Serum and xeno-free, chemically defined, no-plate-coating-based culture system for mesenchymal stromal cells from the umbilical cord

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Abstract

Objectives: Umbilical cord mesenchymal stromal cells (UCMSCs) can be considered to become a new gold standard for MSC-based therapies. A serum and xeno-free, chemically defined and no-plate-coating-based culture system will greatly facilitate development of robust, clinically acceptable bioprocesses for reproducibly generating quality-assured UCMSCs.

Materials and methods: In this study, we report for the first time, such a serum-free, xeno-free, completely chemically defined and no-plate-coating-based culture system for the isolation and expansion of UCMSCs, whose biological characteristics were evaluated and compared with serum-containing medium (SCM) methods.

Results: This culture system not only supported UCMSC primary cultures but also allowed for their expansion at low seeding density. Compared to SCM, UCMSCs in SFM exhibited (i) higher proliferative and colony-forming capacities; (ii) distinctly different morphologies; (iii) similar phenotype; (iv) similar pluripotency-associated marker expression; (v) superior osteogenic, but reduced adipogenic differentiation capacities. In addition, UCMSCs cultured in SFM retained similar immunomodulatory properties to those in SCM.

Conclusions: Our findings demonstrate the feasibility of isolating and expanding UCMSCs in a completely serum-free, xeno-free, chemically defined and no-plate-coating-based culture system and represent an important step forward for development of robust, clinically acceptable bioprocesses for UCMSCs. Further, this provides a superior study platform for UCMSCs biology in a controlled environment.

1 | INTRODUCTION

Due to their immunomodulatory and tissue repair properties, mesenchymal stromal cells (MSCs) have been led to clinical exploitation in numerous clinical trials.^{1,2} MSCs could be isolated from a variety of sources, but source-dependent differences in MSCs properties have recently emerged and lead to different clinical applications.³ Umbilical cord (UC), which is considered clinical waste, is the most stable and readily available source of MSCs. Moreover, umbilical cord mesenchymal stem cells (UCMSCs) have also shown more primitive, proliferative

and immunosuppressive than their adult counterparts.⁴ Therefore, UCMSCs are considered as the new gold standard for MSC-based therapies.

Ex vivo cell culture is mandatory for clinical applications of MSCs, and variables include medium formulation (basal media and supplements), culture surface substrate, cell seeding density and physiochemical environment, along with subculture protocols. Medium is arguably the most critical factors. Classical culture media is supplemented with foetal bovine serum (FBS), which presents a potential risk of infection and immunological reaction.⁵ To reduce these risks, efforts are devoted towards the development of human supplements, including human serum⁶ and platelet lysate.⁷ However, the usage of

TABLE 1 The composition of chemically defined serum-free medium for UCMSCs

Components	Company	Concentration
Iscove's modified Dulbecco's medium	Life Technologies	17.7 g/L
L-glutamine	Life Technologies	5 mM
Sodium bicarbonate	Life Technologies	3.024 g/L
Recombinant human insulin	Sigma	10 mg/L
Recombinant human transferrin	Sigma	10 mg/L
Recombinant human serum albumin	Sigma	4 g/L
β -mercaptoethanol	Life Technologies	55 μ M
Chemically defined lipid concentrate	Life Technologies	0.1%
MEM essential amino acids solution	Life Technologies	2%
MEM non-essential amino acid solution	Life Technologies	1%
Vitamins solution	Life Technologies	1%
Trace elements solution	Corning cellgro	0.1%
Hydrocortisone	Sigma	50 μ g/L
L-ascorbic acid-2-phosphate	Sigma	50 mg/L
Recombinant human fibronectin	Sigma	5 mg/L
Progesterone	Sigma	5 μ g/L
Putrescine	Sigma	10 mg/L
Serotonin	Sigma	2 mg/L
Recombinant human epidermal growth factor	R&D	10 ng/mL
Recombinant human basic fibroblast growth factor	R&D	10 ng/mL
Recombinant human platelet-derived growth factor	R&D	10 ng/mL
Recombinant human insulin-like growth factor	R&D	10 ng/mL

human-sourced supplements is rather controversial, mainly because of its lack of availability and the possibility of disease transmission between donor and patient (unless autologous sources are used). Moreover, the poorly defined nature of human-sourced alternatives could cause inconsistencies in the growth-supporting properties of media and thus make standardization of a cell production process difficult. It is obvious that an ideal scenario would be the exclusion of these ill-defined supplements.⁸ Currently, some commercial defined media for isolation and expansion of bone marrow-derived MSCs (BMMSCs) can be obtained in the market.⁹ Moreover, a study has also demonstrated that a defined embryonic stem cell expansion medium, mTeSR (Stem Cell Technologies), can be used as an alternative to FBS for BMMSCs expansion.¹⁰ Because the composition of these commercial defined media is confidential, it is unclear whether is completely chemically defined or not. Moreover, these defined serum-free and xeno-free media do not appear to be good in supporting clinical culture of BMMSCs because of suboptimal performance.¹¹ Previous

study has showed that MSCs could not be isolated and expanded in StemPro MSC SFM medium, mTeSR or TheraPEAK™ MSCGM-CDTM alone, and the addition of human AB serum is needed for the initial isolation.^{8,12} Moreover, high cell seeding densities are needed for MSCs subculture, and lesser density could slow cell proliferation.^{8,13} Some studies have also reported that the isolation, expansion, phenotype and differentiation potential of MSCs are not well supported in MesenCult-XF.^{11,14} Although several studies have reported that UCMSCs could be successfully isolated and ex vivo expanded using serum-/xeno-free culture media (MesenCult-XF Medium and StemPro XF, SF media),^{15,16} these media do not support the growth of primary UCMSCs cultures in our laboratory.

Recently, several describing defined media and substrate-coating materials for the optimize BMMSC expansion have been published.^{17–19} However, these defined media do not support the growth of primary UCMSCs cultures in our previous work. Moreover, some components of these media are not yet completely xeno-free or extracted from human tissue. All animal- and/or human-derived products should ideally be excluded and synthetic recombinant alternatives used instead.²⁰ In addition, for the expansion of MSCs in defined media, a defined substrate component is also required. All current chemically defined media, to our knowledge, must be used in combination with MSCs attachment solution for the attachment and spreading of cells. It has been demonstrated that MSCs may not divide unless an effective substratum is in place,²¹ but the coating procedure increases workload and costs.

The development of well-formulated culture media for both the isolation and expansion of MSCs is imperative, but has been recognized as an extremely difficult process due to the high complexity of media formulations. It is clear that defined media optimized for MSCs isolation and expansion would greatly facilitate the development of robust, clinically acceptable bioprocesses for reproducibly generating quality-assured cells.²⁰ An ideal MSCs media should meet the following criteria:

1. Serum-free
2. Xeno-free components, and recombinant human origin will be preferable
3. Chemically defined constituents
4. Support the primary cultures of MSCs
5. Support the growth of MSCs passaged cultures at a low seeding density
6. Support the attachment of MSCs without coating
7. Media's performance is similar to or better than FBS-containing medium

In our laboratory, we evaluated a series of basal medium and supplements (including growth factors, attachment factors, binding proteins, hormones and vitamins) based on a Design of Experiment (DoE) strategy and developed a serum-free, xeno-free, completely chemically defined, and no plate-coating-based culture system for the isolation and expansion of UCMSCs. In this study, we systematically analyse the cell morphology, colony-forming unit-fibroblast (CFU-F) efficiency, phenotype, proliferation, pluripotency-associated marker

expression and differentiation capacity in vitro comparison with a classical serum-containing medium (SCM). Morphology, phenotype and differentiation potential have been proposed as minimal criteria to characterize human MSCs, and proliferation capacity is essential for successful commercial translational research to become a viable option for clinical applications.¹ Additionally, increasing evidence supports the notion that the mechanism of functional benefit after MSCs implantation is predominantly dependent on immunomodulatory functions,²² so immunomodulatory activity of UCMSCs in SFM is also assessed.

2 | MATERIALS AND METHODS

2.1 | Media preparation

The chemically defined SFM consisted of basal medium supplemented with xeno-free defined components (Table 1). A classical SCM, consisting of basal medium supplemented with 10% FBS, was used as a control medium. The media were stored at 4°C and used within 4 weeks of production.

2.2 | Sample collection

Human UC tissues (n=7) were collected after full-term deliveries with informed consent of the mothers (age range: 23–31 years, mean: 26 years). Segments from 5 to 10 cm were sectioned and conserved at room temperature into sterile phosphate-buffered saline (PBS) containing 100 U/mL penicillin/100 µg/mL streptomycin (Life Technologies, Rockville, MD, USA), at 4°C for 6–24 hours before tissue processing. Ethical approval was obtained from the Institutional Ethics Committee of Beijing Friendship Hospital.

2.3 | Isolation of umbilical cord-derived mesenchymal stromal cells (UCMSCs)

Each UC was transferred into a sterile laminar flow hood and washed twice in PBS to remove contaminating blood cells. Umbilical arteries and vein were removed, and the remaining tissue was diced into 1–2 mm³ fragments. An enzyme cocktail (hyaluronidase 5 U/mL, collagenase 125 U/mL and dispase 50 U/mL; Sigma, St. Louis, MO, USA) was used to digest the fragments for 60 minutes with gentle agitation at 37°C. The total nucleated cells (TNCs) were plated in 75 cm² flasks (Fisher Scientific, PA, USA) at 37°C, 5% CO₂ for 5 days. After removing non-adherent cells, the adherent cells were passaged when reached 80%–90% confluence.

Cells were fixed using 95% ethanol for 30 minutes, followed by staining with 0.5% crystal violet in ethanol and then washed three times with water before imaging.

2.4 | Proliferation studies

TNCs were cultured in 75 cm² flasks at density of 2×10^5 , 5×10^4 or 1×10^4 cells/cm², and UCMSCs at passage 1 at density of 3.0×10^3 ,

5.0×10^3 or 8.0×10^3 cells/cm² with the two different culture conditions (SCM and SFM). The time was measured when cells reached 80%–90% confluence.

To compare the proliferative potential, UCMSCs in SFM and SCM were serially passaged when reached a confluence of 80%–90%. The mean PD was determined for each passage using the following formula:

$$PD = \frac{[\log_{10}(N_h) - \log_{10}(N_p)]}{\log_{10}(2)}$$

N_h : the collected cell number; N_p : the plated cell number. CPD: the PD for each passage was added to the PD of the previous passages.

2.5 | CFU-F assay

To evaluate the self-renewal capacity of UCMSCs, UCMSCs were seeded in 60 cm² dishes (500, 250, 100 or 50 cells per dish) in SFM and SCM. An additional medium was added after a week. After 14 days, cells were washed with PBS and then fixed with methanol for 15 minutes. The CFU-F were stained with 0.5% crystal violet for 10 minutes and then scored under an optical microscope. Colonies were considered as clusters of more than 50 cells.

2.6 | Flow cytometry analysis

For the analysis of surface antigen expression by flow cytometry, UCMSCs were rinsed twice with PBS and then incubated with a blocking solution (3% FBS in PBS) on ice for 30 minutes. After centrifugation, the supernatant was removed, and then the cells were suspended in the blocking solution at a concentration of 500 000 cells/100 µL. The cells were aliquoted into multiple 15 mL tubes at 100 µL/tube and then stained with antibodies against human CD14, CD19, CD44, CD90, CD105, HLA-DR (SeroTec, Raleigh, NC, USA), CD29 (Beckman-Coulter, Fullerton, CA, USA), CD166 (Fitzgerald, Acton, MA, USA) and CD13, CD34, CD45, CD73 and HLA-ABC (BD Biosciences, Franklin Lakes, NJ, USA). After incubation on ice in the dark for 30 minutes, the cells were washed three times with PBS and then suspended in blocking solution to be analysed. The flow cytometry analysis was performed using the FACSCalibur, and data were analysed using CellQuest Pro software.

2.7 | Multilineage differentiation and staining assay

Adipogenic and osteogenic differentiation and subsequent histochemical staining were performed as previously described.²³ Briefly, osteogenic differentiation was examined by Alizarin Red staining after cells were cultured in the osteogenic differentiation medium containing DMEM-LG, 10% FBS, 0.1 µM dexamethasone, 0.2 µM ascorbic acid and 10 mM β-glycerolphosphat for 21 days. Adipocyte-like cells were identified by Oil Red O staining after cells were cultured in the adipogenic differentiation medium containing DMEM-LG, 1 µg/mL ascorbat-1-phosphat, 10⁻⁷ M dexamethasone and 50 µg/mL indomethacin for 21 days.

2.8 | Reverse transcribed-polymerase chain reaction (PCR) analysis and real-time PCR detection

Reverse transcribed-PCR was performed to confirm the expression of transcription factors. RNA was isolated using the TRIzol total RNA isolation reagent (Life Technologies). The extracted RNA (1 µg) was reverse transcribed by adding 5 mM of random hexamer oligonucleotides, 200 U of SuperScript reverse transcriptase, 0.5 mM deoxyribonucleotide triphosphates and 10 mM dithiothreitol. The cDNA was then amplified by PCR using primer sequences shown in Table S1. After PCR, 10 µL of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining.

Real-time PCR was performed to detect the gene expression indicative of cell differentiation. For quantitative PCR, primers were used in Table S1. Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) was used as the Master-mix. PCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with fast cycling parameters of 50°C for 2 minutes, 95°C for 2 minutes, and then 95°C for 3 seconds and 60°C for 30 seconds for a total of 40 cycles. Results were analysed using the 2-ΔCt method relating gene expression to β-actin.

2.9 | Immune plasticity assay

UCMSCs were primed by IFN-γ with or without TNF-α induction. The immunomodulatory factor expression was analysed.²⁴ Briefly, UCMSCs cultured in SFM and SCM were treated with IFN-γ or TNF-α at 15 ng/mL for 48 hours. Conditioned medium was collected for the evaluation of indoleamine 2,3-dioxygenase (IDO) activity and prostaglandin E2 (PGE₂) and transforming growth factor beta (TGF-β) quantification. PGE₂ and TGF-β concentrations were quantified using ELISA (R&D, Minneapolis, MI), according to the manufacturer's instructions. IDO activity was evaluated by kynurenine level as previously described.⁷ Kynurenine levels are directly proportional to IDO activity.

2.10 | Mixed lymphocyte culture assays

Allogeneic human peripheral blood mononuclear cells (PBMC) were prepared by centrifugation on a Ficoll-Hypaque density gradient, and 4 × 10⁴ cells were co-cultured with 1 × 10⁴ UCMSCs per well in 96-well culture plate with 10 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich). After 48, 72 and 96 hours, 100 µL of cells from each well was transferred to new 96-well plates containing 10 µL Cell Counting kit-8 reagent (Dojindo, Kumamoto, Japan). The absorbance at 450 nm was measured with a Model 450 microplate reader. All experiments were performed in triplicate and were repeated at least twice.

2.11 | Statistical analysis

All data were reported as the mean ± SD of at least three experiments. Data were compared using Student's *t* test. A probability value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Morphology of UCMSCs

The adherent cells in SFM and SCM both exhibited a fibroblast-like (elongated spindle) morphology in parallel or vortex-like patterns, but distinct morphological feature was shown. UCMSCs in SFM exhibited a smaller cell size and more elongated morphology compared with those in SCM (Fig. 1).

3.2 | Proliferation capacity of UCMSCs

In order to choose a suitable initial and subsequent plating density, TNCs and UCMSCs were incubated in SFM and SCM at low, middle or high plating density, and culture times were assessed. The result demonstrated that when cultured at low (1.0 × 10⁴ TNCs/cm²) initial plating density in SFM, TNCs showed a significantly less time than those in SCM (*P* < .05), but were unable to go beyond 60% confluence. However, no significant differences were shown at middle or high plating density between the two expansion conditions (Fig. 2a, *P* > .05). On subsequent passages, UCMSCs in SFM showed a significantly less time than those in SCM when cultured at low (3.0 × 10³ MSCs/cm²) density (*P* < .05), but no significant differences were shown at middle or high plating density between the two expansion conditions (Fig. 2b, *P* > .05). In addition, we also attempted much lower density (2.0 × 10³ MSCs/cm²), but UCMSCs grown in SFM were unable to go beyond 60% confluence.

To evaluate the long-term growth kinetics in SFM and SCM, an aliquot of UCMSCs harvested after primary culture was further propagated for five additional passages. These cultures were performed at the same low (3.0 × 10³ MSCs/cm²) density as the large-scale expansion in the same medium (SFM vs SCM). The result demonstrated that UCMSCs in SFM possessed higher CPD numbers for each passage, compared with those in SCM (Fig. 2c, all *P* < .05), indicating that UCMSCs in SFM have higher proliferative potential than those in SCM.

A CFU-F assay was used to determine whether SFM could support the clonal expansion of MSCs. After 14 days of culture at a low density, it was demonstrated that both SCM and SFM supported colony growth formation. Further, the CFU-F numbers at four seeding density were all statistically lower in SCM than in SFM (Fig. 2d, all *P* < .05). The result showed the higher colony-forming capacity in SFM compared with SCM.

3.3 | Immunophenotypic analysis of UCMSCs

Flow cytometry analysis showed that the UCMSCs in SFM and SCM exhibited a conventional MSCs surface marker profile. Both populations expressed high levels (>90% positive) of CD13, CD29, CD44, CD73, CD90, CD105, CD166 and HLA-ABC and lacked expression (<5% negative) of CD14, CD19, CD34, CD45 and HLA-DR surface molecules (Table 2). Moreover, there were no significant changes in these markers between UCMSCs cultured in SFM and SCM.

FIGURE 1 Morphology of UCMSCs. UCMSCs cultivated in SCM and SFM were assessed via phase-contrast microscopy and crystal violet staining (scale bars=200 μ m)

FIGURE 2 Proliferation and self-renewal capacity of UCMSCs. (a) TNCs culture time. TNCs were cultured in 75 cm^2 flasks at density of 2×10^5 , 5×10^4 or 1×10^4 cells/ cm^2 with the two different culture conditions (SCM and SFM); the time was measured when cells reached 80%–90% confluence (* $P < .05$, $n = 7$). (b) UCMSCs were cultured at density of 3.0×10^3 , 5.0×10^3 or 8.0×10^3 cells/ cm^2 with the two different culture conditions (SCM and SFM); the time was measured when cells reached 80%–90% confluence (* $P < .05$, $n = 7$). (c) Proliferation capacity of UCMSCs. Cumulative population doublings of UCMSCs in SFM and SCM (* $P < .05$, ** $P < .01$, $n = 7$). (d) Self-renewal capacity of UCMSCs. Colony-forming unit-fibroblast (CFU-F) number of UCMSCs in SFM and SCM at different cell number per well (* $P < .05$, $n = 7$)

3.4 | Differentiation of UCMSCs into adipocytes and osteocytes

The osteogenic differentiation of UCMSCs in SFM and SCM was observed by Alizarin Red staining of calcium deposits after 21 days of differentiation. Characteristic staining of bone-like nodules was evident after differentiation of both UCMSCs expanded in SFM and SCM (Fig. 3a). The UCMSCs expanded in SFM exhibited statistically significant increase in mRNA expression of RUNX-2 and alkaline phosphatase compared to those expanded in SCM (Fig. 3b, $P < .05$). These

data indicate that higher osteogenic differentiation potential is maintained in UCMSCs expanded in SFM.

The adipogenic differentiation of UCMSCs in SFM and SCM was observed by Oil Red O staining after 21 days of differentiation. Characteristic staining of the fatty vacuole deposits was evident after differentiation of both UCMSCs expanded in SFM and SCM (Fig. 3c). However, UCMSCs expanded in SFM exhibited a statistically significant reduction in PPAR γ and LPL expression compared with those expanded in SCM (Fig. 3d, $P < .05$). These data indicate that adipogenic differentiation potential is maintained in UCMSCs expanded in SFM,

FIGURE 3 Multilineage differentiation potential of UCMSCs. Osteogenic differentiation potential of UCMSCs was examined by Alizarin Red staining (a) and real-time PCR (b). Adipogenic differentiation potential of UCMSCs was examined by Oil Red O staining (c) and real-time PCR (d) (scale bars=200 μ m; * P <.05, n =7)

but there is a significant reduction in adipogenic capability compared with UCMSCs in SCM.

3.5 | Gene expression of UCMSCs

UCMSCs in SFM exhibited a pluripotency-associated marker Nanog expression, but not Oct4 and Sox2. This result was similar with UCMSCs cultured in SCM (Fig. 4).

3.6 | Immune regulatory properties of UCMSCs

UCMSCs cultured in SFM exhibited lower PGE₂ expression (P <.05, Fig. 5a) and IDO activity (P <.05, Fig. 5b) compared with those in SCM. When UCMSCs were primed by IFN- γ and (or) TNF- α induction, immunomodulatory factor levels of PGE₂, TGF- β expression and IDO activity were increased in both culture conditions, and no significant differences in PGE₂, TGF- β expression (Fig. 5c) and IDO activity were observed between the two culture conditions (Fig. 5, all P >.05).

FIGURE 4 Gene expression of UCMSCs. Oct4, Nanog and Sox2 gene expression in UCMSCs was analysed by RT-PCR. RT+: reverse transcriptase added; RT-: reverse transcriptase not added

FIGURE 5 Immunomodulatory activity of UCMSCs. UCMSCs were primed by IFN-γ with or without TNF-α induction. The immunomodulatory factors PGE₂ and TGF-β expression and IDO activity were analysed (a, b and c). IDO activity was evaluated by kynurenine levels. UCMSCs suppress allogeneic lymphocyte proliferation (d) (**P*<.05, ***P*<.01, #*P*>.05; *n*=7)

TABLE 2 Surface antigen expression levels of UCMSCs expanded in SFM and SCM

Surface antigen	Expression level (%)	
	SFM	SCM
CD13	92.86 ± 1.54	92.05 ± 1.09
CD29	96.87 ± 1.32	95.97 ± 1.04
CD44	93.74 ± 1.38	93.62 ± 1.48
CD73	94.95 ± 0.94	94.71 ± 0.18
CD90	95.38 ± 0.62	95.31 ± 0.82
CD105	98.48 ± 1.13	97.03 ± 2.65
CD166	92.97 ± 1.02	93.04 ± 0.83
HLA-ABC	91.34 ± 1.51	92.94 ± 3.51
CD14	4.23 ± 0.17	4.58 ± 0.20
CD19	3.16 ± 0.17	3.60 ± 0.48
CD34	1.71 ± 0.60	1.59 ± 1.46
CD45	2.50 ± 1.54	2.41 ± 1.25
HLA-DR	1.26 ± 0.07	1.91 ± 1.35

The results demonstrated that UCMSCs in SFM and SCM were able to inhibit PHA-stimulated PBMC proliferation. Notably, UCMSCs in SFM had a similar inhibitory effect on PBMC proliferation with those in SCM (*P*>.05; Fig. 5d).

4 | DISCUSSION

In our previous work, we failed to isolate and expand UCMSCs in describing defined media published in other laboratories and several commercial defined media. Therefore, we developed a serum and xeno-free, chemically defined and no plate-coating-based culture

system for the isolation and expansion of UCMSCs based on a DoE strategy. Previously, we used DMEM/F12, DMEM and α-MEM as basic medium, and more numbers of cells were harvested than when IMDM was used for SFM. MSCs must be adherent to plastic under standard tissue culture conditions, which is a well-described property defined by the International Society for Cellular Therapy (ISCT).²⁵ SCM can support the attachment of MSCs due to the presence of important cell adhesion-promoting proteins, such as fibronectin. To maximize cell adhesion in the absence of serum, a crucial step before seeding MSCs is pre-coated with fibronectin under a defined culture conditions. Fibronectin, a defined xeno-free attachment substrate, has been widely used prior to seeding the cells to provide a favourable environment for the attachment of MSCs.⁵ Taking into consideration the inconvenience of plate-coating, fibronectin was added to the cell culture medium, which could omit plate-coating step. The result showed that the effect of cell adhesion was completely acceptable although it was a little delayed compared with coating culture system, and the morphology and proliferation ability were also similar (Fig. S1). These results demonstrate that our culture system can replace the plate-coating-based culture system for the efficient expansion of UCMSCs in defined conditions. It can reduce risk of contamination and save cost and labour. This media was a true chemically defined medium by replacing serum components with synthetic alternatives without any decrease in the performance of chemically defined medium for UCMSCs. Moreover, this media supported the attachment of MSCs without coating. To our knowledge, this is the first report of a true chemically defined and no plate-coating-based medium for the expansion of MSCs.

Using this SFM, we were able to successfully isolate MSCs from UC (7 of 7), meaning that the isolating frequency by this culture system was 100%. Plating density has emerged as a critical issue for MSCs expansion. Due to the adherent nature of MSCs, plating

density is an important variable to ensure a good expansion rate and to maintain necessary cellular functions. Previous study had reported that the lower the number of cells plated, the more vigorous was the proliferation of the culture.²⁶ Growth at a high seeding density is constrained by density-dependent growth inhibition. Lower seeding densities are probably better for MSCs expansion because of less contact inhibition. Indeed, cultures seeded at a lower seeding density result in lower frequency of interruption by PBS washing, trypsinization and centrifugation during sequential expansion, which enhances the proliferation rates. However, very low seeding densities may not reach confluence. Therefore, 5.0×10^4 TNCs/cm² and 3.0×10^3 MSCs/cm² were recommended as a suitable seeding density. This SFM not only support UCMSCs primary cultures but also allow expansion at a low seeding density. Moreover, a CPD analysis showed that UCMSCs in SFM had higher proliferative potential than those in SCM. The CFU assay is a suitable tool for evaluating the self-renewal capacity of cells.¹ The ability to form distinct colonies from single cells when they are plated at very low densities is one accepted characteristic of adherent MSCs in culture.²⁷ Our result showed the higher self-renewal capacity in SFM compared with SCM. A possible explanation might be that several rich growth factors (fibroblast growth factor [FGF], epidermal growth factor [EGF], insulin growth factor-I [IGF-I] and platelet-derived growth factor [PDGF]) in SFM can stimulate proliferative and self-renewal.²⁸ Previous study had reported that serum-reduced medium with growth factors (EGF, FGF and PDGF) or platelet lysate rich in growth factors revealed moderated proliferation and self-renewal.^{29,30}

The fibroblast-like morphology, phenotype and multilineage differentiation capacity have been proposed as minimal criteria to characterize human MSCs by the ISCT.²⁵ In this study, we analysed all these parameters, and the results showed UCMSCs in SFM fulfilled the definition, but displayed some differences. UCMSCs cultured in this culture system exhibited a thinner and smaller morphology and size compared with those in SCM. This may be related to the several rich growth factors in SFM. Similar results were shown in serum-reduced medium³¹ or platelet lysate.³² UCMSCs cultured in SFM exhibited high expression (>90% positive) of CD105, CD73 and CD90 and lack expression (<5% negative) of CD45, CD34, CD14, CD19 and HLA class II, which was similar with those in SCM. However, this result is lower than the minimal criteria proposed by the ISCT (>95% positive, <2% negative). A possible explanation might be caused by different tissue sources, but independent of the culture conditions. Similar results were also reported in UCMSCs cultured in other serum-/xeno-free culture media.¹⁶ In addition, we further used additional surface markers, and the results also showed that UCMSCs cultured in two culture systems exhibited a similar high expression (>90% positive) of CD13, CD29, CD44, CD166 and HLA-ABC. UCMSCs in SFM exhibited superior osteogenic, but reduced adipogenic differentiation capacity compared with SCM. We hypothesize that these differences on differentiation ability were due to high concentration of PDGF and fibroblast growth factor FGF in SFM. The PDGF and FGF signalling have been shown to be important in MSCs differentiation into the osteogenic lineages.³³ Osteogenic differentiation also appears to be

increased when the FGF signalling pathway of MSCs is activated.^{34,35} Previous study had reported that expression levels of IGF-1 system, including its critical mediator insulin receptor substrate-1, modulated the osteoblastic differentiation of MSCs.³⁶ A reduced adipogenic differentiation might be due to preferential selection of osteogenic precursors in SFM.

Transcription factors including Oct4, Sox2 and Nanog, known to regulate the stem cell properties of embryonic stem cell, have been proposed to regulate the maintenance of the pluripotent state in MSCs.³⁷ We performed a critical evaluation of expression of these three transcription factors and found that Nanog, but not Oct-4 and Sox-2, was expressed in UCMSCs independently from the in vitro culture conditions. This result is in agreement with previous reports,³⁸ but in contrast with data from other laboratory.³⁷

MSCs have been shown to possess immunomodulatory properties.³⁹ Increasing evidence supports the notion that the mechanism of functional benefit after MSCs implantation is predominantly dependent on immunomodulatory functions.^{22,40} Thus, the MSC committee of the ISCT released a position statement paper in 2013 that proposed immunological characterization of MSCs and a perspective in 2015 on immune functional assays for MSCs as potency release criterion for advanced phase clinical trials at the 21st annual meeting.^{41,42} The nature of the immunomodulatory effect of MSCs depends on local immunological conditions, where in particular IFN- γ and TNF- α . In vitro MSC inflammatory "licensing" better recapitulates what likely happens in vivo once MSCs are transfused into patients with dysregulated immune responses or with systemic inflammation.⁴³ Thus, ISCT has suggested that standard immune plasticity assay be based on IFN- γ with or without TNF- α used as a model in vitro priming agent.^{41,42} The immunomodulatory effect of MSCs is for a large extent mediated *via* soluble factors.⁴⁴ Several factors including TGF- β , IDO and PGE₂ have been proposed to play a crucial role in the immunomodulatory effect of MSCs.⁴⁵ Our comparative study showed no significant difference in immunomodulatory factors expression level of PGE₂, TGF- β and IDO between the two culture conditions when UCMSCs were primed by IFN- γ and (or) TNF- α induction, indicating UCMSCs cultured in SFM retain the similar immunomodulatory properties with those in SCM. The proliferation induced by growth factors in SFM did not result in loss of immunosuppressive activity. This result is consistent with other serum-free conditions,⁴⁶ serum-reduced medium³¹ or platelet lysate.⁴⁷

In conclusion, we develop for the first time, in the literature to date, a serum and xeno-free, chemically defined and no plate-coating-based culture system for the isolation and expansion of UCMSCs. Our results suggest that this serum and xeno-free, chemically defined and no plate-coating-based culture system might be a suitable growth medium for culture of UCMSCs for use in clinical transplantation, but further safety (including karyotype, tumorigenicity and genomic stability) and additional function (including cytokine secretion profile, migratory ability and regulation of hematopoiesis) studies are also needed to determine the feasibility of entering into the clinical arena. Further, this provides a superior study platform for UCMSCs biology in a controlled environment.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

ABBREVIATIONS

BM, bone marrow; CFU-F, colony-forming unit-fibroblast; FBS, foetal bovine serum; IDO, indoleamine 2,3-dioxygenase; ISCT, International Society for Cellular Therapy; MSCs, mesenchymal stromal cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGE₂, prostaglandin E₂; SCM, serum-containing medium; SFM, serum-free medium; UCMSCs, umbilical cord mesenchymal stem cells; UC, umbilical cord.

REFERENCES

- Sharma RR, Pollock K, Hubel A, McKenna D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion*. 2014;54:1418–1437.
- Squillaro T, Peluso G, Galderisi U. Clinical trials with mesenchymal stem cells: an update. *Cell Transplant*. 2016;25:829–848.
- Bortolotti F, Ukovich L, Razban V, et al. In vivo therapeutic potential of mesenchymal stromal cells depends on the source and the isolation procedure. *Stem cell reports*. 2015;4:332–339.
- El Omar R, Beroud J, Stoltz JF, et al. Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? *Tissue Eng Part B Rev*. 2014;20:523–544.
- van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, et al. Optimization of chemically defined cell culture media—replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro*. 2010;24:1053–1063.
- Poloni A, Maurizi G, Serrani F, et al. Human AB serum for generation of mesenchymal stem cells from human chorionic villi: comparison with other source and other media including platelet lysate. *Cell Prolif*. 2012;45:66–75.
- Li CY, Wu XY, Tong JB, et al. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. *Stem Cell Res Ther*. 2015;6:55.
- Jung S, Panchalingam KM, Rosenberg L, Behie LA. Ex vivo expansion of human mesenchymal stem cells in defined serum-free media. *Stem Cells Int*. 2012;2012:123030.
- Gottipamula S, Muttigi MS, Kolkundkar U, Seetharam RN. Serum-free media for the production of human mesenchymal stromal cells: a review. *Cell Prolif*. 2013;46:608–627.
- Hudson JE, Mills RJ, Frith JE, et al. A defined medium and substrate for expansion of human mesenchymal stromal cell progenitors that enriches for osteo- and chondrogenic precursors. *Stem Cells Dev*. 2011;20:77–87.
- Al-Saqi SH, Saliem M, Asikainen S, et al. Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells. *Cytotherapy*. 2014;16:915–926.
- Chase LG, Yang S, Zachar V, et al. Development and characterization of a clinically compliant xeno-free culture medium in good manufacturing practice for human multipotent mesenchymal stem cells. *Stem Cells Transl Med*. 2012;1:750–758.
- Badraiq H, Devito L, Ilic D. Isolation and expansion of mesenchymal stromal/stem cells from umbilical cord under chemically defined conditions. *Methods Mol Biol*. 2015;1283:65–71.
- Pal R, Hanwate M, Jan M, Totev S. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J Tissue Eng Regen Med*. 2009;3:163–174.
- Swamynathan P, Venugopal P, Kannan S, et al. Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton's jelly derived mesenchymal stem cells? A comparative study. *Stem Cell Res Ther*. 2014;5:88.
- Simoes IN, Boura JS, dos Santos F, et al. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. *Biotechnol J*. 2013;8:448–458.
- Mimura S, Kimura N, Hirata M, et al. Growth factor-defined culture medium for human mesenchymal stem cells. *Int J Dev Biol*. 2011;55:181–187.
- Jung S, Sen A, Rosenberg L, Behie LA. Human mesenchymal stem cell culture: rapid and efficient isolation and expansion in a defined serum-free medium. *J Tissue Eng Regen Med*. 2012;6:391–403.
- Felka T, Schafer R, De Zwart P, Aicher WK. Animal serum-free expansion and differentiation of human mesenchymal stromal cells. *Cytotherapy*. 2010;12:143–153.
- Dimarakis I, Levicar N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. *Stem Cells*. 2006;24:1407–1408.
- Dolley-Sonneville PJ, Romeo LE, Melkounian ZK. Synthetic surface for expansion of human mesenchymal stem cells in xeno-free, chemically defined culture conditions. *PLoS ONE*. 2013;8:e70263.
- Menard C, Tarte K. Immunoregulatory properties of clinical grade mesenchymal stromal cells: evidence, uncertainties, and clinical application. *Stem Cell Res Ther*. 2013;4:64.
- Wu X, Liu X, Wang S. Implantation of biomaterial as an efficient method to harvest mesenchymal stem cells. *Exp Biol Med (Maywood)*. 2011;236:1477–1484.
- Liu XD, Liu D, Zang CB, et al. IFN-gamma stimulation enhances immunosuppressive capability of human umbilical cord mesenchymal stem cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2014;22:605–611.
- Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315–317.
- Nekanti U, Mohanty L, Venugopal P, et al. Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell Res*. 2010;5:244–254.
- Ylostalo J, Bazhanov N, Prockop DJ. Reversible commitment to differentiation by human multipotent stromal cells in single-cell-derived colonies. *Exp Hematol*. 2008;36:1390–1402.
- Somers P, Cornelissen R, Thierens H, Van Nooten G. An optimized growth factor cocktail for ovine mesenchymal stem cells. *Growth Factors*. 2012;30:37–48.
- Bernardi M, Albiero E, Alghisi A, et al. Production of human platelet lysate by use of ultrasound for ex vivo expansion of human bone marrow-derived mesenchymal stromal cells. *Cytotherapy*. 2013;15:920–929.
- Montzka K, Fuhrmann T, Woltje M, Brook GA. Expansion of human bone marrow-derived mesenchymal stromal cells: serum-reduced medium is better than conventional medium. *Cytotherapy*. 2010;12:587–592.
- Auletta JJ, Zale EA, Welter JF, Solchaga LA. Fibroblast growth factor-2 enhances expansion of human bone marrow-derived mesenchymal stromal cells without diminishing their immunosuppressive potential. *Stem Cells Int*. 2011;2011:235176.

32. Schallmoser K, Bartmann C, Rohde E, et al. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion*. 2007;47:1436–1446.
33. Ng F, Boucher S, Koh S, et al. PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood*. 2008;112:295–307.
34. Kawazoe Y, Katoh S, Onodera Y, et al. Activation of the FGF signaling pathway and subsequent induction of mesenchymal stem cell differentiation by inorganic polyphosphate. *Int J Biol Sci*. 2008;4:37–47.
35. Osathanon T, Nowwarote N, Manokawinchoke J, Pavasant P. bFGF and JAGGED1 regulate alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells. *J Cell Biochem*. 2013;114:2551–2561.
36. Contaldo C, Myers TJ, Zucchini C, et al. Expression levels of insulin receptor substrate-1 modulate the osteoblastic differentiation of mesenchymal stem cells and osteosarcoma cells. *Growth Factors*. 2014;32:41–52.
37. Riekstina U, Cakstina I, Parfejevs V, et al. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Rev*. 2009;5:378–386.
38. Pierantozzi E, Gava B, Manini I, et al. Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem Cells Dev*. 2011;20:915–923.
39. Wang Y, Chen X, Cao W, Shi Y. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol*. 2014;15:1009–1016.
40. Meirelles LdaS, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev*. 2009;20:419–427.
41. Krampera M, Galipeau J, Shi Y, et al. Immunological characterization of multipotent mesenchymal stromal cells—The International Society for Cellular Therapy (ISCT) working proposal. *Cytotherapy*. 2013;15:1054–1061.
42. Galipeau J, Krampera M, Barrett J, et al. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. *Cytotherapy*. 2016;18:151–159.
43. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett*. 2007;110:91–100.
44. English K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol*. 2013;91:19–26.
45. Ben-Ami E, Berrih-Aknin S, Miller A. Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases. *Autoimmun Rev*. 2011;10:410–415.
46. Gottipamula S, Muttigi MS, Chaansa S, et al. Large-scale expansion of pre-isolated bone marrow mesenchymal stromal cells in serum-free conditions. *J Tissue Eng Regen Med*. 2016;10:108–119.
47. Capelli C, Domenghini M, Borleri G, et al. Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from small samples of bone marrow aspirates or marrow filter wash-outs. *Bone Marrow Transplant*. 2007;40:785–791.

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