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Research Article

**EMBRYONIC ENVIRONMENTS MODELS DEVELOPED
THROUGH THE IMPACT OF ESTRADIOL ON INDUCED
PLURIPOTENT STEM CELLS**¹Dr Sidrah Iftikhar,²Dr Mariam Rauf,³Dr Shafaq Arif¹WMO, Civil Hospital, Jlal Pur Jattan, Gujrat., ²MBBS, Foundation University Medical College, Islamabad., ³WMO, BHU Udhowali, Gujranwala.**Article Received:** January 2019**Accepted:** February 2019**Published:** March 2019**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, longterm 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. Endoscopic sinus surgery; Obstructive sleep apnea; Postoperative respiratory complications

Keywords: E2; Brachyury; EB; Gastrulation; iPSC**Corresponding author:****Dr. Sidrah Iftikhar,**

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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. 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 and affects the proliferation and differentiation of neural stem cells via estrogen receptors, 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.

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 variously induced stages including the undifferentiated state, long-term culture without passaged (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.

METHODS:**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

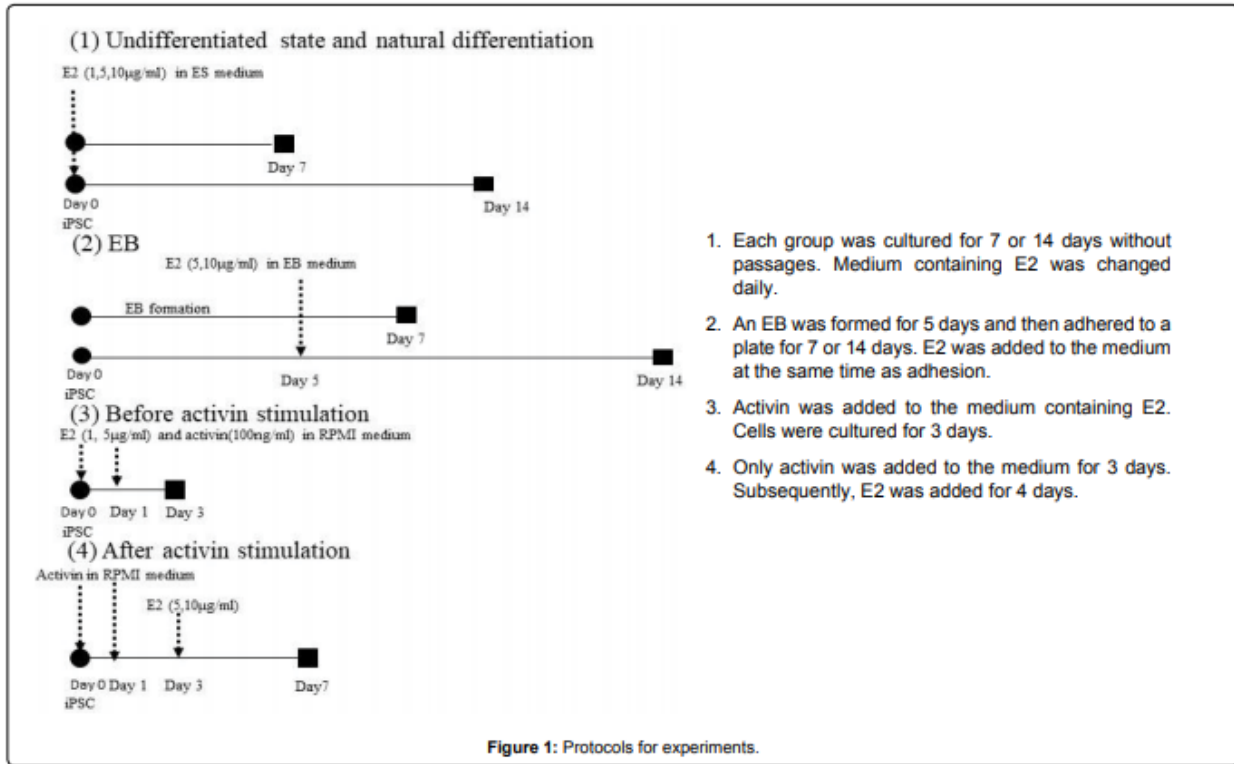
hiPSC colonies were centrifuged and washed with phosphatebuffered saline (PBS). Washed hiPSC colonies were transferred into a non-adherent plate. Suspension cultures were maintained for 5 days in EB medium: 80% knockout DMEM, 100 μ M non-essential amino acids, 2 mM L-glutamine, 100 μ M 2-mercaptoethanol, 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 μ M 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:



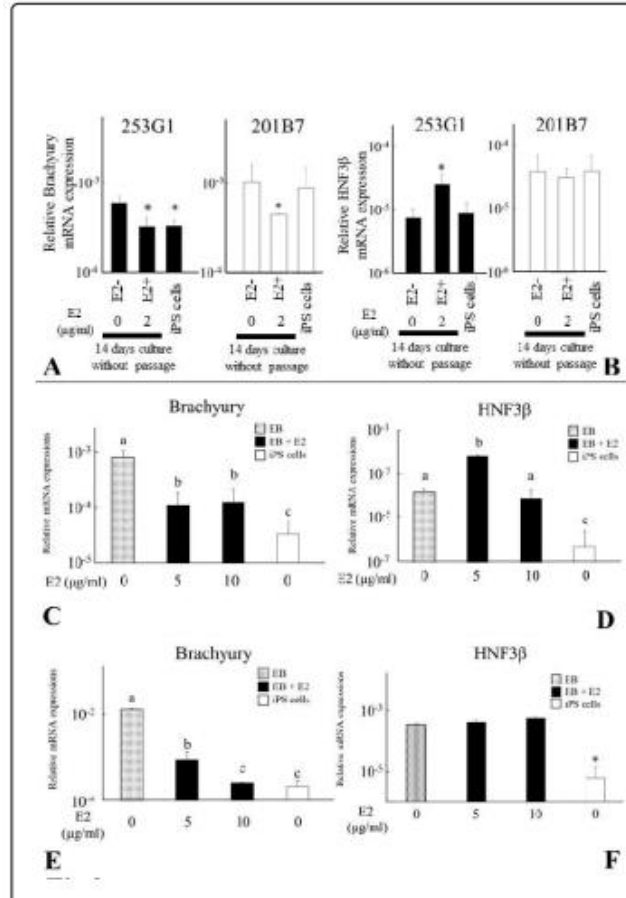
Statistical analysis

All values in figures and the text are expressed as means \pm standard deviation. The significance of differences among mean values was evaluated by Student's t-test. P

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 ER α was detected slightly (Figure 2). Two weeks later, germ layer markers appeared irregularly, depending on whether E2 (5 μ g/mL) was added, but

did not show any tendency. For example, although the expression level of Brachyury seemed to decrease, HNF3 β was up regulated 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 μ g/mL E2 (Figures 2C and 2D). HNF3 β 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 μ g/mL) did not change the amount of Brachyury, but lowered HNF3 β .



After repeated three times, all values are expressed as means \pm 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 HNF3 β increasing followed by the decrease of Brachyury after treatment with E2 appeared in 253G1 cells. A: Relative mRNA expression of Brachyury (* $p < 0.01$).

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 HNF3 β 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.

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 HNF3 β 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.

ER α 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. 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. There is a study claiming that E2 upregulates expression of SIRT1.

CONCLUSION:

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. 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.

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