



Simulation of Embryonic Environments Produced by the Effect of Estradiol on Induced Pluripotent Stem Cells

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Abstract

Estradiol (E2) is one of the sex steroid hormones, whose various well-known functions include control of the female reproductive system, causing breast cancers, and inhibiting arterial sclerosis. In this study to investigate how E2 affected cell differentiation of induced pluripotent stem cells (iPSCs), we used different methods to culture hiPSCs, the undifferentiated state, long-term culture without passages (natural differentiation), embryoid bodies (EBs), and accelerated differentiation by activin, focusing on marker gene expression, including *Brachyury*, *hepatocyte nuclear factor (HNF) 3 β* , *estrogen receptor α* , and other markers. When iPSCs began to differentiate by responding to E2, *Brachyury* expression decreased regardless of the amount of E2, cell line, or protocol. Other markers were up or down-regulated, but did not show any tendency except in the early stage of EBs. The definite timing that E2 had the strongest effect on the aspect of differentiation was the EB stage analogous to gastrulation, in which E2 repressed *Brachyury* expression and induced *HNF3 β* at its restricted amount. These results suggest that the first target of E2 is the gastrulation stage.

Keywords

E2; Brachyury; EB; Gastrulation; iPSC

Abbreviations: E2: Estradiol; hiPSC: human induced Pluripotent Stem Cells; HNF3 β : Hepatocyte Nuclear Factor; ER α : Estrogen Receptor α ; ESRI: Estrogen Receptor 1; EB: Embryoid Body; SIRT1: Sirtuin 1

Introduction

The embryo is exposed to estrogenic environments from the stage of implantation i.e. the blastocyst. Before maturity of fetoplacental circulation, the uterine gland fluid containing estrogen feeds an embryo, because it is secreted from the corpus luteum verum. After fetoplacental circulation is established, maternal blood contains a large amount of estrogen, which increases continuously, because it is secreted from trophoblasts of the placenta. Because of steroid hormone properties, it can pass through the plasma membrane and enter the embryo from the mother's circulation. In the embryo,

17 β -HSD (hydroxysteroid dehydrogenase) type 2, the enzyme that converts estradiol (E2) to estrone (E1), mainly expresses in the fetal liver, gastrointestinal tract and kidney [1]. These facts support that an embryo and/or fetus is continuously exposed to some amount of estrogen after its implantation into the uterus. The effects of E2 on cell differentiation have been studied widely. E2 accelerates morphologic maturation of the fetal rabbit lung [2] and affects the proliferation and differentiation of neural stem cells *via* estrogen receptors [3], but it is difficult to evaluate the effect of E2 on the extremely early stage of development, because research of human embryos is ethically limited.

hESCs (human embryonic stem cells) and hiPSCs (human induced pluripotent stem cells) similar to hESCs overcome these difficulties. Jung et al. indicated that continuous exposure of mES cells to estrogens resulted in increasing octamer-binding transcription factor 4 expression at the transcriptional level, which maintained their undifferentiated state [4]. Hong et al. revealed the ability of E2 to differentiate hESCs into endodermal marker-expressing cell types (α -fetoprotein, α 1-Antitrypsin, GATA-4, and somatostatin) as well as enolase, nuclear factor-68Kda (ectoderm), and Brachyury (mesoderm)-expressing cells from the perspective of gene expression associated with the three germ layers and tissue types [5]. Kim et al. claimed that estradiol had the potential to differentiate hEBs into endodermal or mesodermal cells [6]. In addition, E2 differentiates EBs into osteoblast-like cells [7] hiPSCs, however, have not been target cells so far.

First, according to the above research of differentiation, although the results seemed to have some contradictions e.g. proliferation and maintenance of undifferentiated ES cells as well as promotion of their differentiation at the same time, we tried to differentiate hiPSCs into hepatocytes using E2, but failed to generate mature hepatocytes. This trial was however not meaningless, because we found that estrogen has a definite timing to work at least in early embryonic stages. In the present study, we investigated in detail when E2 affects differential aspects of hiPSCs at various induced stages including the undifferentiated state, long-term culture without passages (natural differentiation), embryoid body formation and outgrowth, before and after accelerated differentiation by activin followed by different concentrations of E2, and focused on marker gene expression and accounted for the role of a part of embryonic environments produced by estrogen.

Materials and Methods

hiPSCs and culture

hiPSC lines 201B7 and 253G1 [8] were cultured on a feeder cell layer of mouse embryonic fibroblasts (MEFs; Oriental Yeast Co, Tokyo, Japan) inactivated with mitomycin C (Kyowa Hakko Kirin Co, Tokyo, Japan) and were maintained by repeated passages. The culture medium consisted of 80% KnockOut™ Dulbecco's minimal Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 20% KnockOut™ Serum Replacement (Gibco), 100 μ M non-essential amino acids (Wako, Chuo-ku, Osaka, Japan), 2 mM L-glutamine (Wako), and 100 μ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA).

EB formation and EB outgrowth

Undifferentiated hiPSCs were cultured for 5 days and treated with collagenase IV (Invitrogen, Carlsbad, CA) for 4 min at 37°C. Detached

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hiPSC colonies were centrifuged and washed with phosphate-buffered saline (PBS). Washed hiPSC colonies were transferred into a non-adherent plate. Suspension cultures were maintained for 5 days in EB medium: 80% knockout DMEM, 100 μM non-essential amino acids, 2 mM L-glutamine, 100 μM 2-mercaptoethanol, and 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). EBs were adhered to a gelatin-coated dish and cultured in the same EB medium for the planned number of days.

Activin stimulation

According to our previous report [9], hiPSC colonies were cultured in Roswell Park Memorial Institute media (RPMI) 1640 medium (Wako) containing 2% FBS (Gibco, BRL, USA) with 100 ng/mL Activin A and 3 μM CHIR99021 for 24 h and then in 2% FBS/RPMI1640 medium with 100 ng/mL Activin A for 48 h.

Treatment with E2

The cells were cultured with E2 (Sigma). The medium was exchanged daily. Acetonitrile was used for the control. The schemes of all experiments are presented in Figure 1.

Quantitative real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) analysis was performed using the Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan). Cycling was performed for 10 min at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C, which was the default conditions of Thermal Cycler Dice Real-Time System software TP 800 (version 5.11B). Primer sequences were as follows: *HNF3β* (Forward: 5'-TAT TGC CCC GTT GAG TGC-

3'; Reverse: 5'-TCC CAG GGA AAC TGC AAG-3'), *brachyury* (5'-GAC AGG TAC CCA ACC CTG AGG A-3'; 5'-AGC ATG GAT AAA CAT GCA GGT GAG-3'), and *estrogen receptor α* (5'-TTA CTG ACC AAC CTG GCA GA-3'; 5'-ATC ATG GAG GGT CAA ATC CA-3'), and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (5'-TGG CAC CCA GCA CAA TGA A-3'; 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'). PCR was performed in triplicate for each sample, and three independent experiments were carried out.

Immunohistochemistry of ERα

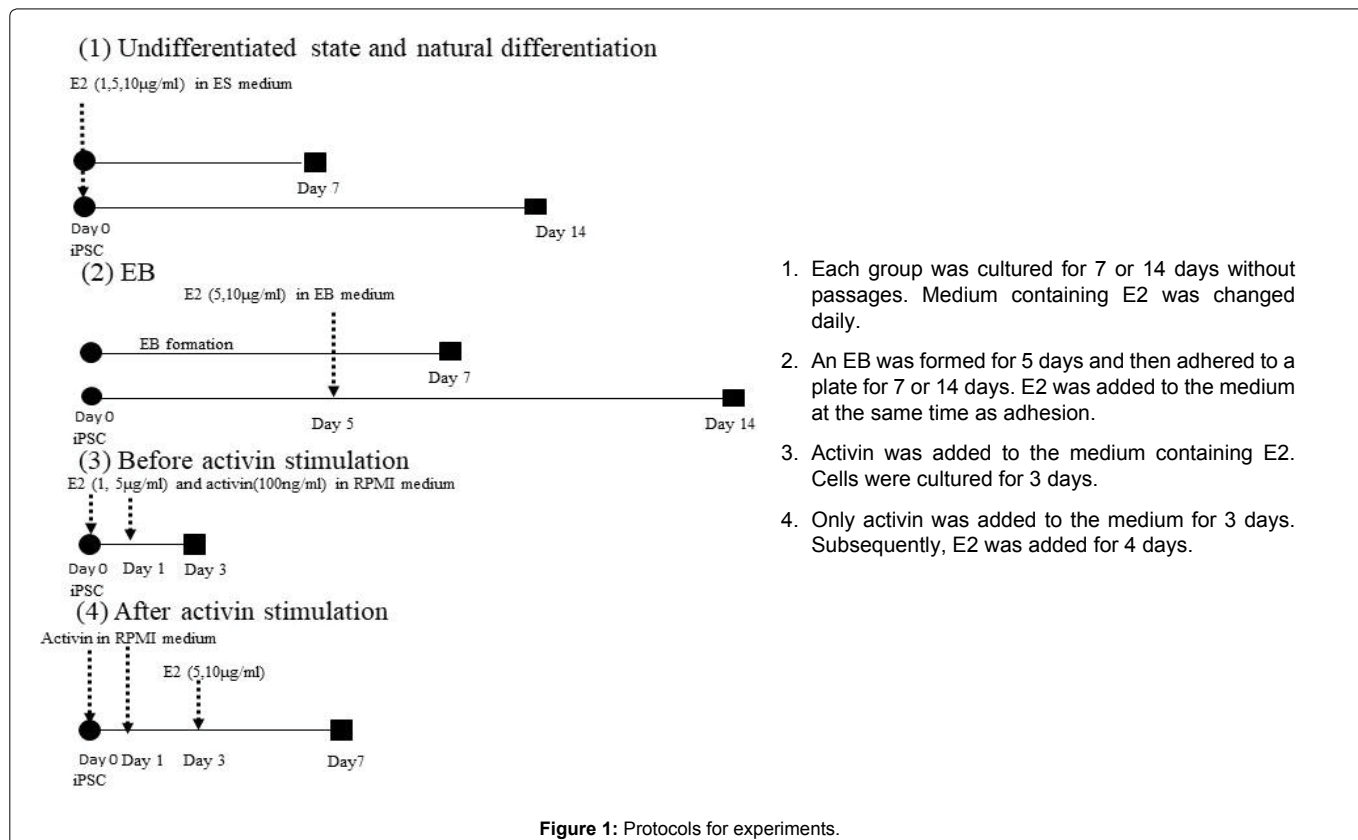
The cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4) for 30 min at room temperature, rinsed three times with 20 mM PBS (pH 7.4), and then prepared for immunostaining. The following primary antibody with an appropriate secondary antibody conjugated with Alexa Fluor 488 as well as 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Eugene, OR, USA) were used: anti-ESR1 (Bethyl Laboratories, Montgomery, TX, USA). For immunohistochemical controls, the primary antibody was omitted.

Statistical analysis

All values in figures and the text are expressed as means ± standard deviation. The significance of differences among mean values was evaluated by Student's t-test. $P < 0.05$ was considered to be significant.

Results

The undifferentiated stage (protocol 1) was near the inner cell mass in the blastocyst and almost showed no response in both cell lines to E2 in terms of cellular differentiation, especially within 1 week, although ERα was detected slightly (Figure 2). Two weeks later,



germ layer markers appeared irregularly, depending on whether E2 (5 µg/mL) was added, but did not show any tendency. For example, although the expression level of Brachyury seemed to decrease, HNF3β was upregulated or did not change according to the cell line (Figures 2A and 2B).

The next stage (EB outgrowth protocol 2) was analogous to gastrulation consisting of three germ layers, when the definite timing for E2 to affect differentiation was found. Some germ layer markers were affected by 5 µg/mL E2 (Figures 2C and 2D). HNF3β expression was significantly (about 100 times) higher than in the control, and Brachyury expression was about 10 times lower than in the control. E2 (10 µg/mL) did not change the amount of Brachyury, but lowered HNF3β.

To confirm an interrelationship between E2 and ERs, ERα expression was investigated in RT-PCR and immunohistochemical analyses (Figure 3). ERα was apparently found at each stage (Figure 3A). It was obvious that EB outgrowth showed more ERα expression than hiPSCs. Moreover, the immunohistochemistry confirmed localization of ERα protein irrespective of E2 addition, which supported its gene expression (Figure 3B).

Before (protocol 3) and after (protocol 4) activin stimulation

E2 was added to RPMI1640 medium together with activin to confirm whether it accelerated definitive endoderm with activin (protocol 3), but cells treated with 5 µg/mL E2 did not survive to the end of the experiment. Therefore, cells were cultured in 1 µg/mL E2-containing medium. The cells survived and expressed only a slightly lower level of Brachyury compared with the expression of other markers, but E2 did not promote differentiation of definitive endoderm.

Next, hiPSCs were stimulated with activin before addition of E2 and then cultured for 4 days in medium containing E2 to confirm whether E2 affected definitive endoderm induced by activin. The marked tendency seen in EBs was lost. Brachyury decreased with E2 treatment, while HNF3β did not change (Figures 2E and 2F).

Thus, addition of E2 decreased the level of Brachyury, but other markers showed only mild or no differences except in the EB stage in which the level of HNF3β was increased by almost 100 times with 5 µg E2 accompanied by the decrease in Brachyury to 1/10 that in the control. Two weeks later, EB outgrowth lost the interrelationship of

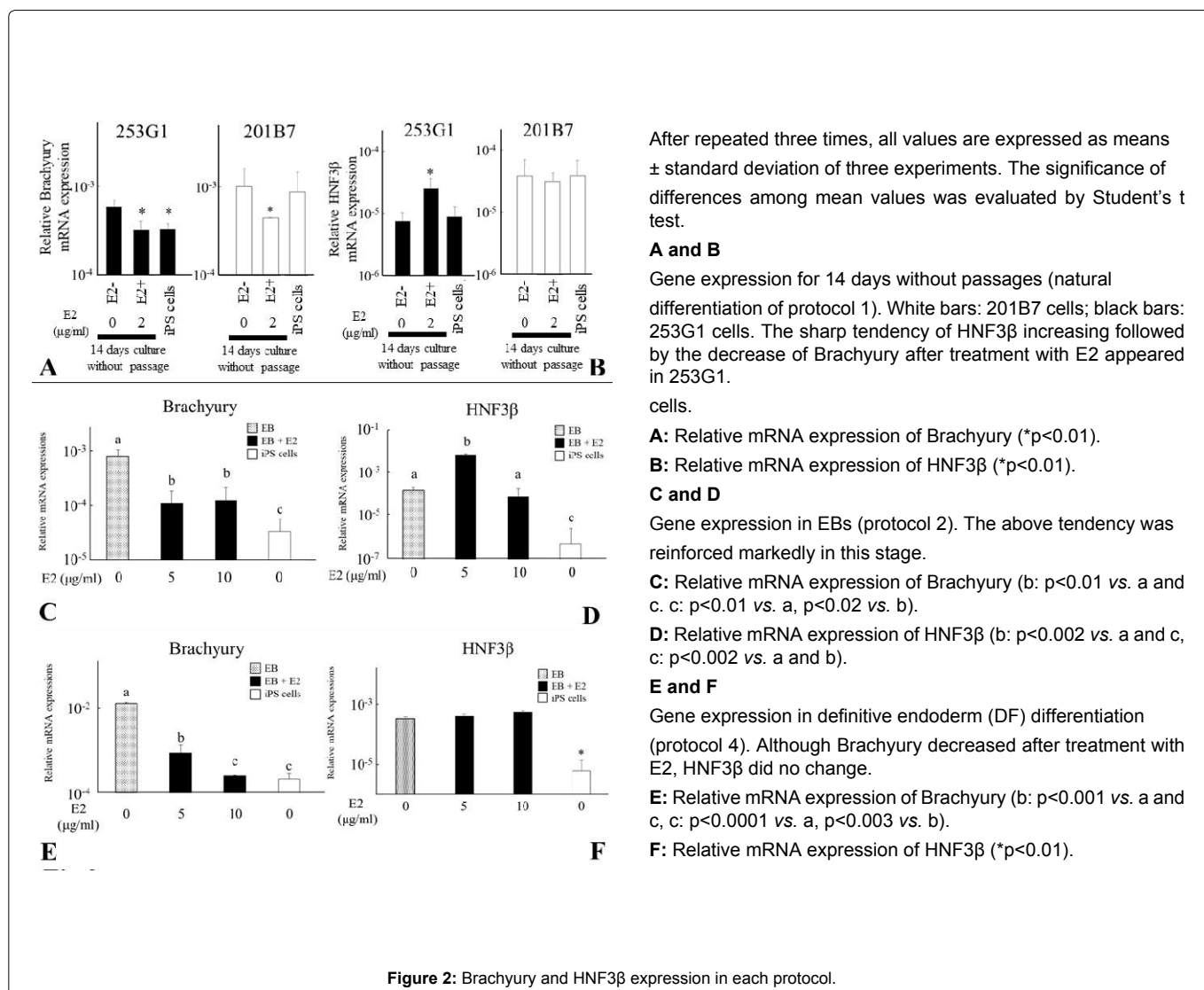


Figure 2: Brachyury and HNF3β expression in each protocol.

After repeated three times, all values are expressed as means ± standard deviation of three experiments. The significance of differences among mean values was evaluated by Student's t test.

A and B

Gene expression for 14 days without passages (natural differentiation of protocol 1). White bars: 201B7 cells; black bars: 253G1 cells. The sharp tendency of HNF3β increasing followed by the decrease of Brachyury after treatment with E2 appeared in 253G1.

C and D

Gene expression in EBs (protocol 2). The above tendency was reinforced markedly in this stage.

C: Relative mRNA expression of Brachyury (b: p<0.01 vs. a and c; c: p<0.01 vs. a, p<0.02 vs. b).

D: Relative mRNA expression of HNF3β (b: p<0.002 vs. a and c; c: p<0.002 vs. a and b).

E and F

Gene expression in definitive endoderm (DF) differentiation (protocol 4). Although Brachyury decreased after treatment with E2, HNF3β did no change.

E: Relative mRNA expression of Brachyury (b: p<0.001 vs. a and c; c: p<0.0001 vs. a, p<0.003 vs. b).

F: Relative mRNA expression of HNF3β (*p<0.01).

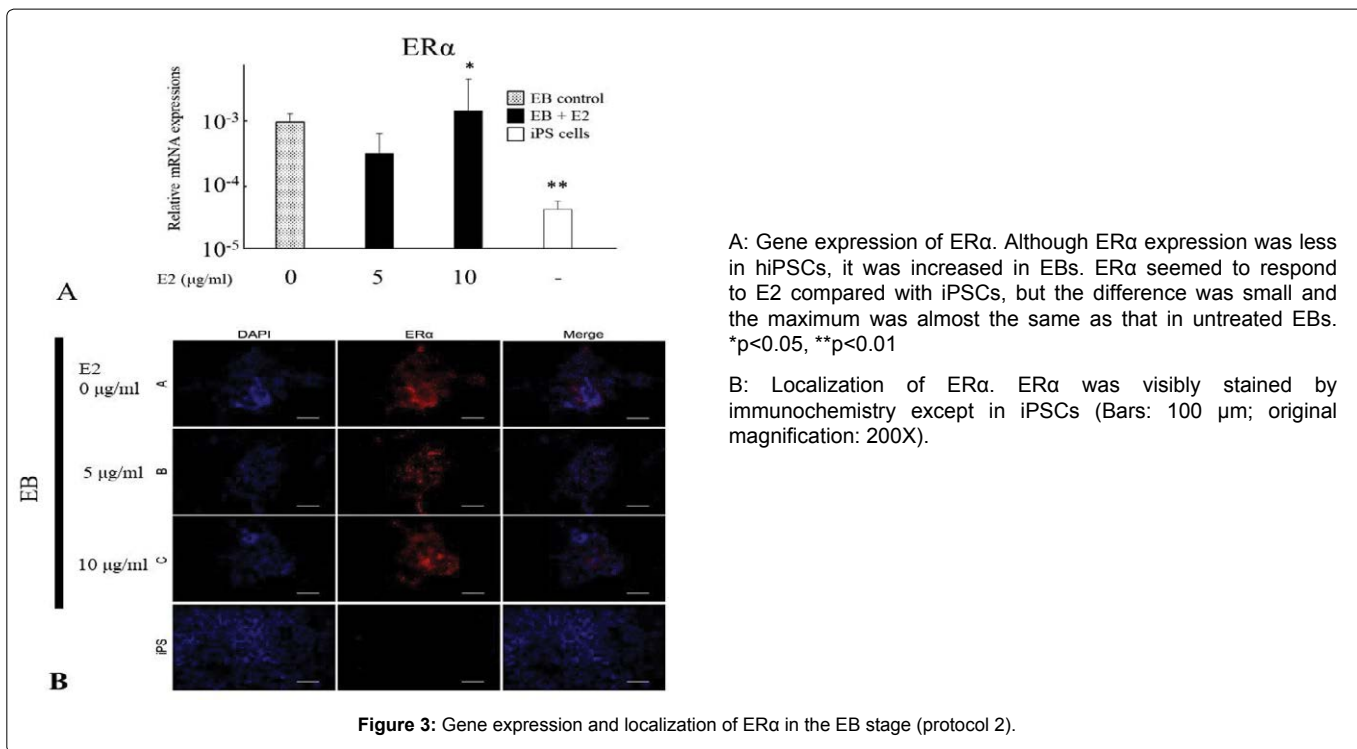


Figure 3: Gene expression and localization of ERα in the EB stage (protocol 2).

the increase in HNF3β with the decrease in Brachyury. Only in EBs, especially at the early stage, the sharp tendency of gene expression was found.

Discussion

Definite effects of E2 seem to be limited to a time during development. Its effects were not all found in iPSCs, i.e. at the epiblast-like stage. Only at the early stage of EBs, E2 addition repressed Brachyury and increased HNF3β drastically. At a later stage or in specific tissue differentiation using activin, expression of differentiation markers was up or down-regulated and lost a defined tendency.

EBs are complex three-dimensional aggregates derived from hESCs or hiPSCs and has the ability to mimic post-implantation embryonic tissues [10]. It may be regarded as before the end of week 3 of human development, around day 17 or 18, when gastrulation begins, which establishes all three germ layers. Invagination, the migration of epiblast cells toward the primitive streak, occurs in this stage. Mesoderm and endoderm originate from these invaginating cells. This mesodermal specification needs regulation of Brachyury, one of the T-box family genes, which is expressed in the node, notochord precursor cells, and notochord, regulates the dorsal mesoderm formation in middle and caudal regions of the embryo, and is essential for cell migration through the primitive streak. Brachyury was repressed and expression of endoderm markers such as HNF3β was marked at the early stage of EBs. Based on these results, we elucidated that E2 plays the role of a switch from the limited epiblasts restricted to the primitive streak to endoderm by repressing Brachyury expression, and there is timing when E2 has the strongest effect i.e. the EB outgrowth stage analogous to the early stage of gastrulation.

ERα appeared clearly at the EB stage, but not on iPSCs. It is certain that the effects of E2 depend on ERs, because gene expression became marked at the EB stage, although it did not increase drastically by

stimulation with E2. Therefore, ER is necessary for the effects of E2, but is independent of E2 stimulation.

Although the mechanism for E2 to convert the limited epiblasts into endoderm is beyond the scope of our study, the following mechanism may be extracted from several reports. According to a study by Wang's group, one of the reasons why some types of breast cancers are tamoxifen resistant lies in overexpression of Brachyury [11]. This mechanism is explained by Brachyury downregulating the expression of sirtuin (SIRT) 1 that represses estrogen/estrogen receptor signaling and cell proliferation in estrogen-responsive breast cancer cells by downregulating B-cell leukemia/lymphoma (Bcl)-2 protein [12]. There is a study claiming that E2 upregulates expression of SIRT1 [13]. Consequently, we suggest the possibility that SIRT1 downregulates Brachyury. In almost all experiments of this study, we showed that expression of Brachyury was lower in E2-treated cells than in the control. This result suggests that SIRT1 inhibits Brachyury and the other way around.

In this short communication, we revealed the definite timing to add E2 most effectively was at the early stage of EBs. E2 may differentiate epiblasts, especially into endoderm, at the early stage of embryonic development through the repression of Brachyury.

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