Erythroblast Differentiation of Human Endometrium derived Induced Pluripotent Stem Cells as a Source of Autologous Transfusion

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Directed by Professor Byung Seok Lee

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ABSTRACT

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Purpose and background: With the consistent increase in life-expectancy, the importance of regenerative medicine cannot be over-emphasized. The objective of this study is to pretreat patient derived endometrial cells with exogenous β estradiol to enhance reprogramming efficiency of endometrial stromal cell derived induced pluripotent stem (iPS) cells. Subsequently, the protocol for committing these cells into hematopoietic and erythroid lineages will be optimized through a two-phase culture system, involving feeder and non-feeder environments.
Method: Discarded endometrial tissues were obtained from women receiving hysterectomy in their 4th to 5th decade due to benign uterine conditions. The endometrial cells isolated were expanded to passage 3-4 to allow stromal cells to dominate in the culture environment. pMIG-human sox2, oct4, klf4 and c-Myc viral vectors, namely the Yamanaka factors were used to transduce the endometrial stromal cells. The impact of exogenous β estradiol on the reprogramming efficiencies of different endometrial donor cells were compared at concentrations of 0, 0.1 and 1μM/ml. Directed differentiation of these established iPS cells were conducted in two phases. The first 9 days involved commitment of the iPS cells to hematopoietic fate with robust expansion on murine bone marrow stromal feeder cells (OP-9). The second phase of differentiation involved feeder free conditions composed of hydrocortisone, stem cell

factor, interleukin-3, recombinant erythropoietin (EPO) and poloxamer 188. After 17 days of differentiation in feeder free environment, the expression profiles of KDR (VEGF-R2), CD235a +, CD34 +, CD43 + and CD 71+ were analyzed by flow cytometry and Wright-Giemsa staining for differential counting based on morphology.

Results: The treatment of donor endometrial stromal cells with β estradiol at a concentration of 0.1μM/ml resulted in iPS cell reprogramming efficiency of approximately 166 ± 5% compared with the non-treated control cells set as 100%. As a result of inducing these cells to hematopoietic fate via a 9 day co-culture with murine stromal fibroblasts, yields ranging from 8-13% was observed depending on the donor. Because the potential of these co-cultured cells to further differentiate into erythroblastic fate may not be confined to defined cell surface markers, all of the OP-9 co-cultured cells were transferred to a feeder-free system composed of hydrocortisone, stem cell factor, interleukin-3, recombinant EPO and poloxamer 188 for 17 days, stably yielding over 80% of polychromatic and orthochromatic normoblasts. Therefore, the protocol for a comprehensive induction of erythroid lineage cells starting from human endometrial tissue via iPS cell reprogramming has been fully demonstrated.

Conclusion: The treatment of exogenous estradiol, unique to endometrial cells yielded higher reprogramming efficiencies of endometrial derived iPS cells compared naïve endometrial cells. From these iPS cell colonies driven from discarded endometrial cells successful induction of hematopoietic cell fate followed by erythroid differentiation up to orthochromatic normoblasts were achieved in an effort to develop autologous transfusion source.

Key words: β-estradiol, endometrium, human induced pluripotent stem cell (hiPSC), hematopoietic, erythroblast, autologous transfusion

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I. INTRODUCTION

In the field of obstetrics and gynecology, often referred to as the 'blood business', transfusion of red blood cell products is a common practice and discovering a better, safer source of transfusion for acute and chronic blood loss is a vital aspect of patient care. Anemia may not always be adequately corrected by conservative means of increased dietary iron intake or supplementation, whereas transfusion, most commonly provided by products of heterologous donation of blood, also has several limitations. With the recent increase in blood-borne diseases and the aging of population in many regions, the number of candidates eligible for blood donation is on the decrease in many regions. In light of this, finding an alternative, preferably an autologous source of transfusion is an important goal to pursue. The human endometrium is an easily replenished and accessible tissue clinically obtainable by means of endometrial curettage and alternatively as discarded tissue after benign hysterectomies.

Due to its abundance, regenerative potential and plasticity, endometrial tissue has been previously described in the literature as a favorable source of tissue regeneration and engineering.[1,2] From the basalis layer, the endometrial gland and stroma has the propensity to expand both quantitatively as well

qualitatively within a window of 2 to 3 weeks in order to accommodate to proper implantation of the fetus. Mesenchymal-epithelial transition (MET), which is known to play a vital role in early reprogramming process leading to induced pluripotency [3], is innately facilitated in the endometrium during the rapid proliferation and differentiation of endometrial gland epithelium. Human endometrium derived induced pluripotent stem cells have been previously described as an efficient cell source for reprogramming into a pluripotent state.[2] Also, resident adult stem cells in the basalis layer of the endometrium has been proposed as a source for in vitro differentiation into various lineages, owing to its high plasticity.[1]

Induced pluripotent stem (iPS) cells (iPSC), first described in 2006 using murine fibroblasts has opened a new era for regenerative medicine. It involves overexpressing fully differentiated somatic cells with factors involved in transcriptional regulation of early embryogenesis. These factors, first defined by Yamanaka in 2006 in a murine model, includes Sox2, Oct4, Klf4 and c-Myc. [4]

Adult stem cells are partially committed with regards to cell fate and displays less plasticity, and have less conspicuous entities in the absence of any universal markers to define them. However, they are considered more predictable and safer in nature when transplantation is considered. On the other hand, embryonic stem (ES) cells are considered truly pluripotent, carrying the hallmark ability to differentiate in vivo and vitro to ectoderm, mesoderm as well as endoderm but ethical concerns prevail since in humans, fertilized embryos have to be destructed at the blastocyst stage to obtain the inner cell mass for ES cell colony expansion. Moreover, ES cells are always heterologous in nature when transplantation is eventually performed.

Another important aspect of donor cells for iPS cell establishment is the innate reprogramming efficiency of the cells to dedifferentiate into a pluripotent state. Skin fibroblasts, keratinocytes, blood and dental pulp have

been described as the source of reprogramming with different efficiencies in various culture conditions. The type and concentrations of ectopic reprogramming factors required and the need for supplementary small molecules which enhance reprogramming efficiencies depend on the basal character of the donor cell of interest.[5]

When iPS cell colonies are actually established from human donors for the purpose of regenerative medicine, maximizing efficiency is of great importance. Human endometrium is uniquely responsive to ovarian steroids and especially 17β-estradiol, the most potent form of estrogen and therefore, exogenous estradiol which is expected to enhance proliferation of endometrial cells may have impact on the reprogramming efficiency of endometrial cells. In an attempt to characterize and develop human endometrial cells as a relevant source of tissue engineering in future medicine, the use of discarded human endometrium from benign hysterectomies is proposed here, where the addition of exogenous estradiol to the defined Yamanka factors is attempted in order to improve reprogramming efficiency.

The second and main aim of this study is to utilize these patient derived iPS cells to differentiate and expand erythroblasts as an attempt for developing a novel source of autologous transfusion. With successful derivation of human endometrium originated erythroblast cells which could be replenished and stored indefinitely, the development of a novel source of autologous transfusion could be made possible.

II. MATERIALS AND METHODS

1. Culture and expansion of human endometrial cells.

Human endometrial tissues were obtained from patients undergoing benign

hysterectomies in their 4th to 5th decade at Department of Obstetrics and Gynecology, Yonsei University College of Medicine, Gangnam Severance Hospital. After obtaining a full-thickness 15x5mm wedge from the hysterectomy specimen excluding any grossly noticeable pathologic lesions, the endometrial layer was dissected from any myometrial fragments under a dissecting microscope to avoid contamination of other cell sources. After mincing the endometrium with a surgical blade (Swann-Morton, England), the fragments were treated with ACK lysis buffer (Lonza, Allendale, NJ, USA) at a concentration of 1ml per 20ml of tissue suspension.

The fragments were then thoroughly washed twice with PBS (Corning, Steuben County, NY, USA) and immersed in 2ml of collagenase IV (Thermo Fisher Scientific, Grand Island, NY, USA) at 37°C in a 5% CO2 incubator (Thermo Fisher Scientific, OH, USA) for 90 to 120 minutes to isolate individual endometrial cells. The cells were carefully pipetted every 15 to 30 minutes via a large bore 1000ul tip to hasten the lysis process. In cases where lysis appeared to be relatively slow, additional collagenase IV (Thermo Fisher Scientific) was added. The cells were then washed and plated on a 100mm culture dish (Thermo Fisher Scientific) in medium containing Dulbecco Modified Eagle Medium (DMEM)-high glucose (Thermo Fisher Scientific), 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific), until almost confluent to 90-95%. The primary cells were split at a ratio of 1:3 for passaging and stored in liquid nitrogen at passage 3 for future hiPSC derivation.

2. Cell proliferation assay

Human endometrial cells namely hENDO1, hENDO5, hENDO7, and ADF-hENDO5' (matching adult dermal fibroblasts from donor of endometrial tissue 5) were suspended in medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), and 1% penicillin/streptomycin (Thermo Fisher

Scientific) and subsequently seeded at a density of 4 X 10^3/well in 96-well plates (Thermo Fisher Scientific) and incubated for 24 hours (h). Afterwards, these wells were assigned into four groups: 1) Control group (no estradiol treatment); 2) 0.1 uM/ml estradiol (E2) (β-Estradiol, Sigma-Aldrich, St. Louis, Mo, USA) group; 3) 1 uM/ml E2 group. These wells were maintained for different durations of 24h, 48h, 72 h, 96 h at 37 ℃ in a humidified atmosphere of 5% CO_2.

After being incubated, 10 ul PrestoBlue Cell Viabillity Reagent (Thermo Fisher Scientific) was added to each well and incubated at 37 ℃ for another 2h. Absorptiometry, with an OD value of absorbance at 570 nm was measured by VERSA MAX Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The results were plotted as means ± standard deviation (SD) of three independent experiments having three determinations per sample for each experiment, based on the previously reported protocols.[6-8]

3. Derivation of iPS cell from donor endometrial cells

Human endometrial hiPSC were driven from passage 3 to 4 endometrial cell cultures. Primary human endometrial cells were reprogrammed to hiPSC using retrovirus reprogramming. Plasmid DNAs of four classic retroviral vectors pMIG-Oct4, pMIG-Sox2, pMIG-Klf4, and pMIG-c-Myc encoding (human) reprogramming factors constructed were obtained for transfection (Addgene, Cambridge, MA, USA). Retroviral supernatants were produced by transfection of 293FT cells (Thermo Fisher Scientific) with a mixture of 3 plasmids: one reprogramming vector, pVSV-G, and pGag/Pol. These endometrial cells were cultured for 3 days before retroviral transduction to observe proliferation. Naïve or β estradiol treated endometrial cells were transduced with a cocktail of retroviral supernatants supplemented with 6 μg/ml polybrene (Sigma-Aldrich) in reduced serum culture medium. The prepared endometrial cells were transduced at 50% confluence for 12 hours with sox2,

oct4, klf4 virus at 10 multiplicity of infection (MOI) and 5 MOI for c-Myc in reduced serum opti-MEM (Thermo Fisher Scientific).

The following day the viral supernatant was removed and infected endometrial cells were washed with fresh medium. At about 48 hours when the transduced endometrial cells reached confluency, passaging is performed and plated on murine embryonal fibroblasts (MEFs) (GlobalStem, Gaithersburg, MD, USA) at an approximate ratio of 1:6 in DMEM supplemented with 10% FBS (Thermo Fisher Scientific), 1% penicillin/streptomycin (Thermo Fisher Scientific). On the subsequent day, the medium was changed to hiPSC medium containing the following: DMEM/F12 (Thermo Fisher Scientific), 10% knockout serum replacement (KOSR) (Thermo Fisher Scientific), 1 mM L-glutamine (Thermo Fisher Scientific), 1% nonessential amino acid (Thermo Fisher Scientific), 0.1mM ß-mercaptoethanol (Sigma-Aldrich), and 10ng/ml FGF2 (R&D Systems, Minneapolis, MN, USA).

The transduced cells were examined daily for the appearance of early colonies and for retrovirus transgene-silenced, GFP-negative hiPSC colonies. Each colonies were manually picked within a short span of 2-3 days to avoid re-plating of the detached and transferred into individual wells containing 10 uM of rho kinase inhibitor Y27632 (EMD chemicals, Gibbstown, NJ, USA). After obtaining a characteristic embryonic stem cell-like morphology the colonies were manually picked and expanded when colony size reached a size between 100 to 200um.

4. Alkaline phosphatase staining

Alkaline phosphatase staining was performed for the hiPSC colonies using the Leukocyte Alkaline Phosphatase kit (Sigma-Aldrich) after 2 full weeks of transduction. Staining was performed directly on the culture vessel, after picking colonies appropriate for propagation

5. Human iPS cell (hiPSC) culture

The hiPSC lines (donor endometrium 1, 2, and 7, passage 10) were maintained in an undifferentiated state by weekly passaging on mouse embryonic fibroblasts (MEF, Global Stem) feeder within the previously described hiPSC medium. When spontaneous differentiation was under 5-10%, they were enzymatically passed with 1 mg/ml collagenase type IV (Thermo Fisher Scientific). When spontaneous approached 10% however, manual picking was performed to maintain standards for hiPSC.

6. Hematopoietic differentiation of hiPSC/OP9 cocultured cells

The hematopoietic differentiation potential of endometrial cell-derived hiPSC was examined by Choi method, as previously described.[9] OP9 cells were plated onto gelatinized 10 cm plates in OP9 growth media (α-minimum essential medium as basal medium supplemented with 20% fetal bovine serum, Thermo Fisher Scientific). After formation of confluent cultures on day 4, the medium was changed and cells were cultured for an additional 4 days to allow for expansion of the extracellular matrix. Undifferentiated hiPSCs were harvested by treatment with 1 mg/ml collagenase IV (Thermo Fisher Scientific) and were detached by scraping to maintain cell clumps with cell lifters.

The hiPSCs were added to OP9 cultures at a density of 1 X 10^6/10 ml per 10 cm dish in differentiation medium (α-minimum essential medium as basal medium; Thermo Fisher Scientific, 10% fetal bovine serum; Thermo Fisher Scientific, 100 μmol/l monothioglycerol; Sigma-Aldrich). The hiPSC/OP9 cocultures were incubated for up to 9 days at 37 ℃ in 5 % CO_2 incubator with a half-medium change on day 1, 4, and 6. To collect cells on day 9, single cell suspension was prepared by treatment of the hiPSC/OP9 cocultures with 1 mg/ml collagenase IV (Thermo Fisher Scientific) for 30 minutes at 37℃, followed by treatment with 0.05 % Trysin/EDTA (Thermo Fisher Scientific) for 15 minutes at 37 ℃. Cells were washed twice with phosphate-buffered saline

(PBS, Corning, Riverfront, NY, USA), then counted and used for fluorescence-activated cell sorter (FACS) analysis as well as for further erythroid differentiation.

7. Erythroid differentiation of hiPSC/OP9 coculture cells

After 9 day coculture of hiPSC/OP9 cells, the cells were seeded at a density of $1X10^6$ cells per ml on T25 plate (Thermo Fisher Scientific) in feeder free conditions for 17 days, based on previously reported protocols.[10,11] Serum-free culture medium was used, which consisted of Stemline II hematopoietic stem cell expansion medium (Sigma-Aldrich) supplemented with 1 % human serum albumin (20% albumin, Green Cross, Seoul, Korea), 200 ug/ml iron-saturated human transferrin (Sigma-Aldrich), 90ng/ml ferric nitrate (Sigma-Aldrich), 50μg/ml insulin (Sigma-Aldrich), 4mM/L L-glutamine (Thermo Fisher Scientific), 1.6×10^{-4} mol/L monothioglycerol (Sigma-Aldrich), and 1 % penicillin/streptomycin (Thermo Fisher Scientific).

The basic culture method consisted of three steps. In the first step, from day 0 to day 6, the cells were cultured in basal medium supplemented with 6 M Hydrocortisone (Sigma-Aldrich), 100 ng/ml stem cell factor (SCF) (Peprotech, Rocky Hill, NJ, USA), and 10 ng/ml interleukion-3 (IL-3) (Peprotech), and 6 IU/ml recombinant EPO (Peprotech). In the second step, from day 7 to day 14, cells were cultured in basal medium along with 50 ng/ml SCF, 10 ng/ml IL-3 and 3 IU/ml recombinant EPO. In the third step, from day 15 to 17, the basal medium was replaced with serum-free conditioned medium with 50 ng/ml of SCF, 2 IU/ml of EPO and 0.05% of poloxamer 188 (Pluronic F 68, Sigma-Aldrich). All cultures were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. These cells were harvested on day 17, and the corresponding cell numbers were counted and used for FACS analysis

8. Fluorescence-activated cell sorting (FACS) characterization of hiPSCs

Cells which have undergone hematopoietic and erythroid differentiation were analyzed by FACS for the presence of hematopoietic and erythroid markers. Hematopoietic differentiated cells were stained with anti-mouse IgG-FITC, anti-human CD34-FITC, CD43-PERCP, CD16-PE, and CD15-PE (BD bioscience, Franklin Lakes, NJ, USA). Cells of erythroid differentiation were stained with anti-mouse IgG-FITC, anti- human CD34-FITC, CD43-PE, CD235a-APC, CD71-FITC (BD Bioscience). For FACS analysis, at least 10,000 events were collected and analyzed. The percentages of selected cell population (base on the comparison with background staining shown when isotype-matched antibodies are used) among the total live cells were displayed. For histogram presentations, mean and standard deviation (SD) were shown. Live cells identified were analyzed using FACS Diva software (BD Bioscience).

9. Analysis of proliferation and morphology

Cultured erythroid cells were counted using a hemocytometer at every splitting (day 4, 7, 10, 12, 14, and 17) comparing each using a seeding density of $1X10^{6.}$ Cell morphology was assessed using slides prepared by cytospin using a cyto-centrifuge (Cytospin 3, Shandon Scientific, Tokyo, Japan) at 1,000 rpm for 5 minutes followed by Wright-Giemsa staining. Pictures of the stained cells were taken with an optical microscope (Nikon Eclipse Ti, Tokyo, Japan).

10. Differential counting of cultured erythroid cells

Proerythroblasts, early and late basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and enucleated RBCs were enumerated by differential counting at 1,000X magnification by optical microscope (Nikon Eclipse Ti) after Wright-Giemsa staining (Sigma-Aldrich).

All statistical analysis was performed with the SPSS software, student’s t-test was performed and p-values of under 0.05 were considered significant.

III. RESULTS

1. Titration of estadiol for maximizing endometrial cell proliferation

In order to titrate the estradiol concentration appropriate for treating donor endometrial cells, β estradiol concentrations of 0, 0.1 and 1 μM/ml was selected.[6-7] Control adult dermal fibroblast obtained from the same donor of endometrium 5 and endometrium 1,5,7 were treated with exogenous estradiol at passage 3-4. β estradiol treatment was performed daily from day 1 to 4, and all experimetns were performed three-folds. Cell proliferation activity was measured by ELISA through MTT assay. (Fig. 1)

2. Reprogramming of endometrial cells

Human endometrial cells from various donors in their 4^{th} to 5^{th} decade receiving benign hysterectomies were successfully reprogrammed to pluripotency using retroviral vectors expressing either pMIG-Oct4, pMIG-Sox2, pMIG-Klf4, and pMIG-c-Myc. After 48-72 hours, retrovirus-transduced cells could be tracked during the early phase with retrovirus LTR promoter-driven GFP expression. One week after introducing the retroviruses containing the pluripotency factors, the human endometrial cells began to lose their original spindle shape and aggregated into colony-like structures, even before being sub-cultured onto MEFs. Multiple iPSC clones from each of the 3 donors were used for future differentiation process. (Table 1)

Table 1. The hiPS cell lines and clones derived from endometrial cells of 3 donors after benign hysterectomies in their 4th or 5th decade

Donor	hiPSC clone
hEndo 1	hiPSC 1 clone 2
	hiPSC 1 clone 3
	hiPSC 1 clone 6
	hiPSC 1 clone 10
	hiPSC 1 clone 14
	hiPSC 1 clone 16
	hiPSC 1 clone 18
hEndo 2	hiPSC 2 clone 3
	hiPSC 2 clone 5
hEndo 7	hiPSC 7 clone 1
	hiPSC 7 clone 2
	hiPSC 7 clone 3
	hiPSC 7 clone 4
	hiPSC 7 clone 5
	hiPSC 7 clone 6

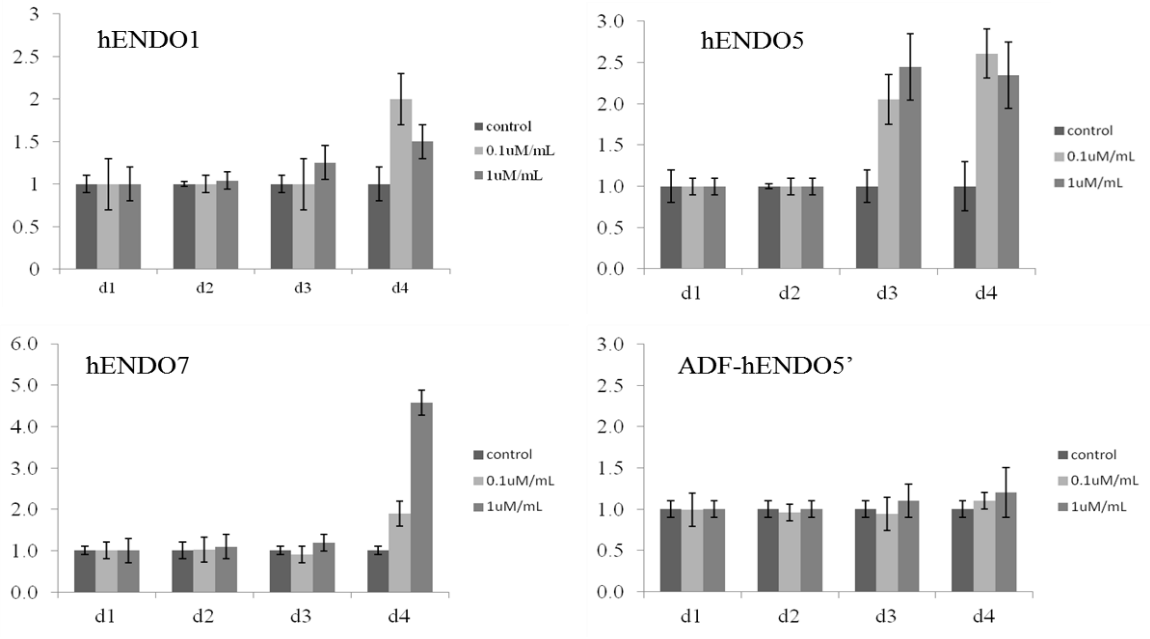

Fig. 1. Titration of estradiol for maximizing endometrial cell proliferation

Donor cells were treated with β estradiol(E2) at a concentration of 0, 0.1μm/ml and 1μM/ml for endometrium 1, 5, 7, ADF (from the donor of endometrium 5) cells every 24 hours for 3 days and analyzed by MTT assay (n=3). Although the response to estradiol was variable depending on the donor endometrial cells, highest proliferative activity was displayed in a majority of the endometrial stromal cells at day 4 of estradiol treatment at a concentration of 0.1μM/ml. (Values: Mean±SD)

3. Comparison of iPS cells reprogramming efficiency before and after β estradiol treatment

Human endometrial stromal cells were reprogrammed with or without β estradiol (E2) pre-treatment and after iPS cell colonies appeared on the co-cultured MEFs, they were maintained up to 21 days for colony counting with alkaline phosphatase staining. Untreated control group yielded mean iPS cell colony numbers of 112±8 and a mean of 186±18 colonies per 10cm dish for the E2 treated group. When the yield was converted to percentage with the control set as 100%, E2 treated endometrial cells yielded 166±5.2% colonies, (n=4) which was significant according to student's t-test (p=0.0001). (Fig. 2)

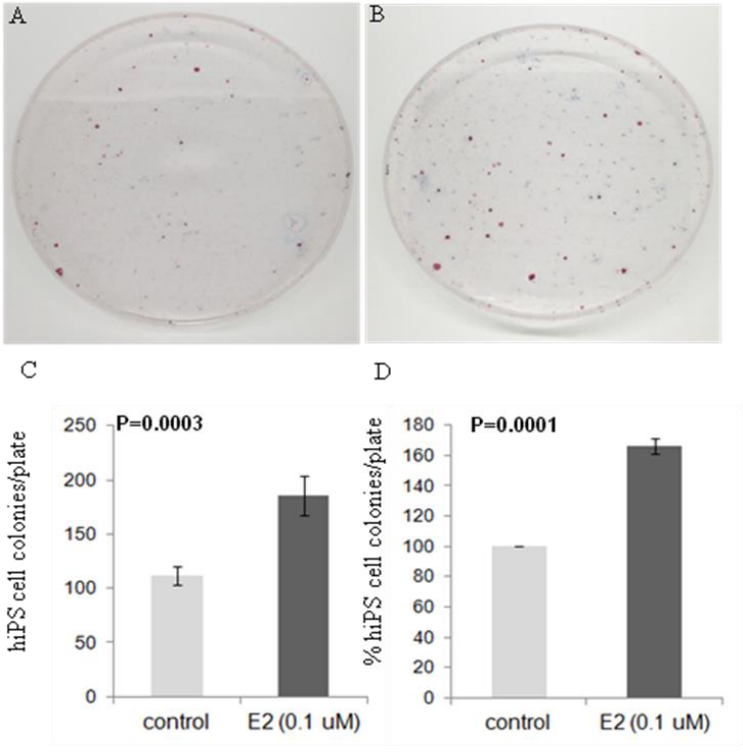

Fig. 2. Comparison of iPSC reprogramming efficiency before (A) and after (B) estradiol treatment

A,B. At reprogramming day 21, iPS cell colonies were counted after alkaline phosphatase staining. Control refers to cells not treated with β estradiol whereas the study group was treated with exogenous estradiol (E2) at 0.1uM/ml for a

duration of 4 days. Significance was evaluated with student's *t*-test. Control yielded mean colonies of 112±8 (100%) vs 186±18 (166±5.2%) when treated with E2 (n=4). (Values: Mean±SD)

4. Hematopoietic differentiation of human endometrial cell derived iPS (hiPS) cells

To induce hematopoietic differentiation in the first phase of differentiation, endometrium derived iPS cells were co-cultured with OP9 bone marrow stromal cell lines (Fig. 3).[9,10] Endometrium derived hiPSC1 (n = 5), hiPSC2 (n = 2), and iPSC7 (n = 2) each established from different donors at passage 10 were used to induce hematopoietic differentiation.

When hiPSC on OP9 cells was in an undifferentiated state prior to hematopoietic differentiation, KDR (VEGF-R2) was detected and when the hiPSC on OP9 began to differentiate, $CD235a^+$, $CD34^+$ and $CD43^+$, markers of hematopoietic multipotent progenitors, began to be detected.[11,12] CD335a (glycophorin A) was first expressed and CD34 was expressed next in the early 3 days of hiPSC/OP9 coculture. The $CD235a^+$ and $CD34^+$ ratios were highest at 4 to 5 days and erythroid progenitor $CD43^+$ was up-regulated at this time. In hematopoietic stem cells, $CD34^+/CD43^+$ is considered to be the hematopoietic multipotent progenitors, whereas $CD34^-/lowCD43^+$ and $CD235a^+$ are the erythroid progenitors and erythroblastoid cells are the most prominent in this population expressing these cell surface markers.[13] $CD43^+$ and $CD235a^+$ cells were cocultured for up to 9 days after differentiation into hematopoietic multipotent progenitors through hiPSC/OP9 coculture prior to inducing erythroblast differentiation.

After 9 days, FACS analysis was performed with induced cell suspensions of hematopoietic multipotent progenitors. As a result, the percentage of $CD43^+$ was 12.3±2.8% (hiPSC1 13.7±1.5, hiPSC2 13.1±1.3 and hiPSC7 8.2±2.3%, respectively) and $CD34^+$ 3.8±1.0% (hiPSC1 3.6±0.9, hiPSC2 3.2±0.04 and hiPSC7 4.9±1.3, respectively). (Fig. 4)

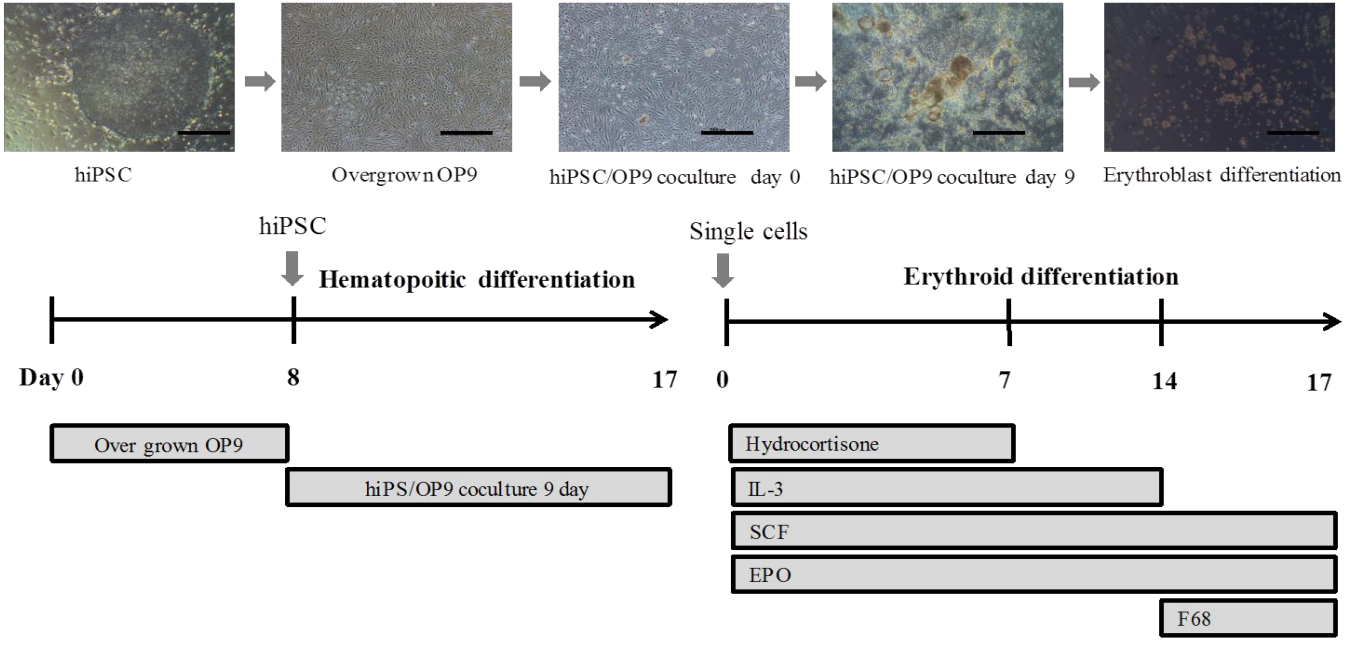

Fig. 3. Experimental protocol for the ex vivo generation of hematopoietic and erythroid cells from hiPSC lines.
For induction of hematopoietic differentiation, undifferentiated hiPSC were cocultured with overgrown OP9. After 9 days of coculture, differentiated hiPSC colonies with radial sac-like structures were formed. For erythroid differentiation, radial sac-like colonies at day 17 were digested into a single cell suspension and transferred to a four-phase liquid culture with changing combinations of growth factors for an additional day 17. (scale bar presents 250 um)

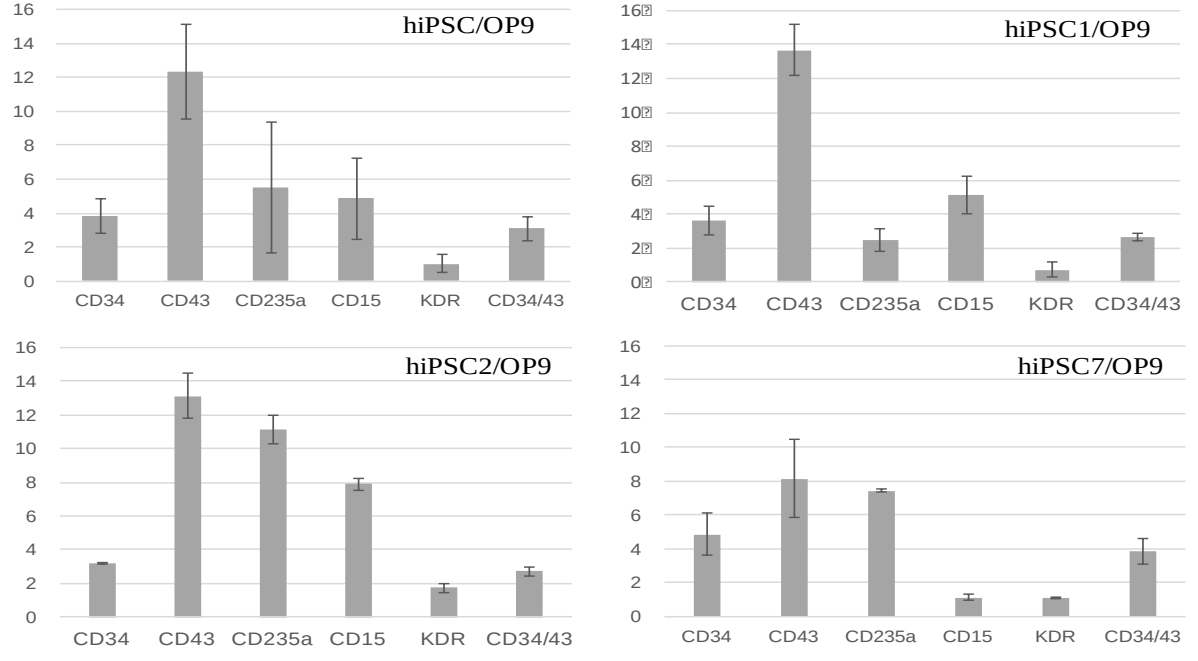

Fig. 4. Hematopoietic differentiation of human iPSC/OP9 on co-culture day 9. Expression of hematopoietic markers as measured by FACS analysis (hiPSC/OP9 CD34, CD43, CD235a, CD15, and KDR n=9, CD34/43 n=6; hiPSC1/OP9 CD34, CD43, CD235a, CD15, and KDR n= 5, CD34/43 n=2; hiPSC2/OP9, and hiPSC7/OP9 CD34, CD43, CD235a, CD15, and KDR n=2). The bars represent the percentages (mean±SD) of positive cells.

The percentage of co-expression of $CD34^+/CD43^+$ was 3.1±0.7% (hiPSC1 2.7±0.2, hiPSC2 2.7±0.2 and hiPSC7 3.8±0.8, respectively), and the percentage of $CD235a^+$ expression was at 5.5±3.8% (hiPSC1 2.5 ± 0.7, hiPSC2 11.1±0.8, and hiPSC7 7.5±0.1, respectively). Late differentiation maker $CD15^+$ was present in 4.9±2.4% (hiPSC1 5.1±1.1, hiPSC2 7.9±0.4, and hiPSC7 1.1±0.1) and KDR^+ in 1.0±0.6% (hiPSC1 0.7±0.4, hiPSC2 1.7±0.3, and hiPSC7 1.1± 0.04, respectively). (Fig. 4)

These results suggest that the yield of $CD34^+/CD43^+$ ratio is fair and $CD34^+/CD43^+$ ratio is compatible to that of erythroid progenitors in the total pool of differentiated hematopoietic multipotent progenitors. $CD34^+/CD43^+$ co-expression is the most reliable marker for hematopoietic multi-potent progenitors and can be expected to readily differentiate into erythroid progenitors. In contrast, a portion of the cells expressing the undifferentiated marker, KDR^+, could be also differentiated into erythroid progenitors. However, the ratios of both $CD235a^+$ and $CD15^+$ cells showed a slightly different trend for the various hiPSC lines. (Fig. 4)

After obtaining a reliable supply of hematopoietic multipotent progenitors through this hiPSC/OP9 coculture, these cells were detached from the feeder environment and transferred consequently to the next feeder free environment to induce erythroblast differentiation.

5. Directed erythroblast differentiation from induced hematopoietic muti-potent progenitors

Induced hematopoietic multipotent progenitors obtained from hiPSCs/OP9 culture were counted on days 4, 7, 10, 14, and 17, in feeder-free conditions. Seeding was performed so that 1 x 10^6 cells/ml were initially plated onto the feeder-free differentiation medium. For all of the hiPSC lines there was a temporary reduction of up to 0.4 x 10^6 cells/ml on day 4 and cell numbers began to increase from day 7. (Fig. 5)

Up to 60% of the hiPSC/OP9 co-cultured cells are lost in the initial phase of

the erythroid differentiation process, owing to the fact that they are grown on erythroblast cell-specific medium, and only those capable of growing into erythroblast progenitors, accounting for approximately 40% of hiPSC / OP9 co-cultured cells survive this selection process.

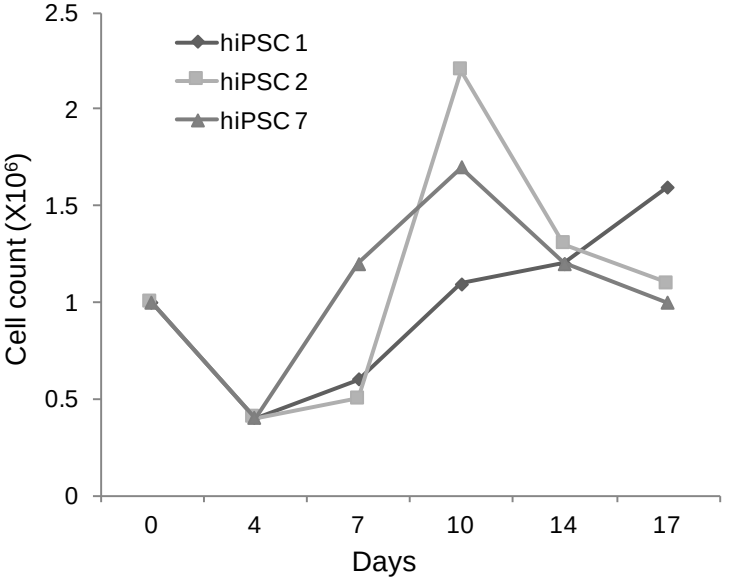

Fig. 5. Changes of cell number according to the days of erythroid differentiation in feeder free culture condition.
Data plotted on days 4, 7, 10, 14, and 17. The number of cells per 1 ml was reduced in all cell lines at day 4, when the hematopoietic multipotent progenitors were transferred from the OP-9 feeder environment to feeder free conditions.

6. Analysis of morphologic changes to erythroid cell differentiation of hiPS cell lines

Cells directed to erythroid differentiation on feeder free conditions were morphologically and differentially counted by Giemsa staining after 7 days, 10 days, 14 days, and 17 days. During the maturation process, erythroid

progenitors are replaced by proerythroblasts, early basophilic erythroblasts, late basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts.[12,13] Erythroblast differentiation at 7, 10, 14, and 17 day demonstrated that basophilic and polychromatic erythroblasts were observable on the 7th day, but only polychromatic erythroblast were observed on the 10th day. On the 14th day, most of the polychromatic erythroblasts are still present, but orthochromatic erythroblasts are more common. On the 17th day, mostly the orthochromatic as well as the polychromatic erythroblasts are present, mixed with minor traces of basophilic erythroblasts. HiPSC1, hiPSC2 and hiPSC7 showed slight difference in their outcome of differentiation but showed similar differentiation rates. (Fig. 6A)

The hiPSC cell lines showed that a total of approximately 76% of the cells displayed late basophilic erythroblast (36±5%) and polychromatic erythroblast (40±9%) morphologies on the 7th day of differentiation by differential counting. The proerythroblasts was observed at a ratio of 3±1% only on 7th day of differentiation and almost none after 10 days. On the 10th day of differentiation, polychromatic erythroblast accounted for the majority (52±4%) and late basophilic erythroblast decreased to 20±2% and orthochromatic erythroblast increased to more than 4% on the 7th day. On the 14th day, orthochromatic erythroblasts were 30±2%, almost two-folds compared to day 10, and basophilic erythroblast were 21% (early 7±1%, late 14±1%) On the 17th day, there were almost no early basophilic erythroblasts (3±1%), whereas orthochromatic erythroblasts accounted for 40±19%, and polychromatic erythroblast about 43±16%. As the time passes, the proportion of orthochromatic erythroblasts increases. (Fig. 6B)

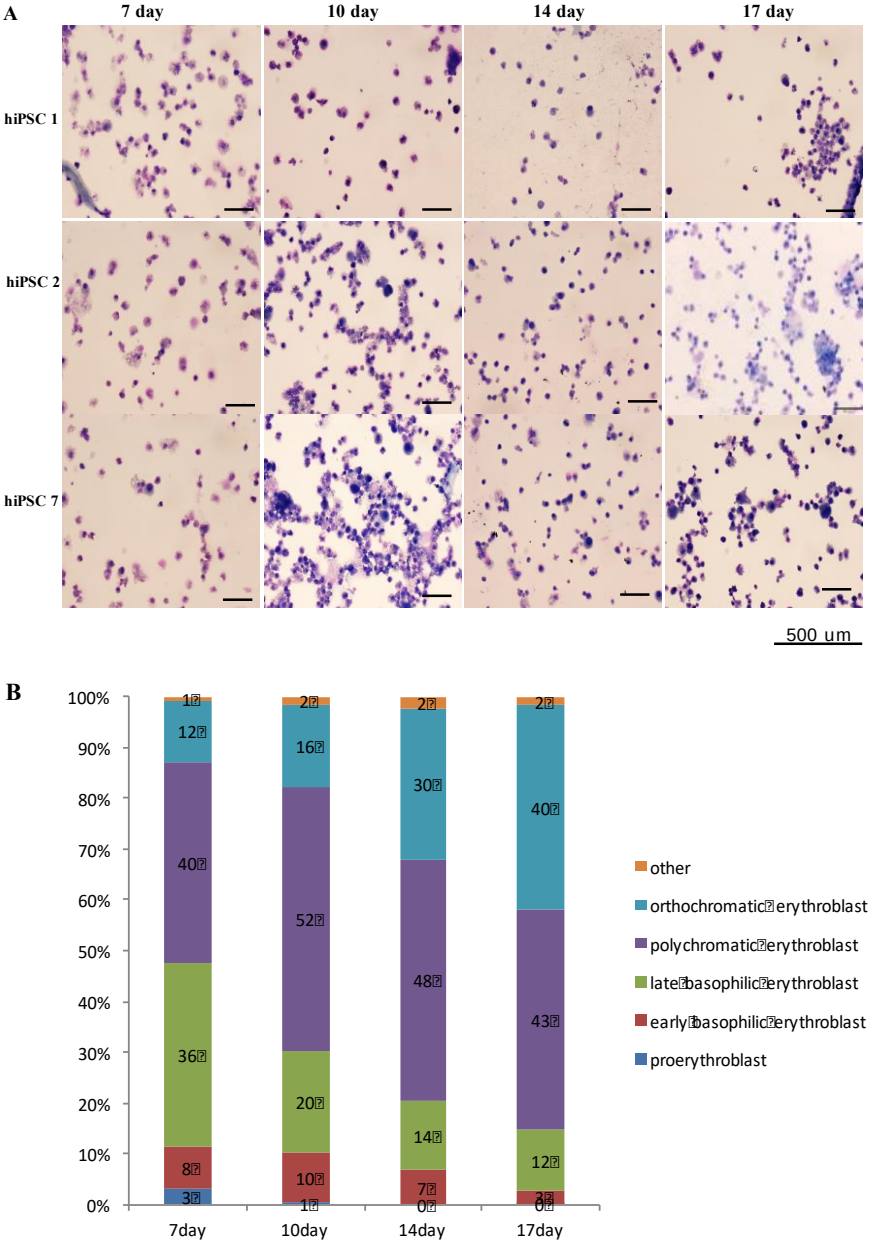

Fig. 6. Comparison of cell morphological changes (A) and cell differential counting (B). A. After Wright-Giemsa staining, erythroid cell types were differentially counted (scale bar presents 500 um). B. The total pooled proportions of the cells from hiPSC 1, hiPSC 2 and hiPSC 7 cell cultures after

17 days of feeder-free differentiation.

7. Analysis of erythroid differentiation cultures by FACS

First, FACS analysis was performed with the erythroid progenitors obtained via the hiPSC7/OP9 coculture method. $CD235a^+$ and $CD71^+$ (transferrin receptor), which are known to be expressed in the erythroid cell lineage, were used as erythroid maturation makers. Since CD43 was expressed in erythroid progenitors but not in mature erythrocytes, undifferentiation makers CD34, CD43 were used as the indicators of hematopoietic multipotent progenitor markers.

$CD34^+/CD43^+$ ratio was 39.8% higher at the beginning of seeding and 7% at the time of differential counting after erythroid differentiation, and the percentage decreased as the erythroblasts matured in the feeder free conditions. (Fig. 7)

In contrast, $CD235a^+$ and $CD71^+$ cells were found to increase in percentage, and especially, the erythroblast marker $CD235a^+$ increased from 3.17% to 45.3%. This is in agreement with previous studies where $CD34^+/CD43^+$ cells are high among the hematopoietic mulipotent progenitors and as for the myeloid progenitors, the ratio of $CD235a^+$ and $CD71^+$ cells begin to increase as of day7 and the $CD235a^+$ ratio increases in the mature erythroblast pool. The presence of $CD71^+$ cells over time, is thought to be due to the presence of various erythroblast cells, owing to the nature of hiPSC-derived hematopoietic multipotent progenitors. [12-14]

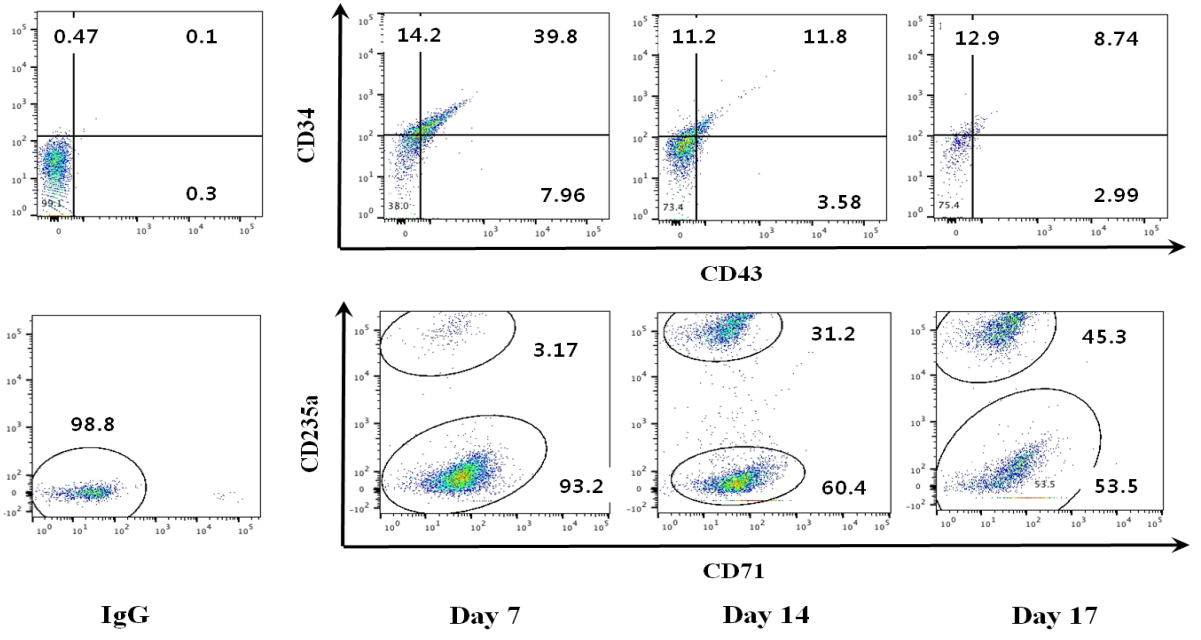

Fig. 7. Flow cytometric analysis of erythroblast differentiation cultures at different time (days) of expansion. Representative experiments with hiPSC7 is shown. As the cells isolated from hiPSC/OP9 begin to differentiate into erythroid fate, the total pool of $CD34^{+/}CD43^{+}$ cells decrease whereas $CD235a^{+}$ and $CD71^{+}$ cells are observed as differentiation is performed.

IV. DISCUSSION

In the era where average life-span extends beyond the 9th decade in many regions of the world, regenerative medicine is a field of great importance alongside the increase in demand for not only improvements in the quantity but also the quality of life. Awareness over various blood-borne diseases have raised concerns over peri-operative transfusions and also for massive or numerous transfusions in patients with chronic diseases where exposure to multiple heterologous blood sources precipitates to irregular antibody formation.

When there is demand for autologous transfusion, whole or fractionated blood components could be sequentially collected over time and stored until acute loss is anticipated. However, such process is not feasible in most patients with chronic or acute anemia, either because of the lack of autologous material or the lack of preparation time. The idea of deriving a safer, easily replenished autologous source of red blood cells has been made possible with the introduction of iPS cells in 2006 for the first time. A counter-intuitive idea of overexpressing somatic cells with a combination of defined factors commonly expressed in the embryo have actually reprogrammed terminally differentiated cells to a pluripotent state.[3] The concept of preserving a self-driven pluripotent cell source which could be indefinitely maintained and expanded has opened numerous possibilities in regenerative medicine and tissue engineering.

Primitive tissues which appear in early embryogenesis are the most suitable preliminary targets for iPS or ES cell differentiation in the field of regenerative medicine, as the signals and environment involved in more complex terminally differentiated tissues are still poorly understood. Neuronal differentiation is one major axis in the field of iPS cell research to overcome neurodegenerative diseases as well as neural injury which cannot be regenerated in vivo.[14,15] The derivation of erythroid lineage cells have also been attempted by many scholars.[8,11-13] Both hematopoietic and other blood progenitors have been derived from iPS cells driven from either skin fibroblasts or peripheral blood

mononuclear cells.

The yields of $CD34^+/CD43^+$ hematopoietic progenitors were highly variable with over 60% for H1 ES cells and generally ranging from 2 to 9% for most iPS cell lines.[16] Other groups have used different iPS cells driven from both $CD34^+$ cells and neural stem cells as well as fibroblasts, claiming that the rate of $CD43^+$ cells is similar regardless of donor origin when differentiation is achieved via embryoid body (EB) formation, with an efficiency slightly below 20%.[13,16] Although our system used, a shortened non-EB two phase protocol, the rates of $CD43^+$ cells using human endometrium derived iPS cells were between 8 to 13% after only a 9-day culture, before erythroid commitment was induced. Unlike ES cells, iPS cells have known to possess epigenetic memory of the donor cell and therefore the efficiency of directed differentiation was reported to be lowered in some and not in other studies.[17,18] Such impairment in differentiation potential becomes attenuated as the iPS cells are passed beyond passage 15, a characteristic of which provides hindrance with regards to time and efficiency.[18] However, endometrial cell driven iPS cells have displayed a reliable yield of hematopoietic and erythroid differentiation potential even at earlier phases at iPS passages under 10.

This current study has significance in several aspects. A complete process of actually deriving iPS cells with discarded patient hysterectomy specimens and consecutively directing differentiation into erythroid lineage was fully described. Beta estradiol, a hormone unique to the endometrium which robustly stimulates its proliferation, actually enhanced iPS cell establishment by approximately 166 ± 5.2%. In the clinical setting, improving efficiency of patient driven iPS cells is still an important issue to minimize failure, and a unique process of supplementing estradiol in the process of reprogramming endometrial cells was described in this study. (Fig. 2)

A significant yield of 52 ± 4% polychromatic erythroblasts on day 7th and 30 ± 2% orthochromatic erythroblasts were observed on the 14th day of differentiation. The fraction of orthochromatic erythroblasts further expanded to approximately 40% on day 17, showing clinically useful yields. (Fig.6)

Moreover, a two-phase culture protocol was optimized and described. Different groups have described protocols either with feeder, feeder-free and/or the involvement of embryoid bodies.[13, 16-18] To maximize the differentiation efficiency a 9-day co-culture of iPS cell clumps was performed on murine bone marrow stromal cells in the initial phase and subsequently transferred to a cocktail of growth factors in a feeder-free system. A period of 26 days is consumed, as opposed to 31 to 41 days to reach similar end-points in previously reported systems.[13] Earlier passages (around 10) of human endometrial cells were used in the study, whereas other donor cells of non-blood cell originated iPS cells were differentiated in later passages beyond 15, which implies that time consumed for iPS cell passage could be shortened. Depending on how urgent the transplantation process should be, such improvements in the time required for directed differentiation becomes an important issue.

The process of retrieving enucleated reticulocytes was not selected as the end-point in this study, and the ratio of polychromatic and orthochromatic normoblasts were evaluated. Also, iPS cells originating from different tissue sources was not tested and only different endometrial stromal cell originated iPS cells were compared to analyze erythroid differentiation efficiency. Further studies will be conducted in the future involving a more diverse origin of cells in order to standardize the process of autologous erythroid differentiation using autologous endometrium driven iPS cells.

V. CONCLUSION

The yield of patient endometrial cell derived iPS cells was considerably improved with the supplementation of β-estradiol in the process of reprogramming with defined Yamanaka factors, and at a relatively early passage, various discarded human endometrium derived iPS cells were successfully and consistently differentiated into erythroid lineages up to the targeted end-point of orthochromatic normoblasts via hematopoietic multipotent progenitors.

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ABSTRACT(IN KOREAN)

환자 자궁내막 세포로부터 제조한 유도만능줄기세포주로부터 적혈구모세포로의 분화유도

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박 주 현

연구의 목적과 배경: 재생의학은 최근의 인구의 노령화에 따른 질병의 발생 증가로 인하여 이를 극복하고 완화하기 위한 새로운 치료방법으로 크게 각광받고 있다. 산부인과에서는 수혈의 빈도가 높은데, 자궁절제술 전후 잉여로 자궁내막조직을 쉽게 얻을 수 있는 것이 착안하여 자궁절제술 환자유래 자궁내막세포를 이용하여 유도만능줄기세포주를 제작하고 이를 자가수혈용 혈액을 생산할 수 있도록 조혈모세포 및 적혈구로 분화시킴으로써 자궁내막을 활용한 자가 수혈용 적혈구 생산의 원천기술을 확보하고자 하였다.

연구방법: 30대 후반에서 40대의 양성 부인과 질환으로 자궁절제를 받은 3명의 환자로부터 잉여의 자궁내막 조직을 얻어 연구에 사용하였다. 유도만능줄기세포주는 자궁내막지지세포를 계대 배양하면서 분획을 팽창시킨 후 sox2, oct4, klf4 그리고 c-Myc을 형질주입 시킨 형광발현 역전사바이러스 벡터를 사용하여 유도만능줄기세포주를 제작하였다. 이 과정에서 자궁내막지지세포의 증식을 자극, 역분화의 효율을 높이기 위해 외인성 β 에스트라다이올을 처리하였다. 적혈구 분화유도는 2단계로 나누어 진행하였다. 첫번째 단계에서는 유도만능줄기세포를 생쥐의 골수지지세포 위에 9일간 공동배양을 한 후 두번째 단계에서는 이를 지지세포가 없이 hydrocortisone, stem cell factor (SCF),

interleukin-3 (IL-3), recombinant erythropoietin (rEPO) 그리고 poloxamer 188이 첨가된 무혈청 배지를 기본으로 하여 적혈구 분화 실험을 17일간 진행하였다. 배양과정에서 분화의 확인은 유세포분석기를 이용하여 KDR (VEGF-R2), CD235a, CD34, CD43, CD71을 측정하였고, 세포수는 hemocytometer를 사용하여 수기로 산정하였으며, 세포의 형태와 백분율은 말초도말 슬라이드를 제작하여 Wright-Giemsa 염색을 하여 현미경하에서 산정하였다.

결과: 자궁내막지지세포에서 유도만능줄기세포를 제작하는 과정에서 에스트라다이올을 전처리한 그룹이 대조군에 비하여 역분화 효율이 평균 166±5%로 증가하였으며, 그 결과에 따라 본 연구의 유도만능줄기세포주 구축과정에서는 0.1um/ml의 농도로 공여세포를 전처리하는 방법을 도입하였다.

구축된 유도만능줄기세포주를 생쥐골수지지세포에 9일간 공동배양한 후 조혈모 전구세포 표지자인 CD43 양성세포를 약 8~13%까지 얻을 수 있음을 확인하였다. 이를 지지세포 없이 적혈구 분화유도 무혈청배지에서 17일간 배양 과정을 통해 약 80%까지 polychromatic 및 orthochromatic normoblast로 분화되는 것을 확인하였다.

결론: 자궁절제술 환자로부터 얻은 잉여 자궁내막지지세포를 고유한 에스트라다이올 처리를 통해 역분화 효율을 증대시킬 수 있었으며, 자궁내막세포로부터 제작된 유도만능줄기세포주로부터 조혈모전구세포로 분화시키고, 이를 다시 적혈구모세포로 성공적인 분화과정을 확립하였다. 이는 무한한 세포원으로서 각광받고 있는 유도만능줄기세포주 구축과정에서 산부인과에서 쉽게 얻을 수 있는 잉여 자궁내막세포를 활용한 연구이며, 이를 통해 수혈 요구가 많은 산부인과 환자를 위해 자가수혈용 확보 기술로서 체외 적혈구 분화 원천기술을 확보하는데 기초 기반 기술을 제공하였다.

핵심되는 말: 에스트라다이올, 자궁내막지지세포, 유도만능줄기세포, 자가수혈제제, 조혈모세포, 체외 분화 적혈구