Embryonic stem cells conditioned medium enhances Wharton's jelly-derived mesenchymal stem cells expansion under hypoxic condition

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Received: 19 March 2013 / Accepted: 19 February 2014 / Published online: 19 October 2014
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Abstract Mesenchymal stem cells (MSCs) are accepted as a promising tool for therapeutic purposes. However, low proliferation and early senescence are still main obstacles of MSCs expansion for using as cell-based therapy. Thus, clinical scale of cell expansion is needed to obtain a large number of cells serving for further applications. In this study, we investigated the value of embryonic stem cells conditioned medium (ESCM) for in vitro expansion of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) as compared to typical culture medium for MSCs, Dulbecco's modified Eagle's medium with 1.0 g/l glucose

(DMEM-LG) supplemented with 10 % FBS, under hypoxic condition. The expanded cells from ESCM (ESCM-MSCs) and DMEM-LG (DMEM-MSCs) were characterized for both phenotype and biological activities including proliferation rate, population doubling time, cell cycle distribution and MSCs characteristics. ESCM and DMEM-LG could enhance WJ-MSCs proliferation as 204.66 ± 10.39 and 113.77 ± 7.89 fold increase at day 12, respectively. ESCM-MSCs could express pluripotency genes including *Oct-4*, *Oct-3/4*, *Nanog*, *Klf-4*, *C-Myc* and *Sox-2* both in early and late passages whereas the downregulations of *Oct-4* and *Nanog* were detected in late passage cells of DMEM-MSCs. The 2 cell populations also showed common MSCs characteristics including normal cell cycle, fibroblastic morphology, cell surface markers expressions ($CD29^+$, $CD44^+$, $CD90^+$, $CD34^-$, $CD45^-$) and differentiation capacities into adipogenic, chondrogenic and osteogenic lineages. Moreover, our results revealed that ESCM exhibited as a rich source of several factors which are required for supportive WJ-MSCs proliferation. In conclusion, ESCM under hypoxic condition could accelerate WJ-MSCs expansion while maintaining their pluripotency properties. Our knowledge provide short term and cost-saving in WJ-MSCs expansion which has benefit to overcome insufficient cell numbers for clinical applications by reusing the discarded cell culture supernates from human ES culture system. Moreover, these findings can also apply for stem cell banking, regenerative medicine and pharmacological applications.

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Keywords Wharton's jelly-derived mesenchymal stem cells · Embryonic stem cells conditioned medium · Hypoxic condition · Cell proliferation

Introduction

Mesenchymal stem cells (MSCs) are first isolated from bone marrow (BM (Friedenstein et al. 1970) and can be isolated from other tissues such as adipose tissue, fetal tissues, amniotic fluid, placenta and umbilical cord (Zuk et al. 2002; In't Anker et al. 2003, 2004; Troyer and Weiss 2008). Currently, the International Society for Cellular Therapy (ISCT) has defined MSCs as plastic adherent cells with a fibroblast-like morphology, positive staining for MSCs surface markers such as CD29, CD44, CD90 and CD105 and capable of differentiation into mesodermal-lineages including adipocytes, osteoblasts and chondroblasts (Dominici et al. 2006). Mostly, BM is a main source of MSCs for research and clinical applications because they can be extensively expanded in vitro and have a broad differentiation capacity into many cell types. However, numbers of isolated cells from BM are rather low and their differentiation capacity depends on the age of donor. Therefore, umbilical cord Wharton's jelly has revealed as an attractive source of MSCs based on their advantages including their primitive origin, non-controversial source, easy collection and enrichment with MSCs. Several studies found that Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) have MSCs characteristics common to those derived from other sources including self-renewal, multi-lineages differentiation capacity and immunomodulatory properties (Witkowska-Zimny and Wrobel 2011). In addition, WJ-MSCs could express the pluripotent embryonic stem cell markers such as *POU1*, *NANOG*, *SOX2* and *LIN28* at low level (Fong et al. 2011), which make WJ-MSCs becoming a good candidate for clinical applications. However, an in vitro expansion of the cells is still needed to obtain sufficient cell numbers for therapeutic purposes. Regarding an in vitro cultivation, MSCs are typically cultured under normal atmosphere of 21 % O₂ tension. However, there are evidences that hypoxic condition (1–5 % O₂) is more suitable for MSCs expansion than normoxic condition. Recent studies have reported that hypoxia or low oxygen tension could increase the proliferation potential while

maintaining multi-lineages differentiation capacity of the cells not only WJ-MSCs but also bone marrow-derived MSCs (BM-MSCs) (Nekanti et al. 2010a, b; Tsai et al. 2012). Moreover, hypoxia has been shown to preserve pluripotency properties of human ES (Ezashi et al. 2005). These data indicate that low oxygen tension plays an important role in regulating both proliferation and stemness properties of stem cells.

In addition to oxygen tension, culture medium is one of the factors that directly affects the biological activities of the cells. Typically, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) is widely used as conventional medium for culturing MSCs. At present, serum-free medium is usually selected for clinical grade expansion of stem cells to prevent prion or xenogeneic proteins transmissions. Although 10 % human serum has shown to support cell proliferation of umbilical cord-derived MSCs under xeno-free culture condition, this medium remains including serum with serves as undesirable source of pathogen contamination (Hatlapatka et al. 2011). Human platelet lysate (hPL) has been shown to support MSCs expansion (Capelli et al. 2007; Xia et al. 2011; Ben Azouna et al. 2012). However, some controversial data have been reported including the reduction potential of MSCs differentiation into osteogenic and adipogenic lineages and decrease of immunosuppressive capacities of MSCs on T cells and NK cells after cultivation MSCs with hPL-containing medium (Gruber et al. 2004; Abdelrazik et al. 2011). Thus, defined serum-free based medium may be promising for an in vitro MSCs expansion. It has been reported that defined serum-free medium for human ES could provide a four to five fold higher proliferation rate of MSCs than those cultured in conventional medium (Battula et al. 2007). In fact, culture medium for ES will be changed every day for preserving their pluripotency properties. Thus, a lot of ESCM will be discarded every day as a waste. This is the first study that investigated whether ESCM can be reused as supportive culture medium for WJ-MSCs in vitro. This study aims to investigate the benefit of ESCM for culturing WJ-MSCs in comparison with the most commonly used basal medium for MSCs as DMEM supplemented with 10 % FBS under hypoxic condition. The expanded cells from both media were further characterized both in cellular and molecular levels including proliferation rate, population doubling times (PDT), cell morphology, cell surface markers,

mesodermal-lineages differentiation capacity, cell cycle distribution and stemness gene expressions. Moreover, we also investigated cytokine components in ESCM obtained from cell culture supernates of our routine human ES culture. The achievement of this goal may serve as an exploration of a new approach for in vitro WJ-MSCs expansion. Moreover, this finding may provide superior benefits in cell expansion such as reduced time-consuming and cost-saving by reusing the discarded cell culture supernates from human ES culture system as an expansion medium for WJ-MSCs.

Materials and methods

WJ-MSCs isolation and culture

Fresh human umbilical cords ($n = 4$) from both sexes were collected after birth by cesarean section in the Department of Obstetrics and Gynecology at Por-Pat Hospital, Nakhon Ratchasima, Thailand. Written informed consent was obtained from the mother under the approval of the Institute of Research and Development of the Suranaree University of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The umbilical cords were collected in phosphate buffer saline (PBS) supplemented with 200 U/ml penicillin (Sigma, St. Louis, MO, USA), 200 µg/ml streptomycin (Sigma) and 4 µg/ml amphotericin B (Bristol-Myers Squibb, NY, USA) prior to storage at 4 °C for further WJ-MSCs isolation. After removal of blood vessels, tissues were dissected into small pieces and then washed with an equal volume of PBS (200 U/ml penicillin (Sigma), 200 µg/ml streptomycin (Sigma) and 4 µg/ml amphotericin B (Bristol-Myers Squibb)). The suspension was centrifuged at 3,200g, 4 °C for 5 min and supernatant was discarded. The precipitate (mesenchymal tissue) was digested with collagenase type I (2 mg/ml) (Invitrogen, Carlsbad, CA, USA) at 37 °C for 16–18 h with agitation. An equal volume of Dulbecco's modified Eagle's medium with 1.0 g/l glucose (DMEM-LG) (Invitrogen) containing 50 % FBS (HyClone, Logan, UT, USA) was added and centrifuged at 3,200g, 4 °C for 10 min. Cell pellet was then resuspended, counted and cells were equally seeded into T25 cm² culture flask (Greiner Bio-One, Monroe, NC, USA) and cultured in two different media as followed 1) DMEM-LG supplemented with 10 % FBS (HyClone), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and

2 µg/ml amphotericin B (Bristol-Myers Squibb) and 2) embryonic stem cells conditioned medium (ESCM). Three to four days after seeding, non-adherent cells were discarded while adherent cells were replaced with their respective fresh medium. The isolated cells were incubated under hypoxic condition with tri-gas incubator (HEPA class 100, Thermo Scientific, MA, USA) at 37 °C, 90 % N₂, 5 % CO₂ and 5 % O₂. Initial culture was maintained for 8 days, thereafter, the cells were subpassaged every 3 days and expanded at a ratio of 1:3 until 4 weeks. Here, we named WJ-MSCs that were cultured by conventional medium and ESCM as DMEM-MSCs and ESCM-MSCs, respectively.

Proliferation and population doubling times analysis

To determine the effect of culture medium on WJ-MSCs proliferation in vitro, cells were plated at a low density of 5×10^3 cells/cm² in T25 cm² tissue culture flask and further cultured for 12 days. Cell proliferation was determined by using haemocytometer and vital Trypan blue staining. Moreover, fold increase in total cell number was calculated by comparison the number of cells at the tested day to day 0. To determine WJ-MSCs doubling time, cells were plated at a density of 5×10^3 cells/cm² in a 6-well plate (TPP) and fed with their respective growth medium as described above. Cells at passage 4 and passage 7 were subjected to PDT experiment. The DMEM-MSCs and ESCM-MSCs were counted daily at the same time for 6 days by using haemocytometer and vital Trypan blue staining. The PDT was calculated by using online formula for PDT (www.doubling-time.com) which is based on the formula: $PDT = t \lg 2 / (\lg NH - \lg NI)$ where NI is the inoculum cell number, NH is the cell harvest number and t is the time of the culture (in h).

ESCM preparation

ESCM was generated from culturing the H9 human ES cell line with fresh ES medium on human foreskin fibroblast feeders. The fresh ES medium was a serum-free medium containing Knockout-DMEM (Invitrogen) supplemented with 20 % Knockout serum replacement (Invitrogen), 1 % non-essential amino acids (Sigma), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen) and 5 ng/ml human basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ,

USA). After 24 h, the culture medium was removed and used as conditioned medium for culture of WJ-MSCs. This conditioned medium was filtrated through 0.2 µm syringe filter before used. For long term storage, ESCM was stored at −20 °C until use.

Cytokine-antibody array analysis

ESCM was collected to determine cytokine components by using the human cytokine array panel A (proteome profilerTM) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The array includes 36 human cytokines panel of C5/C5a, CD40, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α and sTREM-1. Unconditioned medium (fresh ES medium) was used as control to compare the relative expression levels of each cytokine. Data were captured by exposure to Kodak BioMax Light film. Arrays were scanned into a computer and the detected signals were quantified as the pixel density by using Image J software (Image J, 1.45, NIH).

Flow cytometry

The cultured cells were washed twice with 2 % FBS/PBS and suspended in 2 % FBS/PBS with saturating concentration of the following conjugated mouse-anti-human antibodies: CD29-phycoerythrin (PE), CD34-PE, CD44-PE and CD45-fluorescein isothiocyanate (FITC) (all from Becton–Dickinson, San Jose, CA, USA), and the unconjugated antibody: CD90 (Becton–Dickinson) for 1 h at 4 °C. Unconjugated primary antibody was treated with a goat-anti-mouse-FITC-conjugated secondary antibody (Becton–Dickinson) for 1 h at room temperature. Thereafter, cells were washed twice with 2 % FBS/PBS and fixed with 4 % paraformaldehyde in PBS. Control samples were incubated with PBS instead of primary antibody. Samples were analyzed using FACS Calibur (Becton–Dickinson) collecting 20,000 events and the data were analyzed by Cell Quest Software (Becton–Dickinson).

RNA extraction and RT-PCR

The cultured cells were subjected to total RNA extraction by using Total RNA Mini Kit (Tissue

(Geneaid, Taipei, Taiwan) and treated with RNase inhibitor (Invitrogen) according to the manufacturer's protocol. The cDNA was generated by RevertAidTM First Stand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's protocol. The corresponding cDNA was added into PCR master mix containing 10× PCR buffer, 1 U Taq polymerase, 25 mmol/l MgCl₂, 10 mmol/l dNTP mix and 10 µmol/l of each primer set for the corresponding target gene. The primer sequences are shown in Table 1. Amplification conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 55–64 °C for 30 s (see table referring to temperatures used), extension for 1 min at 72 °C and a final extension at 72 °C for 10 min. The samples were separated on a 2 % agarose gel, stained with ethidium bromide and photographed under UV light.

Mesodermal-lineages differentiation assays

The cultured cells were induced to differentiate into adipocytes, chondroblasts and osteoblasts by using STEM PRO Adipogenesis Differentiation Kit, STEM PRO Chondrogenesis Differentiation Kit and STEM PRO Osteogenesis Differentiation Kit (all from Invitrogen), respectively. The medium was changed every 3 days. For adipogenic differentiation, after induction for 4 weeks, cells were fixed in 10 % formalin and stained with fresh oil red O solution (Sigma) to detect fat deposition in the cells. For chondrogenic differentiation, after induction for 3 weeks, cells were fixed in 4 % paraformaldehyde and stained with 1 % alcian blue solution (Sigma) to detect proteoglycan synthesis. For osteogenic differentiation, to detect mineralization (calcium deposits), cells were fixed in 4 % paraformaldehyde and stained with 2 % alizarin red S solution (Sigma) after induction for 2 weeks. The differentiated cells were visualized and photographed under inverted microscope (Olympus CKX41, Olympus Corporation, Tokyo, Japan).

Cell cycle analysis

The cultured cells were washed twice with PBS and fixed with ice cold 70 % ethanol at 4 °C for a minimum of 1 h. After washing two times with PBS, the cell pellets were incubated with propidium iodide/RNase A solution (40 µg/ml propidium iodide, 100 µg/ml RNase A) in

Table 1 List of primer sequences used for RT-PCR analysis

Gene	Primer sequence (5′–3′)	Annealing temp.	Size (bp)	References
<i>Oct-4</i>	GAGCAAAACCCGGAGGAGT TTCTCTTTCGGGCCTGCAC	60	310	Yao et al. (2006)
<i>Nanog</i>	GCTTGCCTTGCTTTGAAGCA TTCTTGACTGGGACCTTGTC	60	255	Yao et al. (2006)
<i>Oct-3/4</i>	CTCACCTGGGGGTCTATT CTCCAGGTTGCCTCTCACTC	60	230	Darr et al. (2006)
<i>C-myc</i>	TGGTCTTCCCTACCCTCTCAAC GATCCAGACTCTGACCTTTTGCC	55	265	Han et al. (2007)
<i>Klf-4</i>	GTTTTGAGGAAGTGCTGAG CAGTCACAGTGGTAAGGTTT	55	332	Chiambaretta et al. (2004)
<i>Sox-2</i>	GCCCCAGCAGACTTCACA CTCCTCTTTTGACCCCTCCCATT	64	170	Tsukamoto et al. (2005)
<i>GAPDH</i>	AGCCACATCGCTCAGACACC GTACTCAGCGGCCAGCATCG	60	302	Yao et al. (2006)

the dark for 15 min at 37 °C. Samples were analyzed by flow cytometry on a FACS Calibur (Becton–Dickinson).

Statistic analysis

All data were presented as mean $\pm$ standard deviation (SD) calculation. Statistical analysis was done by statistical software SPSS17.0 (SPSS Inc., Chicago, IL, USA) and results were analyzed by Student's *t* test with significance at $P < 0.05$.

Results

Proliferation and population doubling time

To determine whether ESCM is appropriate for supporting proliferation of WJ-MSCs, the cells were cultured with ESCM in comparison to the most commonly used basal culture medium for MSCs as DMEM supplemented with 10 % FBS. Furthermore, the expanded cells were subjected to examine the morphology, proliferation and PDT at early (P4) and late passages (P7). To determine the effect of ESCM and DMEM-LG media on cell proliferation, the initial cultures of ESCM-MSCs and DMEM-MSCs were prepared by plating the cells at density of 5×10^3 cells/cm² as described in Materials and Methods. Both populations were then maintained in their particular

medium for 2 weeks. The results revealed that ESCM-MSCs exhibited higher acceleration effect on cell proliferation than DMEM-MSCs. ESCM-MSCs reached a fold increase of 4.11 ± 0.26 (day 3), 15.29 ± 1.19 (day 6), 52.58 ± 2.18 (day 9) and 204.66 ± 10.39 folds (day 12). DMEM-MSCs gave the fold increase of 2.93 ± 0.40 (day 3), 9.27 ± 0.52 (day 6), 32.67 ± 1.73 (day 9) and 113.77 ± 7.89 folds (day 12) (Fig. 1a; Table 2). Both ESCM-MSCs and DMEM-MSCs exhibited normal MSCs morphology as shown in Fig. 1b. No significant difference of cell morphology was observed in the cells expanded in both media. These findings were consistent with faster mean PDT obtained for the cells cultured in ESCM. At early passage (P4), the shortest mean PDT was observed for ESCM-MSCs as 37.13 ± 0.64 h whereas DMEM-MSCs showed longer doubling time as 41.10 ± 0.90 h in average (Fig. 1c). At late passage (P7), the means PDT of the cultured cells from both media were 2 h longer in average as 39.00 ± 1.32 h for ESCM-MSCs and 43.30 ± 0.89 h for DMEM-MSCs (Fig. 1d). Taken together, these data suggest that ESCM could extensively expand WJ-MSCs in vitro especially in a short term which exerts beneficial implications for clinical use.

Immunophenotypes

The expanded cells from both media were further characterized for the expression of MSCs cell surface

Table 2 Fold increase of DMEM-MSCs and ESCM-MSCs cell expansions under hypoxic condition for 12 days

Cell population	D3	D6	D9	D12
ESCM-MSCs	4.11 ± 0.26	15.29 ± 1.19	52.58 ± 2.18	204.66 ± 10.39
DMEM-MSCs	2.93 ± 0.40	9.27 ± 0.52	32.67 ± 1.73	113.77 ± 7.89

All data are presented as mean ± SD (n = 3)

Fig. 1 Cell proliferation, cell morphology and population doubling time (PDT) of DMEM-MSCs and ESCM-MSCs. **a** An in vitro expansion of WJ-MSCs by DMEM-LG supplemented with 10 % FBS and ESCM under hypoxic condition. Fold increases of DMEM-MSCs and ESCM-MSCs cell expansions are presented as mean ± SD (n = 3),

*P < 0.05. **b** Cell morphology of DMEM-MSCs (a–c) and ESCM-MSCs (d–f) at various passages. Magnification ×40. Scale bar 300 μm. **c**, **d** PDT of DMEM-MSCs and ESCM-MSCs at P4 and P7, respectively. All data are presented as mean ± SD (n = 3). D1–D6 = day in culture during P4 and P7 of PDT experiments

markers using flow cytometry analysis. DMEM-MSCs and ESCM-MSCs were positive for CD29, CD44 and CD90 (common cell surface markers of MSCs) and negative for CD34 and CD45 (common hematopoietic stem cell markers). Percent expressions of these markers were quite similar in the cells expanded in both media. At early passage (P4), high positive percentages ranging from 89.87 ± 2.44 to 96.54 ± 1.63 % were observed for CD29, CD44 and CD90 (Fig. 2a, b, e). At late passage (P7), high positive percentages ranging from 94.98 ± 2.13 to 97.56 ± 1.06 % were maintained for CD29 and CD44 for DMEM-MSCs and ESCM-MSCs (Fig. 2c, d, f).

Pluripotency genes expressions

The effect of an in vitro cultivation on pluripotency properties of the expanded cells from both media was also investigated by RT-PCR analysis. These markers are key pluripotency genes essential for maintenance the pluripotency properties of stem cells. All tested pluripotency genes, *Oct-4*, *Oct-3/4*, *Nanog*, *Klf-4*, *C-Myc* and *Sox-2*, were expressed by DMEM-MSCs and ESCM-MSCs at early passage (P4) and late passage (P7) (Fig. 3). However, at late passage (P7), the downregulation of *Oct-4* and *Nanog* was observed in

Fig. 2 Immunophenotype characteristics of WJ-MSCs grown in DMEM-LG supplemented with 10 % FBS and ESCM under hypoxic condition at P4 (**a, b**) and P7 (**c, d**). Positive staining of each marker was shown as percentage in average for DMEM-

MSCs and ESCM-MSCs at P4 (**e**) and P7 (**f**). Shaded histogram represents unstained controls, while open histogram represents positive staining with the indicated antibody. All data are presented as mean $\pm$ SD ($n = 3$)

DMEM-MSCs whereas ESCM-MSCs could maintain the expression levels of all tested pluripotency genes (Fig. 3b). These results indicate that ESCM had more potential to maintain the stem cells pluripotency gene expressions of WJ-MSCs than DMEM-LG both at early and late passage cells.

Cell cycle distribution

To determine whether MSCs expanded in DMEM-LG and ESCM have normal cell cycle or not, the expanded cells were subjected to cell cycle analysis by flow cytometry method. The data showed that both DMEM-

MSCs and ESCM-MSCs have a similar normal cell cycle distribution (Fig. 4). At early passage (P4), DMEM-MSCs and ESCM-MSCs showed a cell cycle distribution ranging from 85.76 ± 0.76 to 91.31 ± 2.77 % for quiescent G_0/G_1 phase, 0.98 ± 0.34 to 1.22 ± 0.31 % for S phase and 5.41 ± 2.90 to 9.74 ± 1.02 % for G_2/M phase (Fig. 4a, b). At late passage (P7), the expanded cells from both media showed a cell cycle distribution ranging from 70.62 ± 1.24 to 74.24 ± 1.56 % for quiescent G_0/G_1 phase, 3.02 ± 0.34 to 3.30 ± 0.91 % for S phase and 18.22 ± 1.94 to 22.21 ± 3.05 % for G_2/M phase (Fig. 4c, d). Altogether, DMEM-MSCs and ESCM-

Fig. 3 RT-PCR analysis of stemness gene expressions of WJ-MSCs grown in DMEM-LG supplemented with 10 % FBS (lane 1) and ESCM (lane 2) under hypoxic condition at P4 (a) and P7 (b). *GAPDH* mRNA expression was detected as PCR positive control and input template control

Fig. 4 Cell cycle characteristics of DMEM-MSCs and ESCM-MSCs at P4 (a, b) and P7 (c, d). Cell cycle distribution of each phase was shown as average percentage in each passage. All data are presented as mean $\pm$ SD (n = 3)

MSCs showed normal cell cycle characteristic of stem cells with majority of the cells staying in quiescent G₀/G₁ phase. These results suggest that ESCM could maintain normal cell cycle distribution of the expanded WJ-MSCs similar to those cultured in DMEM-LG.

Mesodermal-lineages differentiation potential

To investigate mesodermal-lineages differentiation potential of the WJ-MSCs expanded in both media, both early and late passages cells were induced into

adipogenic, chondrogenic and osteogenic lineages by using commercial differentiation media. At early passage (P4), both DMEM-MSCs and ESCM-MSCs population successfully differentiated toward adipogenic, chondrogenic and osteogenic lineages which were identified by oil red O staining, 1 % alcian blue staining and 2 % alizarin red S staining, respectively (Fig. 5a). These cells still maintained their capacity of differentiation into mesodermal lineages until late passage (P7) (Fig. 5b). For adipogenic differentiation, a higher lipid vacuole formation was observed in the differentiated cells derived from DMEM-MSCs as compared to those derived from ESCM-MSCs. These results may imply that long term cultured WJ-MSCs in ESCM provided a reduced effect on adipogenic differentiation potential of the expanded cells. However, chondrogenic and osteogenic differentiation potentials were quite similar in both DMEM-MSCs and ESCM-MSCs at early and late passages. Altogether, these results indicate that WJ-MSCs cultured in

ESCM had a differentiation potential into mesodermal lineages similar to common characteristics of MSCs.

Cytokine components in ESCM

To investigate cytokine components in ESCM which may be involved in enhancing WJ-MSCs proliferation *in vitro*. To this end, cytokine-antibody array was performed in order to determine the existing proteins in ESCM. Among 36 cytokines of interest, ten of them were detected at significantly higher levels in ESCM as compared to unconditioned medium (fresh ES medium) which did not express any cytokine components (Fig. 6a). The detected signals presented on the membrane were translated as pixel density and the relative fold change in cytokine levels between ESCM and unconditioned medium was further calculated. The ten proteins presented in the ESCM were complement component 5/5a (C5/C5a), granulocyte colony-stimulating growth factor (G-CSF), granulocyte-

Fig. 5 Adipogenic, chondrogenic and osteogenic differentiations of WJ-MSCs cultured in DMEM-LG supplemented with 10 % FBS and ESCM under hypoxic condition at P4 (a) and P7 (b). Adipogenic differentiation was evidenced by the formation of lipid vacuoles which were identified by oil red O staining. Chondrogenic differentiation was examined by 1 % alcian blue

staining of formed proteoglycan. Osteogenic differentiation was confirmed by 2 % alizarin red S staining of mineralized depositions. Magnification $\times 200$, $\times 100$ and $\times 40$ for adipogenic, chondrogenic and osteogenic differentiations, respectively. Scale bar = 300 μ m, 100 μ m and 50 μ m for osteogenic, chondrogenic and adipogenic differentiations, respectively

macrophage colony-stimulating growth factor (GM-CSF), growth-related oncogene α (GRO α), soluble intercellular adhesion molecule 1 (sICAM-1), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibitory factor (MIF) and serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), and member 1 (Serpin E1). Among these proteins, the expression levels of GM-CSF, GRO α , IL-6, IL-8, MCP-1, MIF and Serpin E1 were more than 15 fold increase as compared to unconditioned medium (Fig. 6b). These results indicate that the undifferentiated H9 human ES cell line and human foreskin fibroblast feeder layers secrete a variety of cytokines, chemokines and growth factors into their microenvironment. We hypothesize that at least ten secreted factors in ESCM may account for accelerating in vitro expansion of WJ-MSCs in this condition.

Fig. 6 Cytokine components presented in ESCM as compared to unconditioned medium. The table shows the location of the detected proteins on the membrane (a). The relative fold changes in cytokine levels of ESCM were analyzed in comparison with unconditioned medium (b). All data are presented as mean $\pm$ SD (n = 3)

Discussion

At present, WJ-MSCs are widely used as a newsourse of MSCs for research and clinical applications. In clinical use, a large numbers of cells approximately $2\text{--}5 \times 10^6$ cells/kilogram body weights of patients are required for effective therapeutic approach. Thus, an in vitro expansion is still needed to up-scale the cells in order to achieve clinical applications. Typically, MSCs can grow in a commonly used culture medium such as DMEM supplemented with 10 % FBS. However, it has been reported that this medium failed to support MSCs expansion in long term culture (Nekanti et al. 2010a, b). To attempt limitation of using FBS, several studies are focusing on exploration of alternative sources of growth factor for supportive MSCs proliferation in vitro. To date, ESCM has not yet been investigated with respect to its effect on the

proliferation potential of MSCs. Here, we are the first group that opens a new insight of reusing the discarded ESCM from our routine human ES culture system for WJ-MSCs expansion in vitro. We found that ESCM effectively enhanced WJ-MSCs proliferation in maximal fold increase of 204.66 ± 10.39 fold within 12 days whereas the cells cultured in fresh ES-medium containing bFGF could not support their proliferation well and eventually stayed in quiescence stage and died (data not shown). The WJ-MSCs cultured in DMEM-LG gave only an 113.77 ± 7.89 fold increase. These findings were consistent with faster mean PDT of ESCM-MSCs (37.13–39.00 h) as compared to DMEM-MSCs (41.10–43.30 h). Moreover, the cells expanded in both media could preserve MSCs characteristics including cell morphology, MSCs marker expressions and mesodermal-lineages differentiation capacity under hypoxic condition. Although complex medium for culturing human ES has been shown to extensively support WJ-MSCs proliferation (PDT around 24 h), this medium still retains high proportion of 20 % FBS which is not appropriate for use in clinics (Fong et al. 2010). In this study, we used Knockout-DMEM (KO-DMEM) supplemented with Knockout-serum replacement for culturing human ES in order to minimize exposure to animal serum. Thus, ESCM from our system serves as serum-free conditioned medium which offers a great potential for short term expansion of WJ-MSCs at clinical scale.

Based on previous knowledge, hypoxic condition (2–3 % O_2) has been shown improving WJ-MSCs proliferation, upregulation of several stem cell markers and accelerating early expression of mesodermal/endothelial related markers (Nekanti et al. 2010a, b). Similarly, another study reported rapid expansion, inhibition of senescence while maintaining MSCs properties of BM-MSCs by hypoxic culture conditions (1 % O_2) (Tsai et al. 2011). In addition to our results, these data imply that low oxygen tension or hypoxic condition is more suitable for MSCs expansion than typical atmosphere of 21 % oxygen tension. Here, we observed that hypoxia culture could keep pluripotency properties of ESCM-MSCs better than DMEM-MSCs. These data supported by the down regulation of *Oct-4* and *Nanog* expressions in at late passage of DMEM-MSCs culture. These findings may imply the awareness of using DMEM-LG supplemented with 10 % FBS for long term culture of WJ-MSCs under hypoxic condition. *Oct-4* and *Nanog* are transcription factors

required for maintenance of the pluripotency and self-renewal of human ES (Chambers and Tomlinson 2009). It has been reported that ectopic overexpression of *Nanog* and *Oct-4* improved both cell proliferation and stemness properties of BM-MSCs (Liu et al. 2009).

Among cytokine components presented in ESCM, IL-6, IL-8 and MCP-1 seem to be candidate factors for promoting WJ-MSCs proliferation which had been evidenced by previous studies. It has been reported that MCP-1 plays a role in regulating MSCs migration. MCP-1 had ability to trigger MSCs migration into lesions of ischemic cerebral tissues and breast tumor (Rice and Scolding 2010; Li and Jiang 2011). In addition, MCP-1 also induced BM-MSCs homing into ischemic myocardium and facilitated the repairing process (Wu and Zhao 2012). In addition to inducing MSCs migration, MCP-1 provided a trend to increase MSCs proliferation at concentrations of 50 ng/ml and 100 ng/ml (Rice and Scolding 2010). Additionally, there has been evidence that IL-6 plays a role in maintenance undifferentiation state of MSCs. Song et al. (2006) reported the downregulation of IL-6 during MSCs differentiation into specific lineage. Conversely, IL-6 expression was restored upon de-differentiation of MSCs into the undifferentiated state (Song et al. 2006). A subsequent study also revealed that IL-6 was essential for enhancing BM-MSCs proliferation, maintaining the undifferentiated state and inhibiting further differentiation via ERK1/2 pathway (Pricola et al. 2009). Similarly, human placental mesenchymal stem cells showed increasing proliferation in responding to low concentrations of proinflammatory cytokines including IL-6 and IL-8 (Li et al. 2007). Based on these data, MCP-1, IL-6 and IL-8 components in ESCM seem to account for increasing WJ-MSCs proliferation as well as maintaining the undifferentiation state of the cells under hypoxic condition. However, we cannot rule out the effect of other factors which are not included in the cytokine array kit.

Some of the factors presented in the ESCM such as MIF, PAI-1, sICAM-1, IL-6 and IL-8 have been detected in conditioned medium from undifferentiated human H1/H9 (Bendall et al. 2009) and Hsf1 ES cell lines (LaFramboise et al. 2010). Interestingly, undifferentiated human H1 and H9 ES cell lines could produce bFGF and TGF β 1 which have been identified as growth factor for MSCs proliferation. Jian et al. (2006) reported that TGF β 1 induced BM-MSCs proliferation through a novel mechanism of cross-talk

between the TGF- β and Wnt signaling pathways. Additionally, the combination of TGF- β , PDGF and bFGF was shown to support BM-MSCs growth in a serum-free medium up to 5 passages (Ng et al. 2008). Based on these data, we believe that bFGF and TGF β 1 may also be present in ESCM similar to those observed from a previous study because our conditioned medium was obtained from cell culture supernates of the same human ES cell line. Thus, bFGF and TGF β 1 may provide additional effects on promoting WJ-MSCs proliferation in addition to other factors.

In conclusion, our results revealed the value of ESCM in terms of significantly promoting WJ-MSCs expansion as well as preserving common stem cell characteristics of MSCs both at cellular and molecular levels including normal cell cycle, fibroblastic morphology, cell surface markers expression (CD29⁺, CD44⁺, CD90⁺, CD34⁺, CD45⁺) and mesodermal-lineages differentiation capacities into adipocytes, chondroblasts and osteoblasts. In addition, ESCM serves as a rich source of cytokines and growth factors that are required for maintaining WJ-MSCs proliferation. Here, we reported superior benefits of ESCM for extensive WJ-MSCs expansion while still preserving their pluripotency properties with reduced time-consuming and cost-saving approaches. This study opens a new insight of reusing the discarded cell culture supernates from human ES culture system as a culture medium for short term expansion of WJ-MSCs under hypoxic condition. In addition, WJ-MSCs cultured in ESCM can differentiate into hepatocytes (Patcharee and Wilairat. 2013). The beneficial implications of this work can serve for clinical applications, regenerative medicine, stem cell banking, pharmacological applications and other.

Acknowledgments We would like to thanks Por-Pat Hospital, Nakhon Ratchasima, Thailand for providing umbilical cords. This work was supported by Suranaree University of Technology, Thailand.

Conflict of interest None.

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