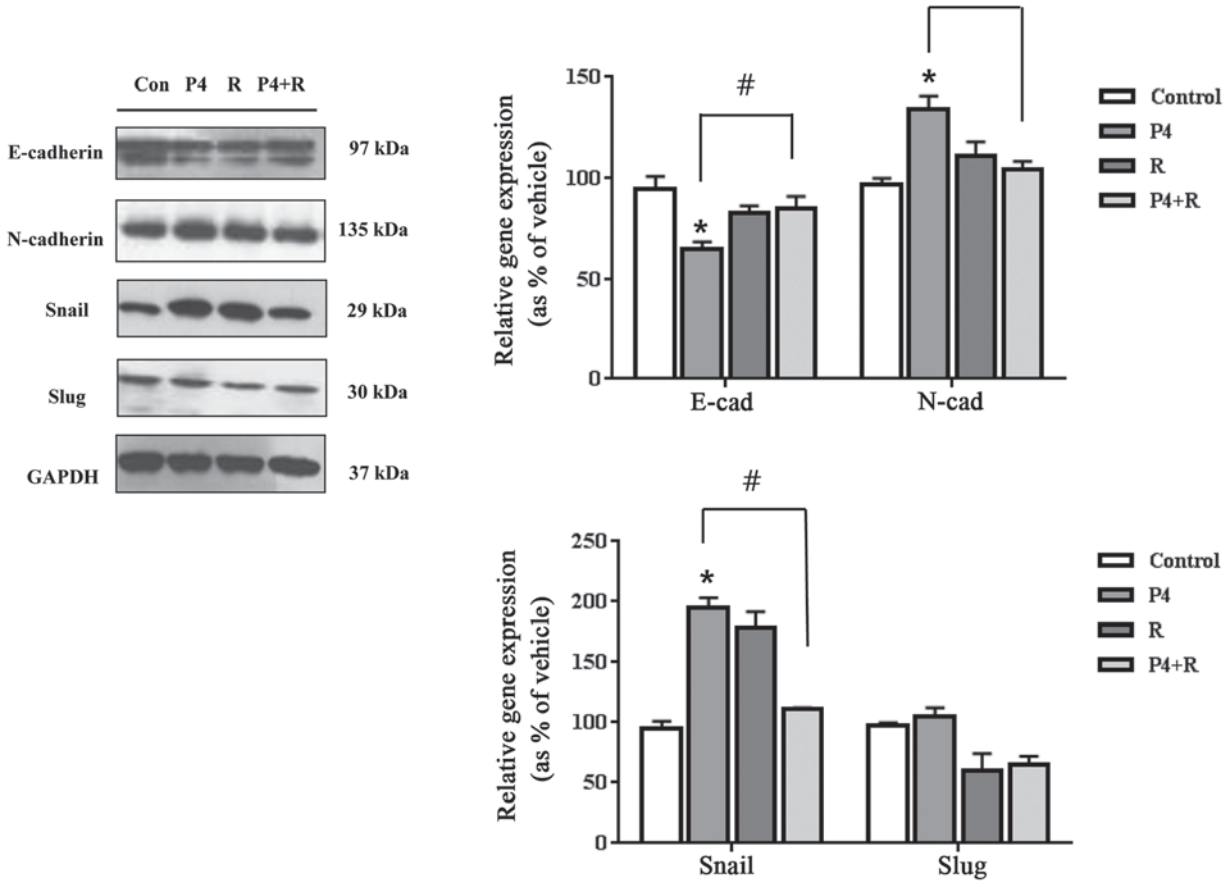

Figure 3. Effects of P4 and its receptor inhibitor on protein expressions of EMT regulatory genes. After the treatment of P4 (10^{-6} M), RU486 (10^{-8} M), and P4 (10^{-6} M)+RU486 (10^{-8} M) in human ES cells for 48 h, total proteins were extracted, and bands corresponding to Snail and Slug were detected by western blotting and quantified using Gel Doc 2000. *Significant elevation or reduction in gene expressions by each treatment comparing with control ($P<0.05$ in Dunnett's multiple comparison test). #Significant elevation or reduction in gene expressions by treatment of P4+RU486 (R) comparing with treatment of P4 ($P<0.05$ in Student's t-test).

removed before use of ES cells in certain applications (31,32). Frequently, the remaining feeder cells cause a problem because they generate confusion in measuring cellular responses of human ES cells to a certain stimulus (33). In the present study, to minimize the possibility of contamination by feeder cells, a feeder-free culture protocol for human ES cells was adopted to properly evaluate the effects of female sex steroid hormones on specific propensities of ES cells such as EMT and maintenance of pluripotency without a concern deriving from the mixed culture of ES cells and feeder cells. The procedure requires Matrigel-coated culture plates with CM from MEF feeder cell culture and monolayer culture of human ES cells. As shown in Fig. 1, these techniques provided a robust culture platform for human ES cells and an environment for the proper expression of stem cell markers without direct intervention of MEF feeder cells.

After establishing the single culture condition for human ES cells, the effects of female sex hormones such as E2 and P4 on specific characteristics of ES cells were investigated. Most animal cells are present in two types. Epithelial cells are composed of basement membrane and held together through several types of interactions, polarity, and immobility (18), while the characteristics of mesenchymal cells are opposite: They are loosely associated each other with no polarity and high mobility (34). Two types of transition, EMT and MET, have been observed during embryonic development, and interconversion between EMT and MET is likely to occur (35,36). During early embryonic development, the mesoderm generated by EMT develops into multiple tissue types, and later in development, mesodermal cells generate epithelial organs such as kidney and ovary via MET (35,37).

In the present study, when E2 and P4 were treated in human ES cells, the expression of epithelial cell marker, E-cadherin, was reduced, but the expression of mesenchymal cell marker, N-cadherin, and EMT regulator genes such as *Snail* and *Slug* was increased, indicating that female sex steroid hormones induce EMT through the regulation of expression of EMT-associated genes through their cognate receptors. However, the single treatment of RU486 also increased the Snail expression, but it had no statistical significance. RU486 is not a specific antagonist for PR. However, it surely has an antagonistic effect on P4. Since the combined treatment of P4 and RU486 restored the Snail expression, which was increased by P4, to the control level as shown in Fig. 3, it can be estimated that P4 induced Snail expression via its receptor, PR.

Next, the effect of sex hormones on the pluripotency of human ES cells was examined. As a result, E2 and P4 were revealed to reduce the pluripotency of human ES cells by inhibiting the expression of pluripotency-associated markers

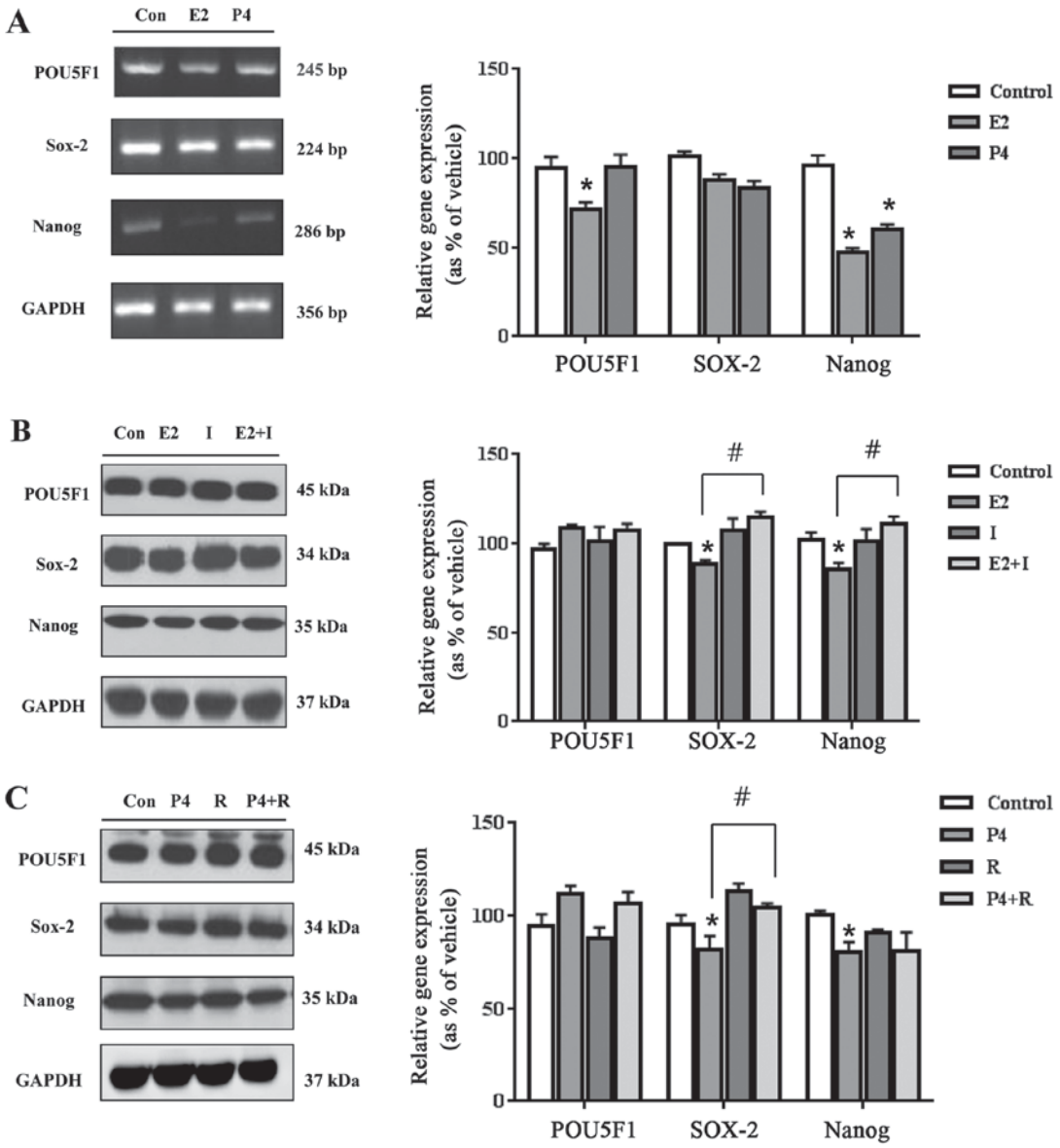

Figure 4. Effects of E2 and P4 on mRNA and protein expressions of pluripotency-related markers. (A) After the treatment of E2 (10^{-8} M) and P4 (10^{-6} M) in human ES cells for 24 h, total RNAs were extracted, and bands corresponding to POU5F1, SOX2, and NANOG genes were detected by RT-PCR and quantified using Gel Doc 2000. *Significant elevation or reduction in gene expressions by each treatment comparing with control (P<0.05 in Dunnett's multiple comparison test). (B) After the treatment of E2 (10^{-8} M), ICI 182,780 (10^{-6} M), and E2 (10^{-8} M)+ICI 182,780 (10^{-6} M) in human ES cells for 48 h, total proteins were extracted, and bands corresponding to POU5F1, SOX2, and NANOG were detected by western blotting and quantified using Gel Doc 2000. *Significant elevation or reduction in gene expressions by each treatment comparing with control (P<0.05 in Dunnett's multiple comparison test). #Significant elevation or reduction in gene expressions by treatment of E2+ICI 182,780 (I) comparing with treatment of E2 (P<0.05 in Student's t test). (C) After the treatment of P4 (10^{-6} M), RU486 (10^{-8} M), and P4 (10^{-6} M)+RU486 (10^{-8} M) in human ES cells for 48 h, total proteins were extracted, and bands corresponding to POU5F1, SOX2, and NANOG were detected by western blotting and quantified using Gel Doc 2000. *Significant elevation or reduction in gene expressions by each treatment comparing with control (P<0.05 in Dunnett's multiple comparison test). #Significant elevation or reduction in gene expressions by treatment of P4+RU486 (R) comparing with treatment of P4 (P<0.05 in Student's t-test).

such as *POU5F1, SOX2*, and *NANOG* genes in both transcriptional and translational levels.

More specifically, previous studies have demonstrated the influence of EMT on characteristics of ES cells. Li *et al* considered EMT as an early and necessary step in lineage specification by showing that stimuli for differentiation induced EMT prior to the differentiation processes and that inhibition of EMT suppressed differentiation of ES cells (38). Likewise, the combinatorial suppression of EMT and apoptotic pathways through many pathways regulated by microRNAs maintained the self-renewal and inhibited the differentiation of ES cells (39). In terms of the interrelationship between EMT process and pluripotency of ES cells, the cell-adhesion molecule E-cadherin is also known to play an important role in pluripotency and reprogramming of ES cells (37). For instance, interference with E-cadherin caused the EMT process and ES cell differentiation, and the expression of E-cadherin in undifferentiated ES cells was decreased immediately after ES cells initiated differentiation (40). In addition, E-cadherin stabilized cortical actin cytoskeletal arrangement in mouse or human ES cells, leading to the prevention of cell surface localization of the promigratory 5T4 antigen, which is

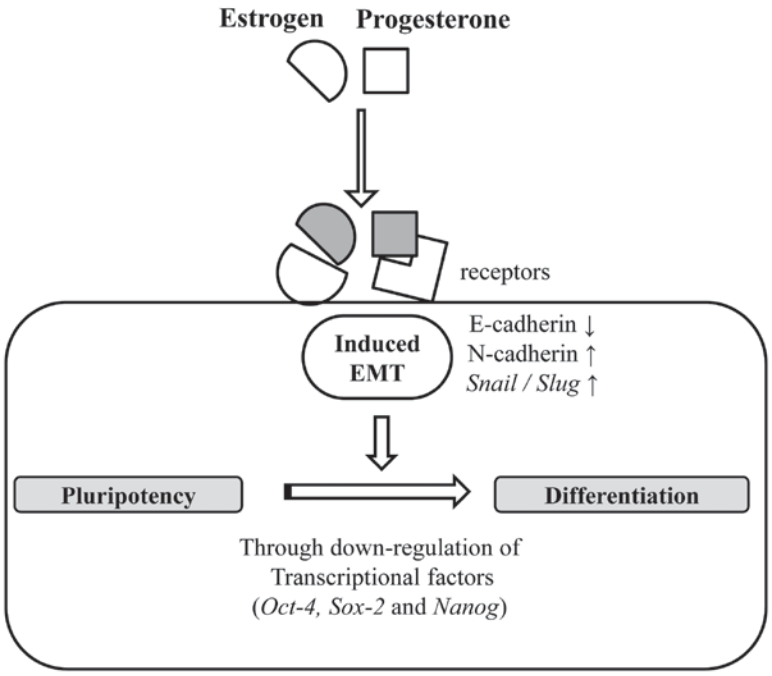

Figure 5. Tentative mechanism of E2 and P4 in regulating the EMT process and pluripotency of human ES cells. In the present study, E2 and P4, female sex hormones, were revealed to lead to the induction of EMT through the up and downregulation of cellular markers and signaling proteins through their cognate receptors. In addition, they down-regulated the expressions of POU5F1, SOX2, and NANOG, which are known to maintain the pluripotency of ES cells. As E-cadherin, POU5F1, NANOG, and others have been known to maintain undifferentiated state of ES cells, E2 and P4 can induce the differentiation process such as embryo development by promote the EMT process and the loss of pluripotency of human ES cells. EMT, epithelial-mesenchymal transition; E2, 17β-estradiol; P4, progesterone; ES, embryonic stem.

associated with very early phase of differentiation and altered motility of ES cells (41,42). Although the absolute requirement of E-cadherin for pluripotency is still under debate, it seems obvious at least that E-cadherin partially contributes to survival, self-renewal, and pluripotency of ES cells (43). Actually, E-cadherin, together with proteins like SSEA1, alkaline phosphatase, POU5F1, NANOG, and others have been used to maintain undifferentiated state of ES cells (44,45). Our results showing the reduced expression of E-cadherin by E2 and P4 to induce EMT process may be also interpreted that these sex hormones regulate the pluripotency of human ES cells by reducing the expression of well-known pluripotency markers such as *POU5F1, SOX2*, and *NANOG* genes as well as E-cadherin. Therefore, these results may also support that EMT process is related with stemness and differentiation of ES cells and can be reflected in the field of application for developing induced pluripotent stem cells (iPS cells).

In summary, we investigated whether female sex steroid hormones influence on EMT and maintenance of pluripotent propensity of human ES cells using a new culture method that can help maintain undifferentiated state of ES cells without the intervention of MEF feeder cells. Collectively, female sex hormones can lead to the induction of EMT through the up- and down-regulation of cellular markers and signaling proteins. In addition, they down-regulated the expressions of *POU5F1, SOX2*, and *NANOG*, which are known to maintain the pluripotency of ES cells. Taken together, E2 and P4 can affect differentiation of human ES cells by inducing EMT and the loss of pluripotency of human ES cells as demonstrated in Fig. 5. It is expected that this study may provide new insights for understanding the roles of female sex hormones in the EMT process and differentiation of human ES cells for embryo developmental processes and for developing more efficient avenues for human ES cell programming.

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