Clean-Up Human Embryonic Stem Cell Lines Using Humanized Culture Condition

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Abstract Human embryonic stem cell (hESC) culture system has been changing culture conditions from conventional to xeno-free for therapeutic cell applications, and *N*-glycolylneuraminic acid (Neu5Gc) could be a useful indicator of xenogeneic contaminations in hESCs because human cells can no longer produce it genetically. We set up the humanized culture condition using commercially available humanized materials and two different adaptation methods: sequential or direct. SNUhES4 and H1 hESC lines, previously established in conventional culture conditions, were maintained using the humanized culture condition and were examined for the presence of Neu5Gc. The hESCs showed the same morphology and character as those of the conventional culture condition. Moreover, they were negative for Neu5Gc within two passages without loss of pluripotency. This study suggested that this method can effectively cleanse previously established hESC lines, bringing them one step closer to being clinical-grade hESCs.

Keywords Human embryonic stem cells · Humanized culture condition · Neu5Gc · Xeno-free

1 Introduction

Human embryonic stem cells (hESCs) have the unique abilities of unlimited proliferation and differentiation potential, and hESCs are attractive sources for drug screening, disease modeling and cellular therapy applications [1–4]. It is known that hESCs have been cultured using basal medium supplemented with animal-derived materials such as fetal bovine serum (FBS) and mitotically

inactivated mouse embryonic fibroblasts (MEFs) or STO feeder cell layers [1, 2, 5]. However, conventional culture conditions use animal-derived materials that may have been directly or indirectly exposed to xeno-contamination during their derivation and propagation [6]. Subsequently, hESCs from conventional culture conditions will have increased risks of immune rejection and xenogeneic pathogen and toxic protein transfer; therefore, xeno-contaminated hESCs are unsuitable for clinical applications [6, 7]. Hence, one of the major challenges for the clinical application of hESCs is the elimination of all animal-derived materials from completely defined culture conditions.

There has been some progress in producing xeno-free culture conditions which does not present risks of transmitting non-human pathogens and immunogenic molecules; FBS has been replaced with KnockOutTM Serum Replacement (KO-SR), and human-derived feeder cells have been used to replace mouse embryonic fibroblasts in hESC culture conditions [8–10]. Recently, various feeder-free culture conditions have also been reported and

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commercialized for hESC culture [11–14]. However, most of these culture conditions contain high concentrations of growth factors, such as basic fibroblast growth factor (bFGF) and leukemia inhibitory factor. In addition, these conditions, usually used Matrigel, a complex mixture of matrix proteins derived from Engelbreth-Holm-Swarm mouse tumors. Moreover, chromosomal abnormalities of hESC lines show a tendency to increase in these culture systems. Furthermore, such systems are laborious, expensive, and on the periphery of hESC culture [15–17].

Neu5Gc is widely expressed in mammalian cells, and it has been identified as an immunoreactive material of contaminated culture cells [18]. However, humans are genetically unable to synthesize Neu5Gc, and Neu5Gc levels are undetectable in human tissue [19, 20]. For this reason, Neu5Gc could be a useful indicator of xenogeneic contamination in hESC culture.

To further promote the optimization of hESC culture conditions for clinical applications, in the present study, we established a humanized culture condition standard protocol that easily and cost-efficiently used commercially available xeno-free materials and xeno-free medium. Additionally, we investigated several characteristics of hESCs cultured in this humanized culture condition. Moreover, we examined the Neu5Gc contamination of hESCs in this condition.

2 Materials and methods

2.1 Conventional culture condition for hESCs

Two karyotypically normal hESC lines, SNUhES4 (46,XY at passage 36; Seoul National University, Seoul, Korea) and H1 (46,XY at passage 45; WiCell Inc., Madison, Wisconsin, USA), were used in this study. Undifferentiated hESCs were cultured using hESC medium (SR medium) containing Dulbecco's Modified Eagle medium Nutrient Mixture F-12 (DMEM/F12; Invitrogen, Grand Island, NY, USA) supplemented with 20% KO-SR (Invitrogen), 4 ng/mL bFGF (Invitrogen), 1% nonessential amino acids (NEAA; Invitrogen), 0.1 mM β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 50 U/mL penicillin and 50 μ g/mL streptomycin (Invitrogen). The STO (ATCC, Manassas, VA, USA) cells treated with mitomycin C (Sigma-Aldrich) were seeded at 2.6×10^4 cells/cm² onto a 0.1% gelatin-coated 35 mm culture dish (STO-gel) or a CELLstartTM-coated culture dish (STO-Cs). All hESCs in this study were maintained at 37 °C and 5% CO₂ in an incubator and were passaged onto fresh feeder cells once per week by mechanical transfer method using glass pipette previously described by Oh et al. [5].

2.2 Humanized culture condition for hESCs

2.2.1 Substrate: CELLstartTM

CELLstartTM (Cs; Invitrogen), a humanized substrate for cell culture, was diluted 1:50 in Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (D-PBS; Invitrogen) and added to a culture dish at a final volume per surface area of 0.078 mL/cm². The Cs was incubated at 37 °C and 5% CO₂ for 2 h. The Cs was aspirated from the culture dish before inactivated feeder cells were seeded.

2.2.2 Feeder cell layer: human foreskin fibroblasts

We used two different methods for culturing human foreskin fibroblasts (hFF; ATCC). One culture condition used Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 50 U/mL penicillin and 50 μ g/mL streptomycin in a 0.1% gelatin-coated flask. The hFF feeder cells were mitotically inactivated by mitomycin C and plated at 1.1×10^4 cells/cm² onto a 0.1% gelatin-coated culture dish (hFF-gel) or a Cs-coated culture dish (hFF-Cs). The other culture condition used IMDM supplemented with 10% human serum (HS; Sigma), 50 U/mL penicillin and 50 μ g/mL streptomycin in a Cs-coated flask. The hFF feeder cells with HS and Cs were mitotically inactivated by mitomycin C and plated at 1.1×10^4 cells/cm² onto a Cs-coated culture dish (Cs-hFF).

2.2.3 Sequential or direct hESC adaptation

The xeno-free hESC culture medium (XF medium) consisted of DMEM/F12 supplemented with 15% Knock-OutTM SR XenoFree (Invitrogen), 8 ng/mL bFGF, 1% NEAA, 0.1 mM β -mercaptoethanol (Invitrogen), 50 U/mL penicillin and 50 μ g/mL streptomycin. We used two different methods for maintaining the SNUhES4 and H1 hESC lines: sequential or direct adaptation. For sequential adaptation (sXF), the hESCs were gradually adapted to the XF medium over the course of the first four passages (ratio of SR medium to XF medium = 25:75, 50:50, 75:25 and 100:0). Direct adaptation (dXF) was accomplished by the directly transfer of hESCs from the conventional culture condition to the humanized culture condition (100% XF medium).

2.3 hESCs substrate test

For the substrate test, the SNUhES4 and H1 hESC lines were cultured on an STO or hFF feeder layer on a 0.1% gelatin- or a Cs-coated dish in SR medium. For three

passages, the hESCs were seeded at 100–120 clumps per 35 mm culture dish and fed every day except for the day after transfer. Attachment rate was calculated as follows: (number of attached colonies/number of seeded clumps) $\times$ 100. Spontaneous differentiation rate was calculated as follows: (number of colonies that have a spontaneously differentiated area more than 80% of the whole colony area/number of attached colonies) $\times$ 100. Attached and spontaneously differentiated colonies of SNUHES4 and H1 lines were counted at the end of each passage, i.e., every 7 days, before being mechanically transferred.

2.4 hESCs in the humanized culture condition with sequential or direct adaptation

The sXF condition was tested with three types of feeder layers: STO-Cs (STO-Cs-sXF), hFF-Cs (hFF-Cs-sXF) and Cs-hFF (Cs-hFF-sXF). The dXF condition was tested in combination with hFF-Cs (hFF-Cs-dXF) and Cs-hFF (Cs-hFF-dXF). For six passages of sequential or direct adaptation, hESCs were seeded at 100–150 clumps per 35 mm culture dish, attached and spontaneously differentiated colonies were counted under a microscope every 7 days before being mechanically transferred.

2.5 Embryoid body (EB) formation

To examine *in vitro* differentiation, undifferentiated hESC colonies at day 7 were treated with 1 mg/mL dispase (Invitrogen) for 15 min at 37 °C and detached from the dish bottom. The detached hESC colonies were cultured in suspension for 2 weeks to allow the formation and spontaneous differentiation of EBs in low-attachment plates (Nalgene Nunc, Rochester, NY, USA). XF medium without bFGF was used for EB culture. After 2 weeks, several EBs were re-attached onto a 0.1% gelatin-coated culture dish in DMEM/F12 supplemented with 10% FBS, they were then cultured for 7 days for further differentiation and subsequent immunocytochemical staining.

2.6 Immunocytochemical staining

The hESCs were washed with D-PBS and fixed with 4% paraformaldehyde (PFA; USB Corporation, Cleveland, OH, USA) for 30 min at room temperature (RT). The fixed hESCs were washed with D-PBS, and then blocked with 3% bovine serum albumin (BSA, Sigma) overnight at 4 °C to inhibit nonspecific binding. Rabbit anti-human Oct-3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human SSEA-4, mouse anti-human Tra-1-60, mouse anti-human Tra-1-81, mouse anti-human Nestin, rabbit anti-human Brachyury and goat anti-human HNF3 β (all from Millipore, Billerica, MA, USA) were used as primary

antibodies for undifferentiated or spontaneously differentiated hESCs. The cells were incubated with primary antibody solutions overnight at 4 °C. The cells were incubated with Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-mouse IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen, Eugene, OR, USA) secondary antibodies for 1 h in the dark at RT. The labeled cells were imaged using an epifluorescence microscope (Nikon, Tokyo, Japan).

2.7 RNA isolation, reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using random hexamer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). PCR was carried out with 3 μ l of cDNA template, 10 pM of each primer, 4 μ l of 2.5 mM dNTP mix, and 0.1 units of rTaq DNA polymerase (Takara Bio, Otsu, Shiga, Japan) in a volume of 50 μ l using the Eppendorf AG 22331 (Eppendorf, Hamburg, Germany). PCR was performed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 55–68 °C for 30 s and extension at 72 °C for 30 s. The PCR products were analyzed by electrophoresis using a 2% agarose gel containing 0.4 μ g/mL ethidium bromide (MP Biomedicals, Santa Ana, CA, USA).

Quantitative RT-PCR was carried out in RotorGene 3000 (Corbett Life Science, Sydney, Australia) using QuantiTect SYBR Green PCR kit (Qiagen). The amplification program included an initial step at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s and extension at 72 °C for 30 s. All reactions were run in triplicate. CT was calculated under default settings for the Rotor-Gene 6.0 software (Corbett Life Science). Relative gene expression was normalized to GAPDH expression. Each primer sequence used for RT-PCR and qRT-PCR is listed in Table 1.

2.8 Bromodeoxyuridine (BrdU)-labeling analysis

Proliferation of the hESCs was determined by FACS analysis of BrdU incorporation using the APO-BrdU TUNEL Assay Kit (Invitrogen) after a 24 h incubation with BrdU, according to the manufacturer's instructions. After they were labeled, the hESCs were detached from the dish bottom using 1 mg/mL dispase, the detached colonies were then incubated with 1 mL Accutase (Chemicon, Temecula, CA, USA) for 5 min for single cell dissociation. Single cells were fixed with 4% PFA for 30 min and blocked with

Table 1 Primer sequences used for RT-PCR and Qpc

Marker	Primer sequence	Products size (bp)	Annealing temperature (°C)
RT-PCR			
GAPDH	F:AGCCACATCGCTCAGACACC R:GTA CT CAGCGGCCAGCATCG	302	60
Oct4	F:CTACAACGCCTACGAGTCCTACA R:TTCTGGCGCCGGTTACAGAACCA	219	60
Nanog	F:CTGAGATGCCTCACACGGAGACTG R:GTCACACCATTGCTATTCTTC	370	55
SOX2	F:GGCAGCTACAGCATGATGCAG R:GCTCTGGTAGTGCTGGGACATG	396	65
TERT	F:AGCTATGCCCCGGACCTCCAT R:GCCTGCAGCAGGAGGATCTT	185	63
Nestin	F:CAGCTGGCGCACCTCAAGATG R:AGGGAAGTTGGGCTCAGGACTGG	214	63
MAP2	F:GCATATGCGCTGATTCTTCA R:CTTCCGTTTCATCTGCCATT	202	55
Brachyury	F:TAAGGTGGATCTTCAGGTAGC R:CATCTCATTGGTGAGCTCCCT	252	58
Enolase	F:TGACTTCAAGTCGCCTGATGATCCC R:TGCGTCCAGCAAAGATTGCCTTGTC	450	68
HNF3P	F:CCATTGCTGTTGTTGCAGGGAAGT R:CACCGTGTCAAGATTGGGAATGCT	196	55
AFP	F:AGAACCTGTCACAAGCTGTG R:GACAGCAAGCTGAGGATGTC	676	68
qRT-PCR			
GAPDH	F:GTCGGAGTCAACGGATTTGG R:AAAAGCAGCCCTGGTGACC		
Oct4	F:TCTCGCCCCCTCCAGGT R:GCCCCACTCCAACCTGG		
Nanog	F:CTGCTGAGATGCCTCACACG R:TGCCTTTGGGACTGGTGGA		

0.05% Tween-20 and 3% BSA for 1 h at RT. After being washed with D-PBS, the cells were labeled with DNA-labeling solution and incubated overnight at 37 °C. After the cells were washed with D-PBS again, Alexa Fluor 488-labeled anti-BrdU was added and incubated for 2 h at RT. These cells were then analyzed using a BD FACS Calibur™ cell analyzer (BD Biosciences, San Jose, CA, USA).

2.9 Karyotype analysis

The hESCs were cultured in medium supplemented with 0.1 mg/mL colcemid (Invitrogen) for up to 4 h to arrest the cell cycle at metaphase. The cells were harvested and fixed for standard chromosome analysis. More than 20 metaphase cells were karyotyped and analyzed using

chromosome image processing via ChIPS (GenDix, Seoul, Korea) and Cytovision (Genetix, New Milton, Hampshire, UK) systems.

2.10 Neu5Gc analysis

The presence of the non-human sialic acid Neu5Gc was tested for using the GC-free basic kit (Sialix Inc., San Diego, CA, USA), according to the manufacturer's instructions. In Brief, all colonies were detached using dispase, and then single cell dissociation was performed using Accutase. Single cells were incubated with a chicken anti-Neu5Gc (1:1000 dilution) antibody or an isotype control antibody (1:1000 dilution) in diluent buffer containing 0.5% of a blocking agent in PBS at 4 °C. The cells were then incubated with a donkey anti-chicken IgY

secondary antibody conjugated to Cy5 (Millipore, 1:100) in PBS containing blocking buffer at 4 °C. A FACS Calibur™ (BD Biosciences) cell analyzer was used for data acquisition.

2.11 Statistical analyses

Mixed model ANOVA or *T* test were used for the attachment and spontaneous differentiation rates data analyses between the different culture conditions. The statistical analyses were performed at the significance level of $p < 0.05$, and data are presented as the mean $\pm$ SEM. The analyses were performed using SPSS software version 21 (IBM SPSS Statistics 21, USA).

3 Results

3.1 CELLstart™ maintained similar hESC colony morphology and attachment/spontaneous differentiation rate compared with the conventional culture condition

Initially, the SNUhES4 and H1 hESC lines were cultured onto STO or hFF feeder cell layers in a 0.1% gelatin- and a Cs-coated dish with SR medium for substrate testing. The colonies on the hFF-gel were angularly shaped compared with those on the STO-gel; however, the morphology of these hESC colonies became similar to the colony morphology observed on hFF-Cs (Fig. 1A). The attachment rate of the H1 cells on hFF-Cs (89.0%) was higher than on STO-Cs (78.3%), while the attachment rate of the SNUhES4 and H1 cells on hFF was lower than on STO, for the most part. However, the spontaneous differentiation rate of each cell line cultured using Cs was low compared with those cultured using 0.1% gelatin (Fig. 1B). The results of these two hESC lines showed no significant differences between Cs and 0.1% gelatin; therefore, we determined that using Cs was comparatively effective for the maintenance of the undifferentiated hESC state.

3.2 Sequentially or directly adapted hESC colonies in XF medium maintained their undifferentiated state

The SNUhES4 and H1 hESC lines were adapted to XF medium using a sequential adaptation method (sXF) with SR medium to XF medium ratio of 25:75, 50:50, 75:25 and 100% for the first four culture passages, according to the manufacturer's instructions. While each cell line was adapting to the XF medium over the course of four weeks, colonies were thinner, and hESC densities were lower than those of the hESCs colonies cultured under the

conventional culture condition (Fig. 2A). However, the hESC colonies gradually compacted and maintained a morphology similar to that of the conventionally cultured hESCs thereafter. The SNUhES4 and H1 cells exhibited reduce attachment rates and increase spontaneous differentiation rates during the period of increasing XF medium ratios; however, after six weeks using the sXF method, both hESC lines exhibited increase attachment and reduce spontaneous differentiation rates (Fig. 2B). These cell lines were analyzed for the undifferentiated state by immunocytochemical staining and RT-PCR (Fig. 2C, D). These results indicated that the hESC lines sequentially cultured in XF medium maintained an undifferentiated state.

The SNUhES4 and H1 hESC lines were also adapted from the conventional culture condition to 100% XF medium directly (dXF). SNUhES4 and H1 colony morphology after direct transfer into XF medium was notably thin, and the hESC density was low (Fig. 3A). However, the hESC colonies gradually compacted and maintained a morphology similar to that of the conventionally cultured hESCs after further culture (data not shown). The hESC colonies exhibited constantly high attachment rates with Cs-hFF-dXF compared with the other condition, hFF-Cs-dXF. Furthermore, spontaneous differentiation rates were inconsistent between different conditions of each cell line. However, each cell line was easily maintained because the rates of spontaneous differentiation were reduced after six weeks (Fig. 3B). The hESCs cultured in the XF medium directly were stained with a panel of immunocytochemical antibodies specific to hESC markers: Oct-4, SSEA-4, Tra-1-60 and Tra-1-81 (Fig. 3C). RT-PCR was performed for the analysis of markers of undifferentiation, including Oct-4, Nanog, Sox2 and hTERT (Fig. 3D). No difference was found in the proportion of cellular expression and formation for the direct XF medium adaptation method.

3.3 Human ESCs cultured for prolonged periods in the humanized culture condition maintained their undifferentiated state, pluripotency and normal karyotype

To confirm that the two hESC lines maintained their undifferentiated state and differentiation potential under the humanized culture condition, we performed qRT-PCR, BrdU-incorporation, RT-PCR, immunocytochemical staining and karyotyping at passages 13–30 of the humanized culture condition (Fig. 4). In qRT-PCR, no significant differences in the expression levels of the Oct-4 and Nanog markers were detected between the hESC lines cultured under the conventional and humanized culture conditions (Fig. 4A). Effects on the proliferation of each hESC line were assessed by FACS analysis of BrdU incorporation. The proportions of positive cells between

Fig. 1 Comparison of culture conditions on STO or hFF feeder cells using either 0.1% gelatin or CELLstart™ in SR medium for SNUhES4 and H1 hESC lines. **A** Morphology of SNUhES4 and H1 cells in each culture condition after three passages, day 6. *a* SNUhES4 on STO-gel, *b* SNUhES4 on STO-Cs, *c* SNUhES4 on hFF-gel, *d* SNUhES4 on hFF-Cs, *e* H1 on STO-gel, *f* H1 on STO-Cs, *g* H1 on

hFF-gel and *h* H1 on hFF-Cs (Scale bar = 500 μ m). **B** Percentage (mean $\pm$ SEM) of attached and spontaneously differentiated colonies ($n = 3$, ANOVA test, $p < 0.05$). *a* Attachment rate of SNUhES4, *b* spontaneous differentiation rate of SNUhES4, *c* attachment rate of H1 and *d* spontaneous differentiation rate of H1

each hESC line in the humanized and conventional culture conditions were not significantly different (Fig. 4B). These cell lines were analyzed by RT-PCR for markers of differentiation: Nestin, MAP2, Enolase, Brachyury, AFP and

HNF3 β (Fig. 4C). To confirm that these cell lines maintained their pluripotency *in vitro*, we tested for the formation of EBs. The EB-derived SNUhES4 and H1 cells expressed markers from the three different embryonic

Fig. 2 Comparison of culture conditions on STO-Cs, hFF-Cs or Cs-hFF in XF medium using the sequential adaptation method (sXF) for SNUhES4 and H1 hESC lines. **A** Morphology of SNUhES4 and H1; each hESC line was transferred from the conventional culture condition to the new culture condition using a stepwise adaptation (Scale bar = 500 μ m). **B** Percentage (mean $\pm$ SEM) of attached and spontaneously differentiated colonies of SNUhES4 and H1 in XF medium after six passages (n = 3, ANOVA test, * p < 0.05). **C** Expression of markers of undifferentiation; Oct-4, SSEA-4, Tra-1-60 and Tra-1-81 (Scale bar = 500 μ m). **D** RT-PCR analysis of RNA from undifferentiated colonies at passage 13, which were continuously cultured after sXF adaptation, showing expression levels of Oct-4, Nanog, Sox2 and hTERT (GAPDH, positive control)

lineages: ectoderm (Nestin), mesoderm (Brachyury) and endoderm (HNF3 β) (Fig. 4D). Karyotype analysis was performed on the hESCs after more than 10 passages of continuous culture using the humanized culture condition; the two cell lines maintained a normal karyotype (Fig. 4E). Our data showed that the humanized culture condition maintained the self-renewal, pluripotency and normal karyotype of the hESC lines.

3.4 Neu5Gc was not detected after culture in the humanized culture condition

We examined the content of the sialic acid, Neu5Gc, under the conventional and humanized culture conditions in the two cell lines by flow cytometry. The expression level of Neu5Gc was high under the conventional culture condition (Fig. 5A(a, d)). However, Neu5Gc expression decreased almost to the levels of the negative control after 1 week,

and no Neu5Gc expression was detected after 10 weeks under the humanized culture condition (Fig. 5A(b, c, e, f)). The SNUhES4 and H1 cells under the humanized culture condition expressed significantly lower levels of Neu5Gc than the cells under the conventional culture condition (Fig. 5B). These results suggested that hESCs cultured under the humanized condition would present decreased xenogeneic risks; furthermore, our developed humanized culture condition did not affect the maintenance of the hESC self-renewal ability.

4 Discussion

The self-renewal and pluripotent capacities of hESCs are promising for hESCs serving as a renewable cell source for future regenerative medicine applications, including insulin-producing cells, neural precursor cells and

Fig. 3 Comparison of SNUhES4 and H1 hESC lines cultured on hFF-Cs or Cs-hFF in XF medium using the direct adaptation method (dXF). **A** Morphology of SNUhES4 and H1 cells; each hESC line was transferred from the conventional culture condition to the new culture condition (Scale bar = 500 μm). **B** Percentage (mean ± SEM) of attached and spontaneously differentiated colonies of SNUhES4 and H1 cells in XF medium after six passages ($n = 3$, T test, $*p < 0.05$). *a* Attachment rate of SNUhES4 in hFF-Cs-dXF, *b* spontaneous

differentiation rate of SNUhES4 in hFF-Cs-dXF, *c* attachment rate of H1 in hFF-Cs-dXF and *d* spontaneous differentiation rate of H1 in hFF-Cs-dXF. **C** Expression levels of markers of undifferentiation; Oct-4, SSEA-4, Tra-1-60 and Tra-1-81 (Scale bar = 500 μm). **D** RT-PCR analysis of RNA from undifferentiated colonies showing expression of Oct-4, Nanog, Sox2 and hTERT (GAPDH, positive control)

cardiomyocytes [4, 21–24]. To generate safe and usable therapeutic stem cell-derived products for clinical cell therapies, it is necessary to eliminate the potential risks of infection posed by animal pathogens from non-human feeders and other animal materials routinely used in hESCs culture [7, 24–26]. Therefore, a transition from xenogeneic supplementation to xeno-free culture conditions would be one of the most important steps forward considering the suitability of hESCs for clinical use.

In our laboratory, we have reported the development of an animal component-free method for culturing hESCs. To determine whether our humanized culture condition generally supports hESC self-renewal, we cultured both SNUhES4 and H1 hESC lines, previously established in

conventional culture conditions, under a xenobiotic-free condition with human foreskin fibroblast feeder cells on CELLstart™ and xeno-free medium containing Knock-Out™ SR XenoFree.

Contact between culture substrates and hESCs is mediated by cell adhesion surface molecules, such as integrins, substrates are mostly human or animal tissue extracts that vary between lots and thus require time-consuming testing [27, 28]. Matrigel™ has become the most widely used substrate of feeder-free culture systems [11, 12]. However, lot-to-lot composition variations of Matrigel can pose problems, inhibiting its effectiveness in maintaining undifferentiated hESCs [24, 29]. In recent years, several types xeno-free substrates have been developed, such as

Fig. 4 Analysis of SNUhES4 and H1 hESC lines under the humanized culture condition using direct adaptation (Cs-hFF-dXF). **A** qRT-PCR analysis of RNA from undifferentiated colonies ($n = 3$) of *a* SNUhES4 at passage 27–29 and of *b* H1 at passage 16–18 under the humanized culture condition. There were no significant differences between the conventional and humanized culture conditions ($p < 0.05$). **B** BrdU-incorporation by undifferentiated SNUhES4 and H1 hESCs at passage 22–25 under the humanized culture condition

($n = 3$). **C** RT-PCR analysis of RNA from EBs cultured for 2 weeks showing expression of Nestin, MAP2, Enolase, Brachyury, AFP and HNF3 β (GAPDH, positive control). **D** Immunocytochemistry analysis from EBs showing expression of Nestin (red, ectoderm), Brachyury (red, mesoderm) and HNF3 β (green, endoderm); blue represents DAPI staining (Scale bar = 500 μ m). **E** Karyotype analysis of *a* SNUhES4 at passage 13 and *b* H1 at passage 12 under the humanized culture condition

recombinant laminin, vitronectin, human-derived ECM and synthetic polymer matrix [30–33]. Nevertheless, some of these are not cost effective for system scale-up and may lead to batch-to-batch variability [24, 28] Consequently, we have used commercially available CELLstartTM, which is humanized substrate containing components only of

human origin, for cell culture with relatively well-defined components. CELLstartTM reduces the risk of cellular contamination by animal-derived pathogens and provides scalable hESC culture that is inexpensive and not labor intensive.

Fig. 5 Detection of Neu5Gc from SNUhES4 and H1 hESC lines cultured under the humanized condition at passages 1 and 10 by flow cytometry. **A** A histogram of detected Neu5Gc. *a* SNUhES4 under the conventional culture condition, *b* SNUhES4 under the humanized culture condition after 1 week (passage 1), *c* SNUhES4 under the humanized culture condition after 10 weeks (passage 10), *d* H1 under

the conventional culture condition, *e* H1 under the humanized culture condition after 1 week (passage 1) and *f* H1 under the humanized culture condition after 10 weeks (passage 10). **B** Comparison of Neu5Gc detection between the conventional and humanized culture conditions ($n = 3$). There were significant differences between the conventional and humanized culture conditions ($*p < 0.05$)

Co-culture with mouse embryonic fibroblasts, such as MEF or STO, was the first culture condition used for the successful establishment of hESC lines [1, 2, 34]. Thereafter, hESC cultures with human foreskin fibroblasts, placental fibroblasts and autologous hESC-derived feeder cells have been reported to be successful for medical applications [26, 35, 36]. Unfortunately, the routine feeder layer culture system is very labor intensive, encounters lot-to-lot inconsistencies between feeder populations, and still use

animal-derived products, which carry potential risks for animal pathogen or antigen transfer [6, 24, 37]. For these reasons, various feeder-free culture conditions have been reported for hESC culture, such as X-Vivo 10, TeSR1 and E8 [11–14]. Rajala et al. [38] tested nine different commercially available or published xeno-free media for hESC culture. They reported that the tested xeno-free reagents were not able to maintain the undifferentiated growth of hESCs [38]. The International Stem Cell Initiative

Consortium et al. (2010) compared defined culture systems for feeder-free propagation of hESCs, and they reported that apart from the commercial preparations, most of the formulations did not support the maintenance of hESCs [39]. Moreover, the feeder-free media required higher concentrations of basic fibroblast growth factor or various other growth factors [10, 40, 41]. In addition, some results using these media showed induced chromosomal changes in the hESCs [12–16]. Therefore, in this study, we used an established cell line of human foreskin fibroblasts for hESC cultivation without materials containing animal components. We also suggested a condition using Cs-hFF, which consisted of feeder cells cultured on a Cs-coated flask instead of a 0.1% gelatin-coated flask in IMDM containing 10% human serum to minimize xeno-transfer potential.

Neu5Gc is expressed on the cell surface of mammalian cells, but humans are unable to synthesize Neu5Gc due to the loss of the human CMAH protein hydroxylase activity, which was caused by an exon deletion/frameshift mutation in the human Cmah gene [18, 19]. For this reason, Neu5Gc levels are undetectable in human tissue, although Neu5Gc is a major sialic acid in most mammalian cells [20]. Our data showed that Neu5Gc was not detected in either cell line using the humanized culture condition, whereas Neu5Gc contamination was confirmed under the conventional culture condition, which animal-derived materials (Fig. 5).

In conclusion, we developed a humanized culture system using commercially available CELLstart™, human foreskin fibroblasts and xeno-free medium containing KnockOut™ SR XenoFree. This humanized culture condition, which changed and simplified system components from animal-derived materials to xeno-free materials, is more cost effective than other defined culture conditions without labor intensive. In addition, this condition can support undifferentiated hESC growth and easily prevent hESC sialic acid contamination for long periods. Moreover, humanized culture system in this study could produce clinical-grade hESC lines from the previously established hESC lines by effectively cleansing them using this system. It means that a lot of results which is studied using previously established hESC lines in conventional culture condition can use after simplifying test of hESCs characterization. Furthermore, the efficient production of clinical-grade hESC lines for clinical applications could be more easily attainable with this method, and we established lots of hESC lines using this system (not shown). As a result, our humanized culture condition is suitable for further optimization of the establishment, culture and differentiation of clinical-grade hESCs and can ultimately serve as a procedure for the production of therapeutic applications.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflict of interest.

Ethical statement This study was approved by the Ethics Committee of the Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University (IRB No. 219932-201510-ER-05-01-01).

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