Establishment of a xeno-free culture system that preserves the characteristics of placenta mesenchymal stem cells

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Abstract Although stem cells are promising candidates for cell replacement therapies, the vast majority are derived using animal sera, which has risk of being contaminated by animal viruses or toxins. To overcome these potential problems, we initially established multiple lines of stem cells from first-trimester human placenta (fPMSC), which were cultivated using human follicular fluid (hFF) instead of fetal bovine serum (FBS). FF provides a very important microenvironment for the development of oocytes. No differences were found in the general morphology, growth rate, karyotype, gene and surface expressions between placental MSCs cultured in 5 % hFF-supplemented medium (fPMSC-X) or 10 % FBS-supplemented medium (fPMSC). Differentiation experiments confirmed similar levels of potency in cells grown in either condition. Since hFF preserved the unique features of the stem cells and is free from potential pathogens, it should be considered as the main culture medium supplement for the propagation of human stem cells for clinical applications.

Keywords Xeno-free · Human follicular fluid · First-trimester · Placental stem cell

Introduction

Proteins, polypeptides, hormones, minerals and metabolites present in serum enable cells to survive in vitro. Serum proteins help cells to attach to the culture dish surface and deliver minerals and fatty acids (Barnes and Sato 1980; Tozer and Pirt 1964; Hynes 1992; Freshney 1994). Various polypeptides possess mitogenic activity on various types of cells (Antoniades et al. 1979; Hollenberg and Cuatrecasas 1973) and hormones, such as insulin and hydrocortisone. Small molecules, including minerals, iron, zinc, copper, and selenium, also present in serum, are essential for the survival and proliferation of cells (Sinha et al. 1990; Frendin et al. 1979; Guner et al. 1977). However, serum of animal origin also has the risk of being contaminated by a wide spectrum of allogeneic pathogens, such as bacterial toxins or viruses, some of which are unidentified. Because of the presence of these contaminants, animal sera should not be considered for the cultivation of stem cells for use in human cell therapies (Wang and Sun 2005; Skottman et al. 2006).

Follicular fluid (FF) is blood plasma that has infiltrated into the antrum of follicle through the basement membrane and granulosa, and is known to support survival, maturation, ovulation of oocytes, fertilization and implantation (Westergaard et al.

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1985; Liu et al. 2003). Aside from its original role, FF is also capable of supporting the survival of endometrial cells in culture (Somigliana et al. 2001); however, the activity has not been widely confirmed, especially for stem cells.

Human placenta is considered a reservoir of adult stem cells that are capable of differentiation into many different cell types (Parolini et al. 2008; Battula et al. 2007). Placenta-derived mesenchymal stem cells (PMSCs) share several characteristics with bone marrow-derived MSCs in terms of gene and surface expressions (Battula et al. 2007; Poloni et al. 2008), but have higher proliferative activities (Barlow et al. 2008). In addition, PMSCs express pluripotency-related genes (Poloni et al. 2008; Strakova et al. 2008) suggesting multiple differentiation potencies. These advantages make PMSCs, although rather recently established, one of the prime candidates for stem cell research and cell replacement therapies.

In this work, we show that PMSCs can be cultivated in human follicular fluid (hFF)-supplemented medium for long periods while maintaining their proliferative activities and differentiation potencies as well as PMSCs maintained in FBS-supplemented medium. These results suggest that hFF has good potential as a supplement for xeno-free culture systems, especially in stem cell research for clinical applications.

Materials and methods

Ethical statement

The institutional review board of Maria Biotech Co. and Maria Fertility Hospital approved all experimental procedures used in this study, and informed consents were secured from each of the study participants according to the guidelines of the Korean Ministry of Health and Welfare.

Collection of hFF

hFF was collected during the in vitro fertilization (IVF) program in the Maria Fertility Hospital (Seoul, Korea), from the patients who were negative for human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis C virus (HCV), hepatitis B virus (HBV) infection and syphilis antigen to prevent infection. During the oocytes aspiration, hFF was

collected from ruptured follicles that were >18 mm in diameter. Cell debris and red blood cells were removed by centrifugation followed by heat inactivation for 35 min at 59 °C. Ten individual hFF were pooled to minimize the potential differences that were identified in our previous study (data not shown) among individuals. These pools were then filtered (0.22 µm) and stored at −80 °C.

Establishment of fPMSC

First-trimester human placental tissue corresponding to decidua of the fetus failing to maintain heart beats at 5–7 weeks after embryo transfer, as determined by ultrasound examination, were removed aseptically using curettage before suction. The aseptically retrieved tissue (~0.1 g) was washed in PBS and minced. The minced tissue was washed, collected, and plated in a 25 cm² culture dish in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA, USA) supplemented with 5 % hFF or 10 % FBS (Life Technologies, Carlsbad, CA, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and antibiotics in a humidified chamber with 5 % CO₂ at 37 °C. The culture was left untouched for 5 days before the first medium change. After the first 5 days, unattached particles were removed by repeated pipettings. The PMSCs from first-trimester (fPMSC) growing out of the tissue particles normally took 2–4 days to become 90 % confluent, by which time the culture was transferred to a larger dish (75 cm²; SPL, Pocheon-si, Gyeonggi-do, Korea).

Karyotype analysis

Cells of approximately 70 % confluency on 75 cm² culture dishes were exposed to 0.1 µg/ml of colcemid (Sigma-Aldrich) for 2 h, harvested with 0.025 % trypsin/1.3 mM EDTA (Sigma-Aldrich) and washed with PBS. The harvested cells were then incubated in hypotonic solution (75 mM KCl; Sigma-Aldrich) for 20 min at 37 °C. After incubation, cells were fixed in 1 ml of cold methanol/acetic acid (3:1; Sigma-Aldrich) fixative, followed by centrifugation at low speed (300×g for 20 min) and resuspension in 1 ml of cold PBS. A drop of the fixed cells was dropped onto a frosted microscopic slide from a height of 30 cm. After air-drying, the slide was exposed to 0.025 % trypsin for 30 s followed by several washes in PBS.

Chromosomes were stained using 10 % Giemsa (Sigma-Aldrich). Cells were cultivated for at least 60 days before karyotype analysis and the obtained raw results were analyzed using computer-based Cytovision (Applied Imaging, San Jose, CA, USA).

Immunocytochemistry

Cells grown on coverslips were fixed in 4 % (v/v) paraformaldehyde (PFA; Sigma-Aldrich) for 15 min at room temperature. After rinsing in PBS, the fixed cells were permeabilized and non-specific epitopes were blocked using 10 % normal goat serum (Life Technologies) in 0.1 % Triton X-100/PBS, followed by incubation in the diluted primary antibody for 1 h at room temperature. After three washes in 0.1 % Triton X-100/PBS, samples were incubated with secondary antibodies diluted in 0.1 % Triton X-100/PBS for 1 h at room temperature or overnight at 4 °C. Prepared samples were then mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) and photographed under fluorescent or confocal microscopes (Olympus, Tokyo, Japan). Oct3/4 goat polyclonal IgG (Santa Cruz, Santa Cruz, CA, USA, Cat #: SC-8630), Nanog goat polyclonal IgG (Santa Cruz, Cat #: SC-30332), C-kit mouse monoclonal IgG1 (Santa Cruz, Cat #: SC-17806), nestin mouse monoclonal IgG1 (Santa Cruz, Cat #: SC-23927), insulin mouse monoclonal IgG1 (Santa Cruz, Cat #: SC-52035). The secondary antibodies used were: Alexa 568 goat anti-mouse IgG (Life Technologies, Cat #: A-11031), Alexa 488 goat anti-mouse IgG (Life Technologies, Cat #: A-11004), Alexa 568 donkey anti-goat IgG (Life Technologies, Cat #: A-11057), Alexa 488 donkey anti-goat IgG (Life Technologies, Cat #: A-11055). Nuclei were highlighted by staining with 10 µg/ml Hoechst 33342 (Sigma) for 10 min at room temperature.

RT-PCR (reverse transcription-polymerase chain reaction)

mRNA was extracted using Dynabeads® mRNA Direct™ Micro kit (Invitrogen, Oslo, Norway). Complementary DNA was synthesized using Superscript II (Invitrogen) and oligo-d(T)₂₀ primers at 42 °C for 1 h, followed by incubation at 72 °C for 15 min. Target sequences from the cDNA were amplified

Table 1 Primers used for RT-PCR

Gene	Primer sequence
OCT4	5'-CGTGAAGTCGGAGAAGGAGAAGCTG-3' 5'-AAAGGCTTCCCCCTCAGGGAAAGGG-3'
Nanog	5'-TGATTCTTCCACCAGTCCC-3' 5'-TGATTCTTCCACCAGTCCC-3'
Tbn	5'-GAAGGCTGCATCCAGAGGCGCG-3' 5'-GGCTTCTTCTGCGGATCTTGGGC-3'
Activin	5'-AGAGCGACCTCACAGCCGTGCTGG-3' 5'-CCGAGGTAGTGCCGTTGACCGACCT-3'
Klf4	5'-CCCGATCAGATGCAGCCGCAAGTC-3' 5'-CTGGCTGGGCTCCTTCCCTCATCG-3'
Rex1	5'-CAGATCCTAAACAGCTCGCA-3' 5'-GCGTACGCAAATTAAGTCC-3'
TERT	5'-TACTGGCAAATGCGGCCCT-3' 5'-TCCCGCACGCTCATCTTCCA-3'
Foxa2	5'-GAGAAGCAGCTGGCGCTGAA-3' 5'-GAACGGGTGGTTGAAGGCGT-3'
Pdx1	5'-CCGCAGGAACCACGATGAGA-3' 5'-CAACGCGCAATCCAGTATCACT-3'
MafB	5'-AACTTGTCTTGGGGCACAC-3' 5'-CTCCTACAATTAGCGCAGGC-3'
Glut2	5'-AGGACTTCTGTGGACCTTAT-3' 5'-GTTTCATGRCAAAAAGCAGGG-3'
Glucagon	5'-AGGCAGACCCACTCAGTGA-3' 5'-AACAATGGCGACCTCTTCTG-3'
Sox1	5'-TCATGTCCGAGGCCGAGAAG-3' 5'-GCTGCGCCTCCTGCATCATG-3'
DCX	5'-CGCCCCAAGCTGGTTACCATC-3' 5'-TTTGTACAACCCCGGTCTCC-3'
GAP43	5'-TTGCCGATGGGGTGGAGAAGAA-3' 5'-TTCAGCCTTGGAGGACGGCG-3'
Otx1	5'-GGCGGCGTGGACTGCAGCTCATAC-3' 5'-GGGCTCACACCTGGAACCGCC-3'
NeuroD	5'-ATCCCAACCCACCACCAACC-3' 5'-TTTCTCAAACCTCGGCGGACG-3'
Map2	5'-TACCAGCCTAAAGGGGGGACAGG-3' 5'-TTGCTGAGTCGTCGGGGTGATG-3'
GAPDH	5'-TGGTATCGTGGAAGGACTCA-3' 5'-CCTGCTTCAACACCTTCTTG-3'

using premixed kits (Bioneer, Daejeon, Korea) with the following conditions; initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53–60 °C for 45 s, and extension at 72 °C for 45 s. Normalized products were

separated on a 2 % agarose (Sigma-Aldrich) gel and visualized by ethidium bromide (Sigma-Aldrich) staining. Sequences of the primers used are summarized in Table 1.

Induction of differentiation

For endodermal differentiation of fPMSCs and fPMSCs-X, approximately 10^6 cells were plated in a 6 cm culture dish in DMEM F-12 (Dulbecco's modified Eagle's F-12 medium; Invitrogen) supplemented with 10 ng/ml bFGF (basic Fibroblast Growth Factor; Life Technologies) and N2 (Stem Cell technologies, Vancouver, BC, Canada). At 7–10 days, cells spontaneously aggregated to form spheres 50–100 μ m in diameter. Once spheres were formed, the induction medium was replaced by DMEM/F-12 supplemented with N2, 10 mM nicotinamide (Sigma-Aldrich), and 4 g/l glucose (Sigma-Aldrich) for another 7 days for maturation. N2 supplement is recommended for use in the in vitro differentiation of stem cells to neural and pancreatic-like cell types.

For ectodermal differentiation, approximately 4.0×10^5 cells/cm² were plated on a polyethyleneimine (Sigma-Aldrich)-coated dish in 10 % fetal bovine serum-supplemented DMEM for 2 days to form spheroids of 100–200 μ m in diameter. Once the spheroids were formed, 10–20 spheroids were transferred to a regular culture dish (25 cm²) and cultivated for 5–10 days in 10 % FBS-supplemented DMEM until they were confluent for expansion.

Flow cytometric analysis

For detection of surface antigens, approximately 90 % confluent cells on the culture dish were harvested using 4 mM EDTA (Life Technologies)/5 % FBS in PBS and rinsed in PBS three times. Washed cells were incubated in 2 % FBS/PBS buffer, containing either mouse IgG (BD, Franklin Lakes, NJ, USA) for reference or predetermined concentrations of FITC- of PE-conjugated antibodies (BD) at 4 °C for 30 min. To exclude dead cells and debris, 5 μ g/ml propidium iodide (Sigma-Aldrich) was added and the samples were analyzed using the BD FACS Vantage SE equipped with Cell Quest software (BD).

Results

Establishment of the fPMSC

During the course of the in vitro fertilization program, a small proportion of embryos transferred failed to maintain their heart beat. These early embryos were removed and proliferating cells were isolated from the tissues corresponding to the decidua. The established cells proliferated for at least 90 days in vitro and expressed Oct4, Nanog, c-kit, Tbn, Activin, Klf4, Rex1 (by immunocytochemistry and RT-PCR). Flow cytometric analysis revealed that the fPMSC were positive for CD13, CD44, CD90, CD105, and HLA-ABC expression, but negative for CD14, CD34, CD45, CD127, and HLA-DR.

Proliferation and general morphology in the xeno-free culture condition

Medium supplementation with 20 % hFF instead of 10 % FBS appeared to enhance the proliferation of cells for the first 2–3 passages, but the rate dropped abruptly thereafter (Fig. 1a), which was accompanied by outstanding morphological changes (data not shown). However, no differences in general morphology and proliferation rate were observed when cultures were maintained in medium supplemented with either 5 or 10 % hFF (Fig. 1a). The proliferation rate of cells was also indistinguishable whether the cells were cultured in 10 % FBS-supplemented medium (fPMSC) or 5 % hFF-supplemented medium (fPMSC-X) for at least 48 days (Fig. 1b). The general morphology of the cells maintained in the xeno-free condition (Fig. 1c) was also indistinguishable from that of the cells grown in FBS-supplemented medium for the same duration (Fig. 1d). Their karyotypes were also similar for both conditions (Fig. 1e, f). This result suggests that hFF can safely replace FBS for the cultivation of cells for the cell replacement therapies.

Maintenance of the gene expressions

Stem cells are often characterized by their gene expressions, of which Oct4, Nanog, and C-kit are some of the genes maintained while in culture (Schöler et al. 1989; Matsui et al. 1990; Mitsui et al. 2003). When fPMSC were cultured in FBS-supplemented medium for 48 days, cells expressing Oct4

Fig. 1 The effect of hFF on the general characteristics of placental MSCs. **a** The effect of three different concentrations (5, 10, and 20 %) of hFF on the proliferation rate of fPMSC-X. 10^5 fPMSC-X at P3 were plated on a 25 cm² culture dish at the beginning of the experiment and trypan-blue negative cells were counted when the cells reached approximately 90 % confluency, which occurred at about day 3. Triplicate sets of experiments were repeated twice for each concentration of hFF. Results were expressed as mean $\pm$ standard error of mean. **b** The effect of hFF and FBS on the proliferation of placental MSCs, as revealed

by the growth curves of fPMSC-X and fPMSC, respectively. Experimental sets and counts were performed as described above except for 48 days and results were expressed as mean $\pm$ standard error of mean. **c** General morphology of fPMSC-X and **d** fPMSC cultivated for approximately 60 days in vitro as shown under phase contrast (DIC) microscopy. Scale bars indicate 10 μ m. **e** Karyotype analysis of fPMSC-X and **f** fPMSC grown for 60 days in vitro confirmed the normal chromosomal compositions and G-banding patterns

Fig. 2 The effect of hFF on the gene expressions of placental MSCs. Expressions of Oct 4 (**a**, **b**), Nanog (**c**, **d**), and c-kit (**e**, **f**) were examined in fPMSC-X (**a**, **c**, **e**) and fPMSC (**b**, **d**, **f**) by immunocytochemistry and counterstaining with Hoechst 33342. Approximately 45.2 ± 2.3 % (**a**), 89.6 ± 1.6 % (**c**), 96.1 ± 1.0 % (**e**) of fPMSC-X expressed Oct4, Nanog, and

c-Kit, respectively, and 37.1 ± 2.0 % (**b**), 82.5 ± 1.3 % (**d**), 98.3 ± 0.5 % (**f**) of fPMSC expressed Oct4, Nanog, and c-Kit, respectively (Scale bar 30 μ m) (**g**) RT-PCR analysis confirmed the expression of the pluripotency-coupled genes, Oct4, Nanog, Tbn, Activin, Klf4, and Rex1, which were well preserved in both culture conditions

(37.1 ± 2.0 %, Fig. 2b), Nanog (82.5 ± 1.3 %, Fig. 2d), and C-kit (98.3 ± 0.5 %, Fig. 2f) and these ratios exhibited no fluctuation when cells were cultured in the xeno-free condition for a similar duration (Oct4: 45.2 ± 2.3 %, Fig. 2a; Nanog: 89.6 ± 1.6 %, Fig. 2c; 96.1 ± 1.0 %, Fig. 2e). Maintenance of the expression levels of these “pluripotency-coupled genes” (Beattie et al. 2005; Voss et al. 2000; Carpenter et al. 2004) normally expressed in embryonic stem cells or embryonic tissues before the onset of gastrulation was further confirmed by RT-PCR (Fig. 2g). Surface expression patterns, which were very similar to those of bone marrow-derived MSCs, were indistinguishable from cells maintained in either of the culture conditions: 10 % FBS-supplemented (Fig. 3a) or 5 % hFF-supplemented (Fig. 3b).

Maintenance of differentiation potencies

The stable gene expression profiles after the extended period of culture in the hFF-supplemented medium

strongly suggests that the differentiation potencies of the cells are also preserved. To test whether this is the case, both of these cells were induced to differentiate into ectodermal lineage. During normal growth, approximately 30–50 % of the fPMSC-X (Fig. 4a) and fPMSC (Fig. 4b) expressed nestin, suggesting an ectodermal tendency. Upon ectodermal differentiation, nestin was induced in almost all of the cells (96.6 ± 2.4 and 94.9 ± 3.1 %, respectively) and no difference was recognizable between fPMSC-X (Fig. 4c) and fPMSC (Fig. 4d). This result suggests that the differentiation potential toward ectodermal lineage was well preserved during cultivation in the hFF-supplemented xeno-free condition. Inductions of the ectodermal-specific genes, Sox1, Dcx, NeurD, GAP43, and Otx1, were also confirmed by RT-PCR (Fig. 4e). In addition, when both cells were exposed to endodermal induction medium for 7 days, fPMSC-X and fPMSC spontaneously formed aggregates and insulin induction was confirmed in both fPMSC-X (Fig. 4h) and fPMSC (Fig. 4i) at 14 days, while no

Fig. 3 The effect of hFF on the surface marker expressions of placental MSCs. FACS analysis revealed that fPMSC-X maintained in vitro for at least 60 days were positive for CD13, CD44, CD90, CD105 and HLA-ABC expression, but negative for CD14, CD34, CD45, CD127, and HLA-DR (a). The surface marker expression pattern was indistinguishable from those of fPMSC (b). The X axis of each diagram shows the relative fluorescence intensity of PE or FITC and the Y axis of each diagram shows the relative cell number

signal was detected in cells cultured in regular medium (Fig. 4f, g). RT-PCR examination further confirmed the inductions of *Foxa2*, *PDX1*, *MafB*, *Glut2*, and *glucagon* mRNA in both of the induced cells (Fig. 4j).

Discussion

The role of the infiltrate of blood serum into the antrum of the follicle, known as FF, is to support survival and proper development of oocytes of various stages within

the follicle (Edwards 1974). This functional activity of the FF may be a result of the fluid composition, and continuous studies have revealed that the FF is a rich reservoir of hormones (Suchanek et al. 1988; Ellsworth et al. 1984; Lanzone et al. 1996), cytokines (Wen et al. 2006; Van Dessel et al. 1996; Chen et al. 1995), proteins (Nandedkar et al. 1992), antioxidants (Attaran et al. 2000), and nutrients, including sugar, minerals, and ions (Revelli et al. 2009). Considering the rich spectra of molecules identified from the FF, in addition to its role in oocyte maturation, it was reasonable to speculate that FF

Fig. 4 The effect of hFF on the differentiation potency of placental MSCs. Differentiation of fPMSC-X and fPMSC into endodermal and ectodermal cells. Approximately 30–50 % of undifferentiated fPMSC-X (a) and fPMSC (b) maintained for at least 48 days in vitro expressed nestin, and the percentage of the expression increased dramatically to 96.6 ± 2.4 and 94.9 ± 3.1 % in both fPMSC-X (c) and fPMSC (d), respectively, suggesting the ectodermal differentiation potency was well maintained in both of the culture conditions. The ectodermal differentiation tendency was further confirmed by

RT-PCR (e). There was no spontaneous induction of insulin in either fPMSC-X (f) or in fPMSC (g) when cultivated for 48 days in their respective culture conditions. However, when the fPMSC-X (h) and fPMSC (i) were subjected to endodermal differentiation for 7–14 days, both types of cells spontaneously aggregated and expressed insulin as confirmed by immunohistochemistry. The endodermal differentiation was further confirmed by RT-PCR (j). Undiff denotes for fPMSC-X or fPMSC cultivated for at least 48 days in vitro and Diff denotes for the differentiated fPMSC-X or fPMSC. Scale bars indicate 30 μ m

has cellular proliferation-supporting activity, which FBS provides in conventional culture systems. In agreement with this speculation, a higher proliferation ratio of endometrial cells was reported when cultivated in hFF-supplemented medium compared with FBS-supplemented medium (Somigliana et al. 2001).

However, few studies have examined whether the FF is also capable of supporting the proliferation of stem cells and maintaining the characteristics of stem cells, thus preserving the differentiation potencies during long-term culture. To address this question, several lines of MSCs were established from first-

trimester human placenta, which were shown to share the surface expressions of bone marrow-derived MSCs and were capable of differentiation into ectodermal and endodermal lineages. When grown in 5 % hFF-supplemented medium for up to 16 passages (approximately 48 days), fPMSC-X was indistinguishable from the fPMSC grown in 10 % FBS-supplemented medium in their general morphology, growth rate, karyotype, gene expressions, and differentiation potencies.

hFFs which are obtained from women undergoing either IVF or gamete intrafallopian tube transfer (GIFT) can be relevant to the defense mechanism against virus and bacteria (Perricone et al. 1992). However, hFFs have to be analyzed for bacteria and viruses, such as HIV, CMV, HCV, HBV, and syphilis infection in order to use allogeneic contamination-free hFFs for media during assisted reproductive technologies (ART) procedure (Devaux et al. 2003). Furthermore, it has been reported that hFF microorganism is a potential cause of adverse pregnancy outcomes in IVF procedure. But treatment with antimicrobials may increase success rates of IVF and GIFT (Pelzer et al. 2013). These reports showed that the hFF is obtained from the woman during ART may be secure against potential risk of containing viruses and other pathogens.

Recently developed stem cells and their derivatives are considered prime candidates for cell replacement therapies; however, during their establishment or differentiation, the majority of stem cells are exposed to unidentified zoonoses originating from xenogenic animal products (Rajala et al. 2010). To avoid these potential risks, mouse feeder cells and animal sera have been replaced by human feeder cells (Mannello and Tonti 2007; Lee et al. 2005) and serum replacement (Gibco BRL) (Parolini et al. 2008), respectively. However, these new protocols are not completely free from the potential risks of exposure to unidentified zoonoses as human feeder cells are routinely grown in FBS-supplemented medium and the serum replacement contains undefined animal-derived products (Rajala et al. 2010). Furthermore, it has been identified that serum replacement could not support the proliferation of PMSC in our previous studies (data not shown). To avoid these risks, we replaced FBS with hFF and showed that hFF can support cell proliferative activity over 16 passages, without any noticeable changes in their general morphology, karyotype,

proliferation rate, gene expressions, and differentiation potencies. Therefore, hFF can be used as an appropriate replacement for animal products for the establishment of stem cells for clinical applications.

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